SCOTTISH HOSPITALS INQUIRY

Bundle of documents for Oral hearings commencing from 19 August 2024 in relation to the Queen Elizabeth University Hospital and the Royal Hospital for Children, Glasgow

Bundle 27 – Volume 17
Miscellaneous Documents

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Subject: Pre Start Meeting, Ward 6A, QEUH
Location: Room L2005, Level 2 Teaching & Learning Building, QEUH

Start: Fri 23/08/2019 09:15
End: Fri 23/08/2019 10:00

Recurrence: (none)

Meeting Status: Meeting organizer

Organizer: MacLeod, Calum
Required Attendees: Jones, Brian; Rodgers, Jennifer; Redfern, Jamie; Steele, Tom; Deighan, Chris; Devine, Sandra; Kennedy, Iain
Optional Attendees: JONES, Brian (NHS GREATER GLASGOW & CLYDE); Inkster, Teresa (NHSmail)

Good morning

A pre start meeting is being held before the Incident Management Team meeting regarding Ward 6A, QEUH on

Date: Friday 23rd August
Time: 0915
Venue: Room L2005, Level 2, Teaching & Learning Building, QEUH

Kind Regards

Calum MacLeod
Infection Prevention & Control Administrator
Level 2, Zone 1, Office Block
Queen Elizabeth University Hospital
G51 4TF

[redacted]

Calum.MacLeod[redacted]

From: INKSTER, Teresa (NHS GREATER GLASGOW & CLYDE)
[redacted]
Sent: 22 August 2019 19:55
To: Devine, Sandra [redacted]; Inkster, Teresa
[redacted]
Subject: [ExternaltoGGC]RE: Imt

I am hoping to be in. Who is chairing the IMT?
Kr
Teresa

Dr Teresa Inkster
Lead Infection Control Doctor NHSGGC
National Training Programme Director Medical Microbiology
Dept of Microbiology
Queen Elizabeth University Hospital
Glasgow
Direct dial : [redacted]

From: Devine, Sandra [redacted]
Sent: 22 August 2019 17:21
To: INKSTER, Teresa (NHS GREATER GLASGOW & CLYDE);
[redacted]
Subject: Imt

Hi Teresa
Alison has indicated that you might be back tomorrow. IMT for 6a is at 10am with a pre meet at 9.15. Details in e mail from Calum.
Sandra

Sent from my BlackBerry 10 smartphone on the EE network.

Schedule of Accreditation

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United Kingdom Accreditation Service

2 Pine Trees, Chertsey Lane, Staines-upon-Thames, TW18 3HR, UK

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NHS Greater Glasgow and Clyde

Issue No: 009 **Issue date**: 07 March 2023

Microbiology Department
New Lister Building
Glasgow Royal Infirmary
10-16 Alexandra Parade
Glasgow
G31 2ER

Contact: David Jordan
Tel: +44 [redacted]
E-Mail: David.jordan [redacted]

Testing performed by the Organisation at the locations specified below

Locations covered by the organisation and their relevant activities

Laboratory locations:

Location details		Activity	Location code
Address New Lister Building Glasgow Royal Infirmary 10-16 Alexandra Parade Glasgow G31 2ER United Kingdom	**Local contact** David Jordan Tel: [redacted] Fax: [redacted] E-Mail: David.jordan [redacted]	Microbiology	A
Microbiology Department Facilities Management & Laboratory Medicine Building Queen Elizabeth University Hospital 1345 Govan Road Glasgow G51 4TF	As above	Microbiology	B

Site activities performed away from the locations listed above:

Location details	Activity	Location code
Sandyford (sexual health) Clinic 2/6 Sandyford Place Sauchiehall Street Glasgow G3 7NB	MICROBIOLOGY: Plate inoculation and Microscopy	C

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NHS Greater Glasgow and Clyde

Issue No: 009 **Issue date:** 07 March 2023

Testing performed by the Organisation at the locations specified

DETAIL OF ACCREDITATION

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
HUMAN BODY FLUIDS AND TISSUES	Microbiology examination activities for the purpose of clinical diagnosis General Bacteriology	In House documented methods based on related UK Standards for Microbiology Investigations' (SMIs)	
Blood cultures	Detection of growth of microorganisms of clinical significance	Automated blood culture using colorimetric methodology on the BioMerieux BacT/ALERT Virtuo and SOP LP 509	A and B
Human body fluids, tissues and swabs	General isolation & characterisation of microorganisms of clinical significance Including Trichomonas vaginalis (TV) and Bacterial vaginosis (BV).	Manual SOP's per section, key references: LP547 Routine processing LP545 CSF LP502 Respiratory procedures LP514 Genital sample processing	
Swabs and urine	MRSA screening	Manual culture on chromogenic agar LP503 MRSA screening And Semi-automated culture using WASPlab-LP557 including reading from produced images use of WASPlab and LP558 WASPlab Web App	A & B
Swabs	MSSA screening	Manual culture on chromogenic agar LP537 MSSA screening	B
Swabs and fluids	CRO screening	Manual culture on chromogenic agar LP515 CRO screening	A and B
Swabs and urine	General isolation, detection & characterisation of microorganisms of clinical significance	LP511 Urine processing using manual methods and And Semi-automated culture using WASPlab-LP557use of WASPlab and LP558 WASPlab Web App	A & B

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Testing performed by the Organisation at the locations specified

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
HUMAN BODY FLUIDS AND TISSUES (cont'd)	Microbiology examination activities for the purpose of clinical diagnosis (cont'd)	In House documented methods based on related UK Standards for Microbiology Investigations' (SMIs) (cont'd)	
Heat fixed smears and inoculated culture plates for the purposes of sexual health screen	Detection of bacteria of clinical significance for purposes of sexual health screen including Trichomonas vaginalis (TV) and Bacterial vaginosis (BV).	LP500, detected by microscopy	C
CSF, Ascitic Fluid and CAPD fluids	Quantification of white and red cells and bacteria	LP545 CSF processing LP504 Sterile fluids	A and B
Faeces	General isolation & characterisation of microorganisms of clinical significance	LP507 Enteric Pathogens using traditional culture	
	Detection of Clostridium difficile GDH Antigen & Toxins A&B	LP510 C Difficile Using the DS2 ELSIA analyser	
	Detection of clostridium difficile GDH Antigen & Toxins A&B	LP555 C Difficile Using Alere Rapid Membrane Enzyme Immunoassay	
Blood cultures	Identification and antimicrobial susceptibility testing from 6 hour positive cultures	Using positive Virtuo bottles, Vitek MS(MALDI-ToF) and Vitek AST SOP LP 509	A & B
Bacterial & Yeast and fungal Cultures (isolated in-house)	Identification, Antimicrobial susceptibility testing & determination of minimum inhibitory concentration (MIC) of bacteria, yeast and fungi to antimicrobial agents	Manual Antimicrobial Susceptibility Testing following EUCAST guidelines including disc diffusion and determination of MIC using Micronaut broth microdilution and Gradient MIC strip methods. LP531 Sensitivity Procedures using Manual Methods. Automated Antimicrobial Susceptibility Testing and identification of bacteria and yeasts using Vitek 2 and Vitek MS (Maldi). LP548 Use of Vitek 2 LP527 Use of Vitek MS (Maldi)	

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Issue No: 009 **Issue date:** 07 March 2023

Testing performed by the Organisation at the locations specified

Materials/Products tested	Type of test/Properties measured/Range of measurement	Standard specifications/ Equipment/Techniques used	Location Code
HUMAN BODY FLUIDS AND TISSUES (cont'd)	Microbiology examination activities for the purpose of clinical diagnosis (cont'd)	In House documented methods based on related UK Standards for Microbiology Investigations' (SMIs) (cont'd)	
Sputum, BAL, Urine, Tissue, PUS, Fluid, FNA	Detection of positive Mycobacteria culture	LP502 Respiratory Procedures LP505 TB Procedures using BacTAlert MGiT analyser Phenol Auramine & Ziehl Neelson Microscopy	
	Parasitology		
Faeces, Urines and rectal swabs	Examination for the presence of parasites (ova, cysts, trophozoites and worms) of clinical significance.	LP507 Enteric Pathogens Using Mini Parasep system, wet preparations	A and B
	Cryptosporidium oocysts	Modified Ziehl Neelsen & Auramine Phenol Microscopy	
	Mycology		
Human samples including skin scraping, hair and nail clipping	General isolation & characterisation of fungi of clinical significance	Detection of pathogenic fungi using manual methods & direct microscopy of KOH using Calcofluor White LP560 Non Superficial Mycology LP523 Investigation of Superficial Specimens	A
BAL & Serums samples	Detection of Aspergillus Galactomannan Antigen	Semi quantitative detection using DS2 ELISA analyser LP559 Detection of Apsergillus Galactomannan Antigen	
CSF & Serum	Detection of Cryptococcal Antigen	LP544 Cryptococcal Antigen IMMY Lateral Flow Assay	A and B
END			

Client/Site	NHS GG&C – QEUH & RHC	**RAMS No.**
Activity	Microbiological Sampling & ClO_2 Testing (Inc. Intertek Sampling Sweep and Wards 2A & 2B)	V5 1904 ClO_2 & Micro Sampling **Review**
Date Works to Be Carried Out	Commencing April 2019	August 2019
Method Statement Created By	David Watson/Allan McRobbie	Operator review prior to works commencing

Operators On Site	**Name**	**Signature**	**Date**
Lead Operator	Craig Guyer		
Additional Operators	Jamie Clayforth		
	Robert Taylor		
	David Watson		
	Stuart Kelly		

Lead Operator is responsible for Implementing MS/RA and Managing Works on site
All operators to review Method Statement and Risk Assessment prior to works commencing.
Lead Operator to produce appropriate works record upon completion of works.

COSHH Details

DMA Canyon Operators should ensure copies of all relevant COSHH sheets are carried on site, either electronically (on Laptop/Smartphone) or paper copy.

Emergency, First Aid, Fire Safety, Welfare Arrangements and Site Rules

It is the responsibility of DMA Canyon Operators in conjunction with Site/Client to familiarise themselves with site/local Emergency, First Aid, Fire Safety and Welfare arrangements and to ensure compliance with all local site safety rules prior to works commencing. First Aid Kits should be carried in vehicles at all times.

DMA Canyon Escalation Details:

In the event of an emergency DMA Canyon Operators should contact their Supervisor/Manager.
In the event of Line Manager being unavailable then DMA Canyon Directors should be contacted.
Site/Client should alert DMA Canyon Operators to any sites specific or local emergency contact procedures.

DMA Canyon Office	[redacted]
Allan McRobbie	[redacted]
Craig Morning	[redacted]
Graeme McCullie	[redacted]
David Watson	[redacted]
Mike Kinghorn	[redacted]

DRAFT

Method Statement

Method Statement for the Microbiological Sampling of Outlets and Testing Protocol in Water Systems (including Intertek Sampling and Wards 2A 7 2B Sampling)

Prior to work commencing

1. Operators will compile necessary components required to take the samples required prior to commencing and move them to the depart/ward.

2. Prior to starting work the operators shall consult with the Charge Nurse to explain the works which are to be carried out and to receive any additional instructions with regards to isolation rooms (or other similar) where additional infection control protocols are required. Operators must adhere to these additional protocols in addition to the general hygiene protocols already covered by this method statement).

3. Prior to handling sample bottles, (and any filters, spouts etc.) operator shall ensure hands are thoroughly washed and alcohol gel used on hands.

4. Sampling from Optitherm Taps

    a) Samples shall be taken from tap set on cold first and then a second sample shall be taken the hot/mixed outlet.

    b) Ensure any dirt/debris visible on the tap is removed using disinfectant wipes and then spray both the internal and around ends of the tap spout with isopropyl alcohol (70%) and leave for a period of 2 minutes.

    c) The outlet shall then be flushed on cold setting for a period of 30 seconds to remove disinfectant from spouts, minimising splashing around WHB as far as is practical

    d) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

    e) Record temperature of water from cold outlet immediately after sample taken and again after a total of 2 minutes after outlet turned on (Including time taken to collect samples) and record ClO_2 reading from the cold outlet and record on sampling sheet.

    f) Then spray both the internal and around ends of the tap spout with isopropyl alcohol (70%) and leave for a period of 2 minutes.

    g) The outlet shall then be flushed on hot/mixed setting for a period of 30 seconds to remove disinfectant from spouts, minimising splashing around WHB as far as is practical

    h) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

    i) Record temperature of water from hot/mixed outlet immediately after sample taken and again after a total of 60 seconds after outlet turned on (Including time taken to collect samples) and record ClO_2 reading from the hot/mixed outlet and record on sampling sheet.

5. Sampling from Markwik Panel Taps

    a) Samples shall be taken from tap set on cold first and then a second sample shall be taken with tap set to the full hot/mixed position.

    b) Ensure any dirt/debris visible on the tap is removed using disinfectant wipes and then spray both the internal and around ends of the tap spout with isopropyl alcohol (70%) and leave for a period of 2 minutes.

    c) The outlet shall then be flushed on cold setting for a period of 30 seconds to remove disinfectant from spouts, minimising splashing around WHB as far as is practical

    d) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

    e) Record temperature of water from cold outlet immediately after sample taken and again after a total of 2 minutes after outlet turned on (Including time taken to collect samples) and record ClO_2 reading from the cold outlet and record on sampling sheet.

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f) Then spray both the internal and around ends of the tap spout with isopropyl alcohol (70%) and leave for a period of 2 minutes.

g) The outlet shall then be flushed on full hot/mixed setting for a period of 30 seconds to remove disinfectant from spouts, minimising splashing around WHB as far as is practical

h) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

i) Record temperature of water from hot/mixed outlet immediately after sample taken and again after a total of 60 seconds after outlet turned on (Including time taken to collect samples) and record ClO_2 reading from the hot/mixed outlet and record on sampling sheet.

6. Sampling from Direct Hot and Cold Outlets (Taps)

a) Both hot and cold tap shall be sampled, sampling the hot outlet first then repeating the procedure for the cold.

b) Ensure any dirt/debris visible on the tap is removed using disinfectant wipes and then spray both the internal and around ends of the tap spout with isopropyl alcohol (70%) and leave for a period of 2 minutes.

c) Outlet shall then be flushed for a period of 30 seconds to remove disinfectant from outlet.

d) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

e) Completely fill each bottle directly from the tap to the level of the neck. Once full, immediately replace the screw cap.

f) Record temperature of water and ClO_2 reading from the outlet immediately upon completion of taking sample and record on sampling sheet.

7. Sampling from Shower Connections

a) A mixed hot and cold sample shall be taken from the shower hose by setting the shower halfway between full hot/mixed and full cold setting.

b) Shower connection shall be sprayed both internally and externally with Isopropyl alcohol and left for a period of 2 minutes.

c) Outlet shall then be flushed for a period of 30 seconds to remove disinfectant from shower connection.

d) 1 x 1000ml and 2 x 500ml sterile sample bottles shall then be filled in turn for legionella, pseudomonas and potable analysis (For samples being submitted to Intertek 1 x 1000ml and 1 x 500ml sterile sample bottles shall be used)

e) Completely fill each bottle directly from the tap to the level of the neck. Once full, immediately replace the screw cap.

f) Record temperature of water and ClO_2 reading from the outlet immediately upon completion of taking sample and record on sampling sheet.

8. Sampling from Filtered Outlets

a) Where outlets are to be sampled which have filters fitted and sample is to be taken through the filter then sampling procedure(s) as described above are to be followed with the filter in-situ.

b) Where outlets are to be sampled which have filters fitted and sample is to be taken with the filter removed then all flushing required prior to sampling is to be completed with the filter in-situ.

c) Filter to be then removed in accordance with DMA RAMS for Filter replacements.

d) Sample bottles shall then be filled as required without flushing any water from the outlet – all water, so far as is reasonably practical, to be collected into the sample bottle(s).

e) Filter shall then be replaced with a new filter in accordance with DMA RAMS for Filter replacements

9. Once all samples completed samples to be submitted to NHS Labs at GRI for analysis, as per instruction to lab from Mr I Powrie and Dr T Inkster. or delivered to Estates Dept for collection by courier for Intertek.

10. DMA Sample paperwork to be completed and submitted to both DMA offices and GRI Lab/Intertek as appropriate.

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Additional Information

Ward 2A & 2B routine sampling outlets locations

From Markwik 21 Panel mounted taps are:

Ward	RISER	ROOM REF	Room Name
2A	M36	TCT-010	Room 6
2A	M36	TCT-003	Room 3
2A	M39	SCH-063	Old Treatment Room
2B	M39	DCU-024	Consultant Room 1
2B	M39	DCU-018	Dirty Utility

Outlets to be sampled using the Direct Hot and Cold Taps are:

Ward	RISER	ROOM REF	Room Name
Anaesthetics	M36	SCH-092	Hospital at Night (Anaesthetics Dept)
2A	M36	SCH-062	Play Room (Stainless Steel Sink – Direct hot and cold)
2A	M39	SCH-087	DSR (Cleaners)

Outlets to be sampled from the shower hoses are:

Ward	RISER	ROOM REF	Room Name
2A	M36		Room 6 Bathroom
2A	M36		Room 16 Bathroom
2A	M39		Room 17 Bathroom
2A	M39		Room 22 Bathroom

DRAFT

Safety Risk Assessment

Hazard Type	Hazard Effect	Who Might be Harmed	S	L	R	Control Measures	Residual Risk S	Residual Risk L	Residual Risk R
Manual Handling	Physical Injury caused by lifting heavy and or awkward loads	Operators	3	2	6	Split equipment into small easily handled parts. Decant chemicals into small amounts/containers. Individual should not lift or carry loads beyond their own capability and for bulky or heavy objects obtain assistance. Ensure work area and pathways are clear and suitable for lifting loads.	2	1	2
Exposure to Chemicals/Chemical Splash	Chemical burns to skin, eyes, respiratory/ingestion system (refer to COSHH Sheets for further info)	Operators	3	3	9	Use of Suitable PPE (e.g. Gloves, Goggles, Overalls) Decant chemicals into small amounts and suitably labelled containers or use suitable transfer equipment (e.g. pumps). Follow instructions on MSDS/COSHH sheets.	2	2	4
Chemical Spill	Environmental impact of chemicals being released into water course	Environment	4	2	8	Spill Kits to be carried to clean up chemical spills (refer to above for personal protection measures). Spill kit to be suitable bagged and returned to DMA for proper safe disposal.	4	1	4
Exposure to Biological Contamination	Risk of contracting Legionnaires Disease or other infections (e.g. Weils Disease, e.coli, coliforms)	Operators	5	2	10	Minimise creation of aerosols. Ensure are is free of bacterial contamination (e.g. rodent bird droppings). Suitable PPE to be worn if general area contaminated (e.g. Gloves, Masks)	5	1	5
Cuts/Abrasions	Cutting / trapping hands or fingers on tools & equipment or on sharp materials in work area	Operators	3	3	9	Wear suitable protective footwear, clothing, gloves and goggles where appropriate, and follow manufacturer's instructions for equipment. Inspect all equipment and ensure is fit for purpose prior to use.	2	2	4
Working at Height	Physical injury from falling off ladder roof or platforms, or equipment being dropped from height	Operators & others in area below	5	2	10	Ladders to be inspected prior to use to ensure fit for purpose. Only tasks of short duration to be carried out from ladders, or for access only. Roofs/platforms to be inspected to ensure safe prior to accessing.	5	1	5
Scald Risk	Burns & Scalds to hands/skin when working on hot pipework and with hot water	Operators	3	3	9	Ensure isolation valves are fitted and in working order. If necessary wear suitable PPE, (e.g. gloves, goggles) to protect skin.	2	2	4
Slip/Trip	Physical injury from falling slipping on wet/slippery surfaces or tripping over equipment/obstacles	Operators	4	2	8	Safety boots provide grip and ankle support and must be worn at all times Ensure that work area is kept tidy at all times. Ensure that hoses and cables do not present a trip hazard. Mop up any spills immediately. Ensure work area is adequately lit. When draining water systems ensure water is drained to a safe are (Drain) and does not contribute to a slipping or any other type of hazard.	3	1	3
Asbestos	Inhalation of asbestos fibres	Operators & others in area if disturbed	5	3	15	Consult Asbestos Register. Visual inspection of work area prior to commencing work. Stopping of all work if disturbed asbestos is suspected in any areas being worked in or could be disturbed by works being carried out.	5	1	5
Weather Conditions	Potentially increases the risk potential of other hazard types and weather conditions should be considered prior to works commencing.	Operators	N/A	N/A	N/A	Operatives will remain vigilant where early morning dew frost is evident. Safety boots provide grip and should be worn at all times. Care will be taken when handling GRP panels and other equipment/materials to ensure they are dry and frost free before handling. No work in high winds or driving rain when working at height or in situations which may cause unsafe working practices. Where access to site cabins is restricted then comfort/shelter can be taken in the company vehicle	N/A	N/A	N/A

ADDITIONAL SITE SPECIFIC RISKS NOT PREVIOUSLY IDENTIFIED OR SITE CONDITION WHICH AFFECTS THIS ASSESSMENT SHOULD HAVE A DYNAMIC RISK ASSESSMENT FORM COMPLETED.

SEVERITY	Remote	Possible	Likely	Very Likely	Inevitable
Fatal/Permanent Disability	5	10	15	20	25
Long Term Absence	4	8	12	16	20
Injury - >3 days lost time	3	6	9	12	15
Injury - <3 days lost time	2	4	6	8	10
Negligible – No lost time	1	2	3	4	5
	LIKELIHOOD				

Final Assessment

Provided company personnel have fully assessed area/task and follow the control measures and carry out tasks in a safe manner as detailed above, the level of risk ass°Ciated with the tasks identified will be reduced to an acceptable low level of risk. Site operators must assess the site/work area and tasks to be carried out on site prior to works commencing. Any additional risks not covered by the initial assessment must be added, assessed in the relevant area on following page prior to works commencing and suitable safety precautions recorded and taken in order to maintain acceptable level of risk.

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Cupriavidus spp. and other waterborne organisms in healthcare water systems across the UK

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SUMMARY

Background: *Cupriavidus pauculus* is a rare clinical pathogen, cases of which have been linked to contaminated hospital water systems. An outbreak of three cases of *C. pauculus* and other waterborne organisms was reported in a Glasgow hospital in 2018.
Aims: To determine whether *Cupriavidus* spp. are present in hospital water systems elsewhere in Scotland and the UK, and to ascertain the optimal laboratory methodology for detection. This study also sought to establish where in the water system these organisms are detected, and whether a selective media could be developed for isolation. In addition, water samples were tested for the presence of other Gram-negative water-borne organisms.
Methods: Water samples were received from 10 UK National Health Service hospitals and from various parts of the water system. Isolates were plated on to tryptone soya agar (TSA) and Pseudomonas isolation agar, and were further identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S polymerase chain reaction.
Findings: *Cupriavidus* spp. were detected in four of 10 hospitals tested, and all five isolates were from the periphery of the water system. All hospitals had evidence of other opportunistic premise plumbing pathogens. *Cupriavidus* spp. were identified using TSA, with some isolates growing on Pseudomonas isolation agar; as such, they may be detected inadvertently when testing water specifically for *Pseudomonas aeruginosa*.
Conclusion: Isolation of *Cupriavidus* spp. was not unique to the Glasgow incident, and these bacteria are present in hospital water systems elsewhere in the UK. Water testing in response to clinical cases is recommended. Consideration should also be given to water testing following bacteraemias due to other rare and unusual water-borne pathogens.
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Introduction

UK mains drinking water is supplied to premises at a high standard, but does contain small numbers of organisms which, if allowed to multiply, may be a risk to susceptible hospitalized patients. To date, the main organisms thought to pose a risk

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include *Legionella* spp., *Pseudomonas aeruginosa* and atypical mycobacteria. There is specific guidance and methods for testing for these organisms in water samples [1,2]. Whilst there are reports of *Cupriavidus* spp. in hospital water systems [3], the incident at Queen Elizabeth University Hospital in Glasgow, where *C. pauculus* was isolated from both the water system and patient blood cultures, was the first report of an outbreak of healthcare-acquired *C. pauculus* [4].

There is little knowledge regarding the prevalence of *Cupriavidus* spp. within healthcare water systems. There is no guidance on managing these bacteria, and no recognized testing methodology. Until recently, difficulties in laboratory identification are likely to have hampered recognition of this organism in clinical practice. The advent of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (for the rapid and accurate identification of bacteria) has revolutionized the ability to identify many organisms accurately, including *Cupriavidus* spp. Prior to MALDI-TOF being widely adopted in clinical microbiology laboratories, biochemically inert organisms including *Cupriavidus* spp. may not have been identified accurately to genus or species level, resulting in them being dismissed as unidentified environmental organisms of dubious clinical significance. Moreover, MALDI-TOF is still not necessarily widely available in environmental laboratories which test water samples. Thus, even when isolated from clinical specimens, the possible link between these *Cupriavidus* spp. and water systems may still be unnoticed.

The aims of this study were: to determine whether *Cupriavidus* spp. were present in other healthcare water systems in Scotland and other parts of the UK; to assess whether features of Scottish water systems, such as steel pipes, might affect the ecology; to determine where in healthcare water systems (incoming mains, water tanks, expansion vessels, outlets) *Cupriavidus* spp. could be detected; to determine the extended antibiotic susceptibility profiles of *Cupriavidus* spp.; and to develop a protocol for detecting *Cupriavidus* spp. in water systems, and to detect any other organisms (especially Gram-negative bacteria) in water samples.

Methods

Hospitals were approached by the chief investigator to participate, based on geographical location to ensure a representative sample of mainland UK. From January to June 2021, system-wide pre-flush water samples were collected across the water system from 10 healthcare facilities. Sample sites included water storage tanks, expansion vessels and outlets (a maximum of 15 samples per hospital). Water samples were transported to the ISO 17025 UKAS-accredited Glasgow Royal Infirmary Microbiology Environmental Laboratory under temperature-controlled conditions to minimize specimen deterioration in bottles (containing thiosulphate to neutralize chlorine) pre-supplied by the laboratory.

In the absence of an established methodology for detecting *Cupriavidus* spp. and many other waterborne Gram-negative bacteria (with the exception of *Legionella* spp. and *P. aeruginosa*), 100-mL water samples were filtered on to:

- chromogenic coliform agar (E&O Laboratories, Bonnybridge, UK) incubated for 24 h at 36 °C;
- tryptone soya agar (TSA; Oxoid, Basingstoke, UK) incubated for 48 h at 36 °C;
- Pseudomonas isolation agar (E&O) incubated for 48 h at 36 °C;
- carbapenemase agar (Colorex mSuperCARBA E&O) incubated for 24 h at 36 °C; and
- CHROMID Carba Smart (bioMérieux, Marcy l'Etoile, France) incubated at 24 h for 36 °C.

After incubation, Gram-negative bacteria were identified using MALDI-TOF. Any bacteria that were not identified after repeat testing by MALDI-TOF were then tested using VITEK2. Bacteria that could not be identified by either technology were classed as unidentified Gram-negative bacilli. The identities of *Cupriavidus* spp. isolates were confirmed by independent reference laboratories.

Cupriavidus isolates underwent assessment of antibiotic susceptibilities for amoxicillin, aztreonam, co-amoxiclav, ciprofloxacin, gentamicin, meropenem, tazocin, temocillin, cotrimoxazole, meropenem and minocycline by disc testing, or determination of minimum inhibitory concentrations (MICs) using gradient strips.

Total viable counts of water samples were carried out using a pour plate method, with 1-mL volumes of water mixed into yeast extract agar. Plates were incubated at 22 °C and 37 °C; total viable counts of $<$100 colony-forming units (cfu)/mL were quantified.

Results

All 10 sites (Figure 1) obtained pre-flush samples from outlets, and eight sites also collected storage tank samples. Only three hospitals were able to sample expansion vessels. In total, 157 samples were tested (Figure 2). Pre-flush samples were collected to be representative of both systemic and peripheral contamination. A total of five samples from four of the 10 hospitals contained *Cupriavidus* spp. There were three isolates of *C. pauculus*, and one each of *C. gilardii and C metalladurans*. Other than one expansion vessel sample, isolates were obtained from the outlets. Three of the five samples had counts of *Cupriavidus* spp. $>$100 cfu/100 mL. All these isolates grew on TSA, and three also grew on Pseudomonas agar plates. No isolates grew on either of the carbapenamase agars.

Antimicrobial susceptibility testing results are shown in Table I. There are no interpretative criteria for *Cupriavidus* spp.; previous publications have used the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *Pseudomonas* spp. or the criteria of the Clinical and Laboratory Standards Institute (CLSI) for non-Enterobacterales where EUCAST breakpoints do not exist. Of note, four of the five isolates had meropenem MIC $>$32 mg/L and would be classed as resistant according to the EUCAST interpretative criteria for *Pseudomonas* spp. All five isolates were sensitive to co-trimoxazole (by EUCAST breakpoints) and minocycline (by CLSI interpretative criteria).

Table II shows the pattern of detection of all Gram-negative bacteria at each hospital. The percentage positivity ranged from 26.7% to 95%, and the percentage of positive samples with counts $>$100 cfu/100 mL ranged from 37.5% to 100%. In addition to the five isolates confirmed as *Cupriavidus* spp., a further four isolates that were identified as *Cupriavidus* spp. by VITEK2 alone were not confirmed as *Cupriavidus* spp. by the UK Health

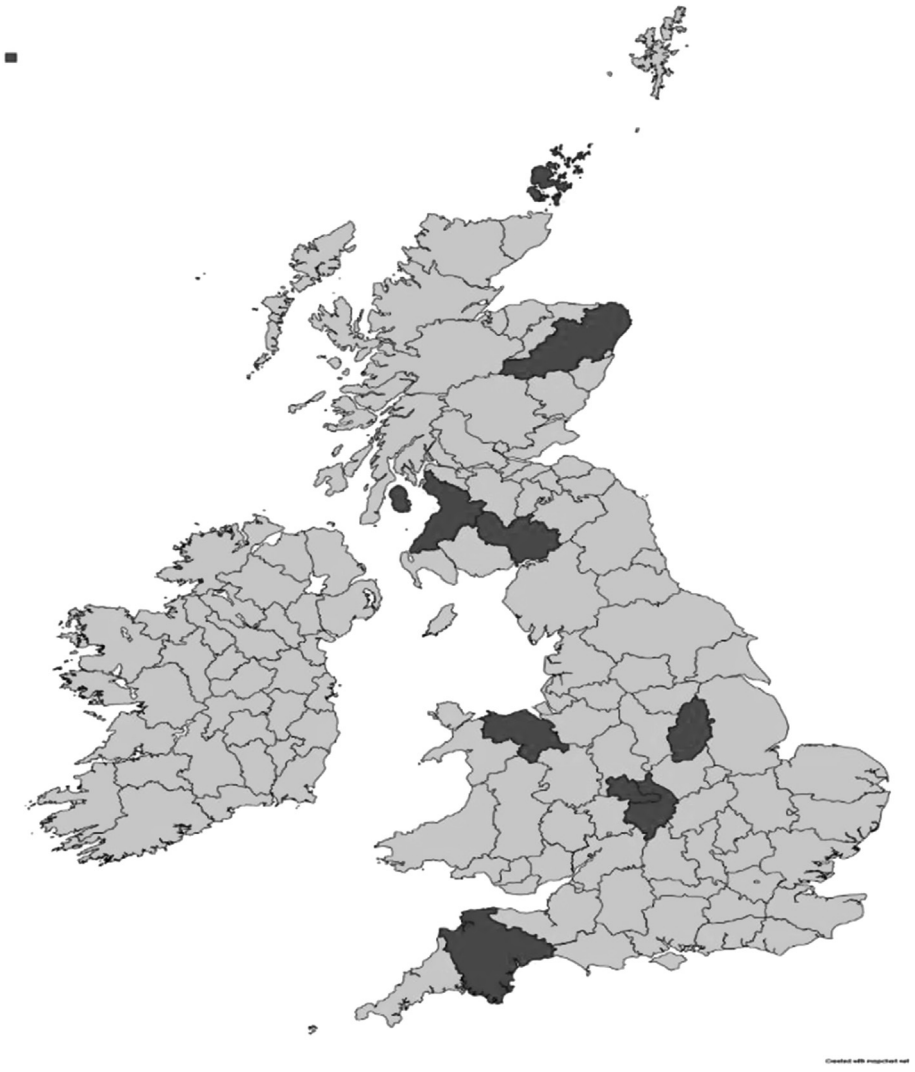

Figure 1. Map displaying geographical location of hospital sites sampled.

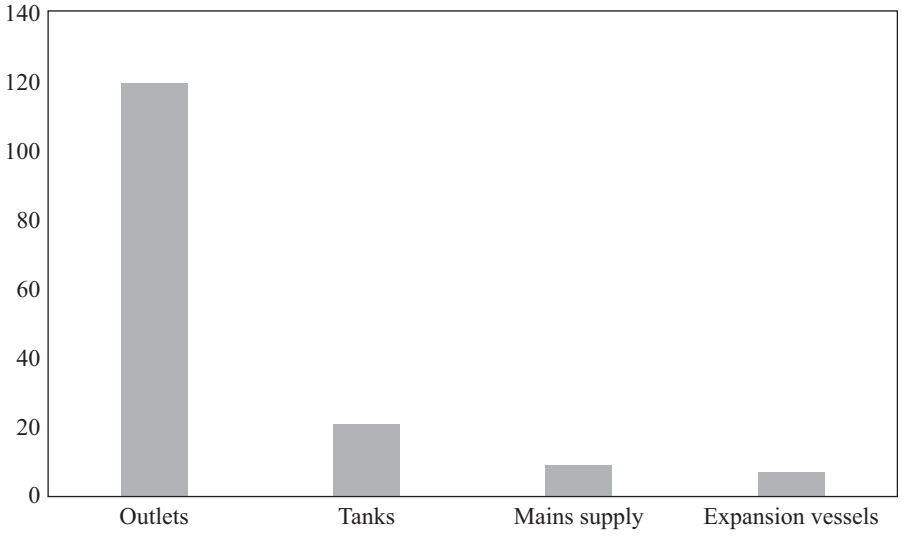

Figure 2. Number of samples by location within the water system.

Table I
Antimicrobial susceptibility testing of five isolates of *Cupriavidus* spp.

	Minocycline MIC[a] S ≤4 mg/L	Meropenem MIC S ≤2 mg/L	Cotrimoxazole MIC[a] S ≤2 mg/L	Meropenem disc S ≥24 mm R <18 mm	Ciprofloxacin disc S ≥50 mm R <26 mm	Aztreonam disc S ≥50 mm R <18 mm	Tazocin disc S ≥50 mm R <18 mm	Gentamicin disc[b]	Coamoxiclav disc[b]	Amoxycillin disc[b]	Temocillin disc[b]
Isolate A	0.19	>32	0.5	0 mm	32 mm	17 mm	33 mm	0 mm	0 mm	0 mm	12 mm
Isolate B	0.125	0.38	0.38	34 mm	34 mm	12 mm	38 mm	22 mm	38 mm	32 mm	0 mm
Isolate C	0.19	>32	0.38	0 mm	33 mm	0 mm	30 mm	0 mm	10 mm	0 mm	0 mm
Isolate D	0.19	>32	0.5	0 mm	33 mm	0 mm	29 mm	0 mm	12 mm	0 mm	0 mm
Isolate E	0.25	>32	0.38	0 mm	32 mm	0 mm	35 mm	0 mm	13 mm	0 mm	0 mm

MIC, minimum inhibitory concentration; S, sensitive; R, resistant.
[a] Criteria of the Clinical and Laboratory Standards Institute.
[b] No zone interpretative criteria.

Table II
Numbers of outlets testing positive in each hospital for Gram-negative bacteria and with total viable count >100 colony-forming units (cfu)/100 mL

Hospital	*N* (%) of samples testing positive for Gram-negative bacteria	*N* (%) of positive samples with counts >100 cfu/100 mL
A	9/15 (60)	7/9 (77.8)
B[a]	11/15 (73.3)	6/11 (54.5)
C[a]	8/16 (50)	3/8 (37.5)
D[a]	11/16 (68.7)	9/11 (81.8)
E	8/15 (53.3)	8/8 (100)
F	19/20 (95)	10/19 (52.6)
G	4/15 (26.7)	2/4 (50)
H	13/15 (86.7)	12/13 (92.3)
I	11/15 (7.3.3)	6/11 (54.5)
J[a]	5/15 (33.3)	1/15 (6.7)

[a] Hospitals isolating *Cupriavidus* spp.

and Security Agency reference laboratory, and were subsequently identified as *Xenophilus aerolatus* using 16S rDNA sequencing (Micropathology Ltd, Coventry, UK). The range of other opportunistic premise plumbing pathogens (OPPPs) isolated from water samples is shown in Figure 3. As expected, OPPPs were isolated in all hospitals. *Blastomonas ursincola* was the most commonly isolated Gram-negative pathogen, followed by *Acidovorax* spp., *Sphingomonas* spp. and *Brevunidimonas* spp.

Discussion

Incoming mains water naturally contains micro-organisms, but provided water systems are maintained and used correctly (to minimize the risk of biofilm formation), these will be of little consequence to most patients at the point of exit. However, additional precautions may be required to prevent infection in patients who are highly immunosuppressed or who have other risk factors for waterborne infections (e.g. intravascular lines).

Cupriavidus spp. are aerobic oxidase-positive Gram-negative bacilli that belong to a group of organisms known as OPPPs [5]. These organisms possess a number of characteristics in common; disinfectant resistance, pipe surface adherence and biofilm formation, growth in amoebae, growth on low organic concentrations, and growth at low oxygen levels [5]. They are normal inhabitants of incoming mains water (in low numbers), but can multiply in premise plumbing to levels capable of producing infections in individuals (usually) with predisposing conditions. For many OPPPs, there is no recognized method for detection in water systems, even for species such as *Stenotrophomonas maltophilia* whose association with water systems is long established. For such a heterogeneous group of bacteria, it is likely that optimum detection may require different media and growth conditions.

Cupriavidus spp. are known to contaminate hospital water systems with hydro pools, and extracorporeal membrane oxygenation delivery systems [6]. They are rare clinical pathogens, but have been reported to cause sepsis, abscesses, tenosynovitis, osteomyelitis, pneumonia and peritonitis [6,7]. In the new-build Glasgow hospital, cases of bacteraemia in paediatric haemato-oncology patients with *C. pauculus* and other Gram-

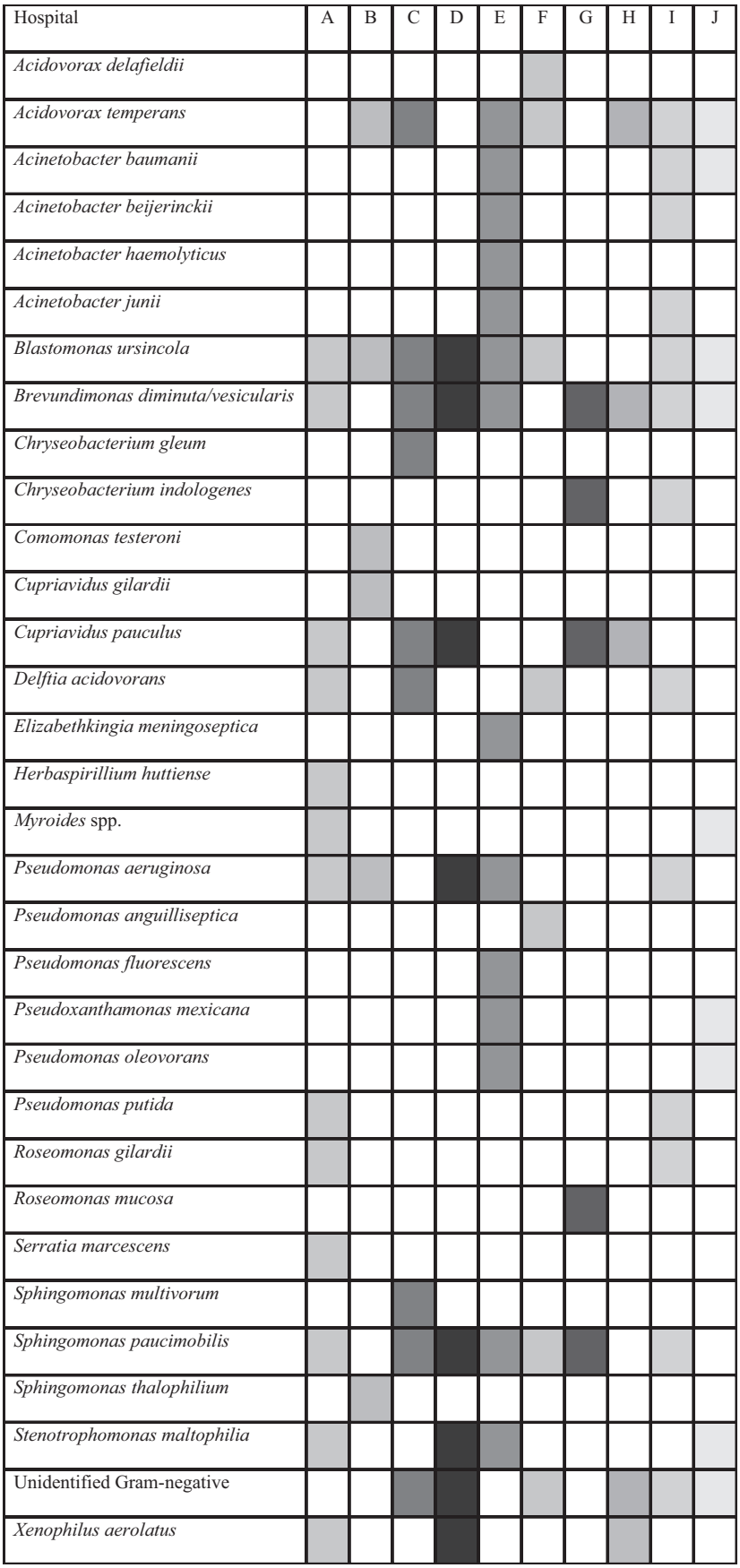

Figure 3. Presence of environmental Gram-negative organisms at study hospitals.

negative organisms were associated with a contaminated water supply [4]. Seventy-five of 98 outlets from the haemato-oncology unit tested positive for *C. pauculus* (76.5%), and the counts exceeded 100 cfu/100 mL in 37 cases [4]. Outlets tested positive in wards throughout the children's and adult hospitals. The organism was detected further back within the water system, with pre- and post-filtration storage tanks, risers and expansion vessels testing positive for *Cupriavidus* spp. and other OPPPs [4]. However, *Cupriavidus* spp. were not detected in mains water samples. Investigation into why the contamination occurred revealed numerous factors including incorrect selection of hospital water system components, and inadequately managed construction and commissioning processes which provided an environment conducive to biofilm formation [4,8].

This study aimed to ascertain whether cupriavidus is commonly found in hospital water systems. The results indicate that the organism is also present in hospital water elsewhere in the UK, although both the numbers of affected outlets and concentrations of *Cupriavidus* spp. were significantly less than found during the Glasgow incident. Moreover, the organism was predominantly detected at the periphery of water systems. All hospitals tested had evidence of other OPPPs or environmental Gram-negative pathogens in the water. The most common isolates were *Acidovorax* spp., *B. ursincola* and *Brevundimonas* spp.; these organisms have been reported in previous studies investigating hospital water system biofilms [9,10]. The present authors were unable to find any published reports of clinical infection due to *B. ursincola* or *Acidovorax* spp. *Blastomonas* spp. have been detected in hospital water systems that have been treated, suggesting they are relatively resistant to chlorination and chlorine dioxide [10]. *Brevunidmonas* spp. are rare opportunistic pathogens that have been linked to bacteraemia, liver abscesses, peritoneal dialysis-associated peritonitis, septic arthritis, endocarditis and keratitis [11]. *Sphingomonas* spp. are also uncommon causes of infection, but have been linked to prosthetic valve endocarditis, spondylodiscitis and bacteraemia [12—14].

Clinical isolates of *C. pauculus* remain rare in the UK. Data obtained from Public Health England showed 14 cases of bacteraemia in a 6-year period (2015—2020) with no evidence of clustering. During the same time frame, there were 12 cases of bacteraemia reported in Scotland, which included the three cases linked to the Glasgow outbreak. Outbreak cases aside, the incidence would appear to be higher in Scotland, given that its population is approximately one tenth of that of England. It is not clear whether this is a true increased incidence or could reflect differences in case ascertainment (i.e. is it possible that laboratories were detecting the organism but dismissing *Cupriavidus* spp. environmental contaminants?).

Based on the antibiotic susceptibility testing results for *Cupriavidus* spp., it was not considered possible to develop a selective culture medium based upon antimicrobial incorporation. Of the media that we investigated, only TSA reliably supported growth of *Cupriavidus* spp. It is important to note that some isolates of *X. aerolatus* were misidentified as *Cupriavidus* spp. using VITEK2. *X. aerolatus* is a rarely reported Gram-negative organism first identified from air samples in South Korea [15]. The present authors only found one published case report of a clinical case of infection with this bacterium, which was a child with peritoneal dialysis-associated peritonitis [15], and did not find any reports of isolation of *X. aerolatus* from water samples.

Based on the authors' experience during the Glasgow outbreak, and the findings of this study, TSA agar is recommended for laboratories wishing to isolate *Cupriavidus* spp. in water systems. Cultures should be maintained for 48 h, and isolates should be investigated by MALDI-TOF rather than VITEK2.

No interpretative criteria exist for determining the antibiotic susceptibilities of *Cupriavidus* spp., and previous studies have used interpretative criteria for *P. aeruginosa* [16]. All five isolates in the present study were sensitive to co-trimoxazole and minocycline. These findings broadly accord with the findings of Massip *et al.*, who undertook susceptibility testing on 34 clinical isolates of *Cupriavidus* spp. [16] and concluded that meropenem and aminoglycosides were unreliable, and minocycline and cefepime exhibited the best in-vitro activity. Sixty-two percent of their isolates were co-trimoxazole-susceptible, whereas all five of the isolates in the present study were susceptible. Clinical cases of *Cupriavidus* spp. bacteraemias and other infections have been treated successfully with ceftazidime, cefipime, tazocin and ciprofloxacin [17,7,18].

Currently in Scotland, water testing is recommended for *Legionella* spp. as per the code of practice approved by the Health and Safety Executive, and for *P. aeruginosa* specifically in augmented care units (at least 6 monthly) [1,2]. A separate 2019 document advises that if there may be an association between infection and water or water-related equipment, consideration should be given to environmental sampling [19]. Given that *Cupriavidus* spp. is not associated with endogenous carriage, and published literature to date has only established water as a source, there may be a strong argument for testing water when even a single patient case is detected, without waiting for further linked cases. Indeed, in the Glasgow incident, the three cases were separated by several months. The infrequency of cases, even in an outbreak setting, likely reflects that specific host factors are necessary for the development of infection.

The main limitation of this study was that only a small number of UK hospitals submitted water for testing. Nevertheless, *Cupriavidus* spp. were found in hospital water systems elsewhere in Scotland and the UK. Although *Cupriavidus* spp. were mainly found in the peripheries of systems, this observation may have been subject to sampling bias.

In conclusion, it is recommended that *Cupriavidus* spp. should be classed as alert organisms to act as a stimulus for water testing in the event of a patient having healthcare-associated infection with these bacteria. Consideration might also be given to water testing following infection with other rare and unusual waterborne pathogens, such as *Delftia acidovorans*, *Sphingomonas* spp., *Brevunidomonas* spp., *Comamonas* spp. and *Elizabethkingia* spp. [19]. However, further work is required to link clinical isolates of these bacteria to environmental sources, and to determine optimum methodologies for recovery of these bacteria from water systems.

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Conflict of interest statement
None declared.

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Gut to lung translocation and antibiotic mediated selection shape the dynamics of *Pseudomonas aeruginosa* in an ICU patient

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Bacteria have the potential to translocate between sites in the human body, but the dynamics and consequences of within-host bacterial migration remain poorly understood. Here we investigate the link between gut and lung *Pseudomonas aeruginosa* populations in an intensively sampled ICU patient using a combination of genomics, isolate phenotyping, host immunity profiling, and clinical data. Crucially, we show that lung colonization in the ICU was driven by the translocation of *P. aeruginosa* from the gut. Meropenem treatment for a suspected urinary tract infection selected for elevated resistance in both the gut and lung. However, resistance was driven by parallel evolution in the gut and lung coupled with organ specific selective pressures, and translocation had only a minor impact on AMR. These findings suggest that reducing intestinal colonization of *Pseudomonas* may be an effective way to prevent lung infections in critically ill patients.

Bacteria often colonise multiple anatomical sites in human hosts, but the dynamics of within-host translocation and its consequences for pathogenesis and host adaptation remain poorly understood[1–3]. For example, advances in microbiome profiling methods have shown that the gut microbiome can transmit to the lungs of critically ill patients[4,5], and translocation is associated with poorer outcomes in mechanically ventilated patients[6]. While gut-to-lung translocation has been demonstrated at the microbiome level, the dynamics and consequences of translocation for individual pathogens and antibiotic resistance are not well understood.

P. aeruginosa is an opportunistic pathogen that is a major cause of healthcare-associated infections worldwide[7,8], most notably in patients with compromised immunity[9,10]. *Pseudomonas* is not considered to be a typical member of the gut microbiome, and intestinal colonisation with *Pseudomonas* is associated with an increased risk of developing lung infections[11–13] and mortality[14]. Gut colonisation usually precedes lung infection, and the same strain is often found in the gut and lungs, suggesting that the gut acts as a reservoir of *Pseudomonas* that can be transmitted to the lung and other infection sites[15–17]. However, direct evidence for gut-to-lung transmission of

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P. aeruginosa is lacking, and it is possible that intestinal carriage simply reflects an innate susceptibility to *Pseudomonas* infection or proximity to a source of *Pseudomonas* that can independently colonise the lung and gut.

One of the major challenges of dealing with *Pseudomonas aeruginosa* is antibiotic resistance. *P. aeruginosa* has high levels of intrinsic resistance to antibiotics[18–20] and a remarkable ability to evolve resistance de novo in hospitalised patients[21–23]. In classic population genetic models, migration can accelerate evolutionary adaptation at a local scale by increasing the genetic diversity that selection acts on[24]. In this case, translocation could play a role in antibiotic resistance by moving resistance determinants between bacterial colonisation sites. In an extreme example, bacterial populations in one organ (for example, the gut) could act as a source of resistant mutants that are then disseminated to other organs (i.e. the lung).

To test the importance of gut-to-lung transmission in *Pseudomonas* colonisation and antimicrobial resistance (AMR), we carried out an in-depth case study on a single intensively sampled ICU patient over a 30-day period. We used phylogenetic approaches to test for translocation, and a combination of genomic and phenotypic methods to study the link between AMR and within-host transmission.

Results

Clinical timeline

The focal patient was admitted to ICU of Hospital Universitari Germans Trias i Pujol in Badalona, Spain with a primary diagnosis of seizure. Mechanical ventilation was started on ICU admission and was continued for a total duration of 39 days. The patient was immediately treated with amoxicillin clavulanate, which is not active against *P. aeruginosa*, due to suspected aspiration of oropharyngeal or gastric contents into the lower respiratory tract (bronchoaspiration). The patient was enrolled in ASPIRE-ICU trial[25] at 48 h post admission (hereafter day 1). Meropenem was started on day 12 and continued for 10 days to treat a suspected urinary tract infection. Over the course of stay in ICU, a total of 52 *P. aeruginosa*

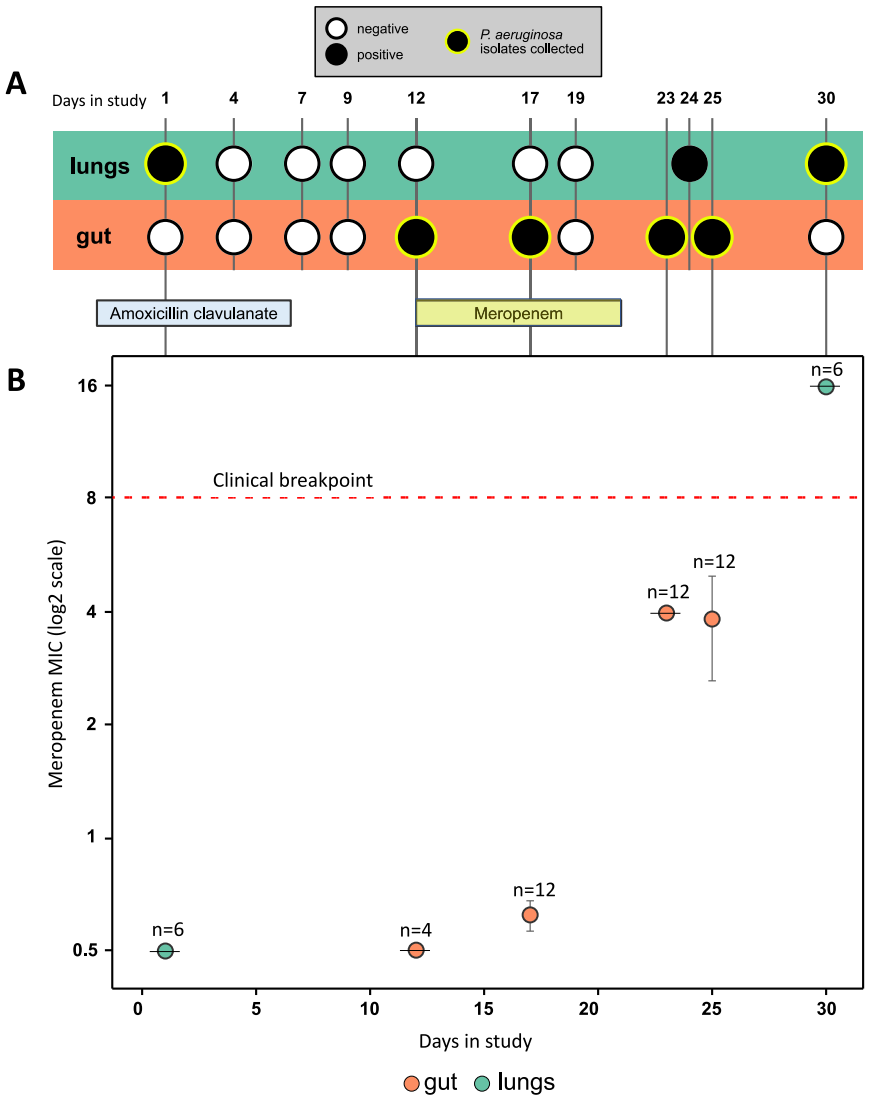

Fig. 1 | Clinical timeline and resistance phenotyping. A Timeline of patient sampling, showing samples that tested positive or negative for *P. aeruginosa* colonisation by culturing. Sampling points from which isolates were collected are highlighted with a green ring. The patient was treated with amoxicillin clavulanate from 2 days prior to enrolment until day 6 and with meropenem from day 12 to day 21. **B** Meropenem minimum inhibitory concentration (MIC) (mean ± s.e.m μg/mL) for isolates ($n = 4–12$, as labelled on plot) from each sampling point from the gut (orange) and lung (green). Each isolate had a median meropenem MIC calculated from $n = 3$ biologically independent replicates. Meropenem resistance increased over time, and *P. aeruginosa* isolates from the final lung sample were above the EUCAST clinical breakpoint for meropenem resistance (red dashed line). Amoxicillin clavulanate resistance was not measured as this antibiotic is not active against *P. aeruginosa*. Source data are provided as a Source Data file.

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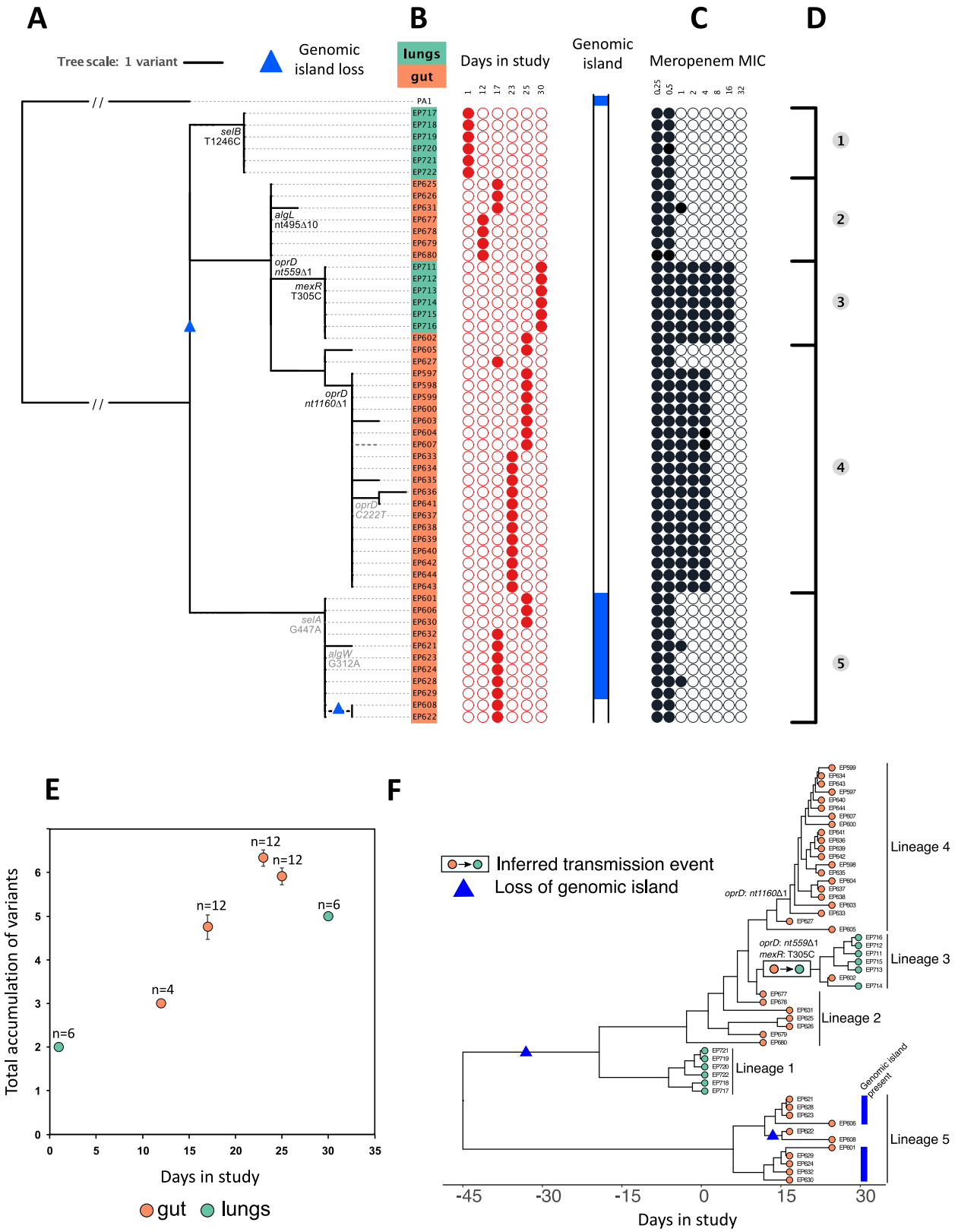

isolates were collected, from endotracheal aspirate (ETA) samples ($n = 12$) and peri-anal swabs ($n = 40$) up until day 30 (Fig. 1), which was the end-point of the ASPIRE-ICU trial. Culture screening of patient blood samples from day 2, day 11 and day 21 all reported no growth of *P. aeruginosa*. *P. aeruginosa* colonisation was detected in the lungs at day 1. Gut colonisation was detected following meropenem treatment, and meropenem resistant *P. aeruginosa* ultimately colonised the lung. This complex clinical timeline suggests that translocation between the gut and lung may have occurred (Fig. 1), but clinical data and isolate phenotypes alone provide limited insights into the underlying drivers of within-host translocation and AMR.

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Fig. 2 | Genome sequencing and phylogenetic analysis. A Phylogenetic reconstruction of lung ($n = 12$) and gut ($n = 40$) isolates rooted using *P. aeruginosa* PA1, another ST782 clinical isolate sampled from a respiratory tract infection[72], as the outgroup. Putatively adaptive polymorphisms in genes or pathways showing parallel evolution are annotated on the phylogeny. Protein altering mutations are shown in black and silent mutations are shown in light grey. A polymorphism in a known multi-drug efflux pump regulator (*mexR*) is also highlighted. Variation in the presence/absence of a 190kB genomic island is shown, and inferred losses of the genomic island are identified with blue triangles in the tree. **B** Isolate name, lung (green) or gut (orange) origin, and day in study of collection. **C** Susceptibility to meropenem for each isolate is presented with filled black circles in $\log_2$ scale of the minimum inhibitory concentration (MIC). **D** The topology of the tree suggested five distinct groups based on the identification of small polymorphisms. **E** The accumulation of variants over time (mean ± s.e for isolates ($n = 4$–12, as labelled on plot) from each sampling point from the gut (orange) and lung (green) suggests within-host evolution of a clone rather than recurrent episodes of colonisation. **F** Dated genealogy of isolates constructed with BactDating[73]. The inferred instance of gut to lung transmission and the acquisition of meropenem resistance mutations have been annotated on the genealogy. Source data are provided as a Source Data file.

Genomic insights into pathogen colonisation and evolution

To characterise the genetic diversity within this patient, we used long and short read sequencing to construct a hybrid assembly for a single isolate, yielding a ~6.3 Mb ST782 reference genome distributed across 5 contigs. Short-read sequences of lung ($n = 12$) and gut ($n = 40$) isolates were mapped to this reference genome, and we identified polymorphic SNPs ($n = 17$), indels ($n = 7$), and variation in presence/absence of a 190 kb genomic island (Supplementary Table 1 and Supplementary Fig. 1).

The genetic diversity found in this patient could reflect either (i) recurrent colonisation/infection by multiple clones or (ii) within-host evolution of a single clone. To discriminate between these processes, we reconstructed the phylogeny of isolates using *P. aeruginosa* PA1, a closely related ST782 genome, as an outgroup (Fig. 2A–D). All of the isolates were closely related and the number of variants per isolate correlated strongly with the day of isolate collection, supporting the idea that within-patient diversity emerged as a result of in situ evolution of a single founding clone (Fig. 2E; $r^2 = 0.62$, $F_{1,50} = 82$, $P < 0.0001$). No other patients within the ASPIRE-ICU cohort at this hospital were colonised by *P. aeruginosa* ST782 during the trial, providing further support for the within-host diversification as opposed to repeated colonisation.

We estimated the onset of colonisation using a time-scaled genealogy of isolates with the most-recent common ancestor (MRCA) of all isolates predicted to be at least 3 weeks prior to ICU admission (Fig. 2F; Supplementary Fig. 2; MRCA from BactDating: 22–74 days before day 0)[26].The rate of evolution in this patient was ~18 ± 10 SNPs/year[26], which is higher than the typical evolutionary rate of bacterial pathogens of 1–10 SNPs/year[3]. However, this elevated evolutionary rate is in line with the rate reported from another patient in this trial[23], highlighting the high in vivo mutation rate of *P. aeruginosa* in critically ill patients. This phylogenetic approach, which strongly supports continuous host colonisation, suggests that culture-based approaches (i.e. Fig. 1A) have limited ability to detect *P.aeruginosa* colonisation.

Signatures of parallel evolution provide a simple way to identify putative beneficial mutations that underpin adaptation to the novel environment of the human host[3,27]. Parallel evolution occurred in 3 genes or operons that have functional roles in resistance to carbapenem antibiotics (*oprD*)[27], alginate biosynthesis (*algW, algL*)[28], and selenocysteine biosynthesis (*selA and selB*)[29]. These putative pathoadaptive mutations accounted for 7 of the 24 variants, providing strong evidence for rapid adaptation to the host environment. Interestingly, 3 of these 7 mutations were synonymous, suggesting that transcription efficiency may have been a key target of selection[30]. The presence of the variable genomic island in the outgroup and in lung isolates (Fig. 2, lineage 5) implies that island was lost on two independent occasions, suggesting that loss of this element was adaptive. Inferring the selective advantage of large scale deletions is difficult, but it is worth noting that this island carries pyoverdine biosynthesis genes that are selected against in the host environment[31]. Isolates in possession of the genomic island showed similar levels of meropenem resistance (~0.5 µg/mL MIC) to isolates from the same phylogenetic lineage (lineage 5) with loss of the genomic island (Fig. 2), suggesting that the loss of this island was not driven by antibiotic treatment.

Bacterial phylogenies are a powerful tool to detect transmission events, particularly when combined with isolate sampling dates[3,32,33]. To reconstruct within-host translocation, we used our time scaled isolate genealogy to infer translocation events (Fig. 2F). Secondary lung colonisation was driven by the growth of a clone with mutations in the *oprD* porin, which is a key carbapenem sensitivity determinant, and *mexR*, which regulates the expression the MexAB-OprM multidrug efflux pump[34] (Fig. 2A – lineage 3). This lineage is nested within a broader clade of gut isolates, providing strong evidence of gut to lung transmission (Fig. 2F). The dated genealogy suggests the MRCA of lineage 3 existed between day 18 and day 24 (Supplementary Fig. 2)[26], giving an approximate time frame for gut to lung translocation. However, this genealogy does not provide any insight into whether lineage 3 acquired the carbapenem resistance mutations before or after transmission to the lung. Interestingly, a single lineage 3 isolate was recovered from the gut. The presence of this lineage in the lung and gut implies that either meropenem resistance evolved in the gut prior to transmission to the lung, or that this lineage secondarily transmitted from the lung to the gut after evolving meropenem resistance in the lung.

Beyond this clear-cut case of gut to lung translocation, inferred patterns of translocation depend strongly on the assumptions made regarding initial colonisation by the ancestral clone. If *Pseudomonas* initially colonised the gut, then the phylogeny implies that initial lung colonisation by lineage 1 was driven by an earlier gut-to-lung translocation event that could have been associated with broncoaspiration upon ICU admission. Alternatively, the ancestral clone may have colonised the lungs. Under this model of host colonisation, the phylogeny suggests that lung to gut transmission occurred in lineages 2 and 5, implying that at least one episode of translocation occurred at some point prior to ICU admission. Finally, it is possible that the ancestral clone independently colonised both the lungs and gut. According to this model of host colonisation, it is not necessary to invoke any additional translocation (beyond the clear gut to lung translocation in lineage 3) in order to explain the phylogenetic distribution of lung and gut isolates. Unfortunately, it is not possible to clearly differentiate between these models because the lack of pre-ICU admission isolates hinders our ability to reconstruct the early history of *Pseudomonas* colonisation in this patient. All three models of host colonisation lead to a similar number of inferred translocation events (1–3), suggesting that they are similarly parsimonious from a phylogenetic perspective. Culturing patient samples found evidence of lung colonisation prior to gut colonisation (Fig. 1A), but we argue that this culture data provides limited insights into early host colonisation, which likely occurred >20 days prior to ICU admission. A further challenge of using culture data to infer host colonisation is that the limits of detection from culturing samples from different tissues are unknown, and it is conceivable that ETA samples and peri-anal swabs simply differ in their sensitivity to detect *Pseudomonas* in the lung and gut.

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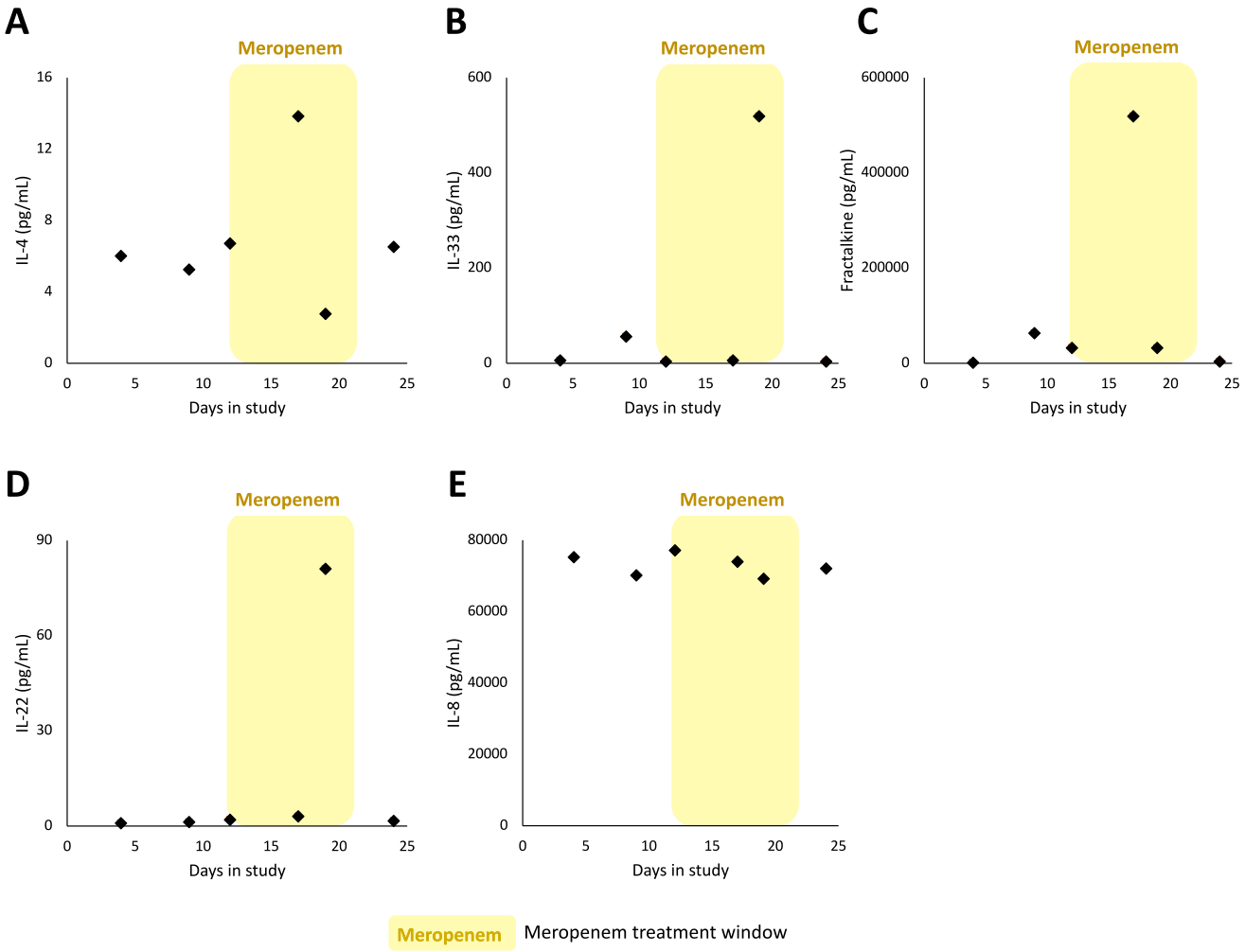

Fig. 3 | Cytokine concentrations were measured in ETA samples collected over the course of the study at days 4, 9, 12, 17, 19 and 24. The following cytokines were measured: **A** IL-4, **B** IL-33, **C** Fractalkine, **D** IL-22, **E** IL-8. Yellow shading indicates meropenem treatment window on timeline (day 12 to day 21). Source data are provided as a Source Data file.

Immune response to lung colonisation

Lung colonisation provides *P. aeruginosa* with the opportunity to establish infection by adhering to the mucosal surface and penetrating the epithelial barrier, leading to the development of pneumonia, which is associated with a very high mortality rate in ICU patients[7]. To investigate the role of host immunity in preventing infection, we measured the abundance of a panel of host immune effectors in samples of endotracheal aspirate (ETA; Fig. 3). The dated genealogy suggests that secondary lung colonisation by lineage 3 occurred between day 18 and day 24. Interestingly, ETA samples from days 17 and 19 were associated with spikes in the expression of IL-33, Fractalkine, and IL-4[35,36], which have previously been to shown to enhance the clearance of *P. aeruginosa*[37]. Colonisation was also associated with a > 10-fold increase in the concentration of IL-22 (day 19 sample), which protects against infections caused by attaching and effacing bacterial pathogens by increasing mucous production and by limiting excessive inflammation mediated by neutrophil influx[38,39]. Measuring cytokine levels in ETA samples from the lungs provides a direct measurement of immune response, but one concern over this approach is that it is possible for individual samples to give high concentrations of all cytokines, for example as a result of patient dehydration. However, in this case levels of IL-8 remained essentially constant across samples, supporting the idea that spikes of protective cytokines were not an artefact (Fig. 3E). These cytokine data provide further support of the idea that secondary lung colonisation occurred at some point between day 12 and day 17, and they suggest that the host immune response may have prevented colonisation from progressing to pneumonia.

Drivers of antibiotic resistance

P. aeruginosa has high levels of intrinsic antibiotic resistance and a remarkable ability to evolve increased resistance under antibiotic treatment[18,21]. Given the possibility of translocation between the gut and lung, we next sought to understand the relative contributions of migration, mutation and selection to the origin and spread of meropenem resistance in this patient. The phylogeny clearly shows that elevated meropenem resistance evolved on 2 separate occasions due to mutations in *oprD* and *mexR* (Fig. 2, lineages 3 and 4). However, it is challenging to follow the dynamics of meropenem resistance mutations using isolates alone due to the limited number of isolates sequenced ($n = 52$) and the gaps in the sampling of isolates. To try and overcome this problem, we combined isolate sequencing data with amplicon sequencing of *oprD* using DNA extracted directly from ETA samples and peri-anal swabs, some of which were not screened for isolates according to ASPIRE-ICU protocol (Fig. 4A, B). Amplicon sequencing revealed that *Pseudomonas* was present in the lung from day 17 onwards, which coincides with the inferred time of secondary lung colonisation from the isolate phylogeny (Fig. 2F) and cytokine profiling (Fig. 3). *oprD* amplicon sequencing from DNA isolated from

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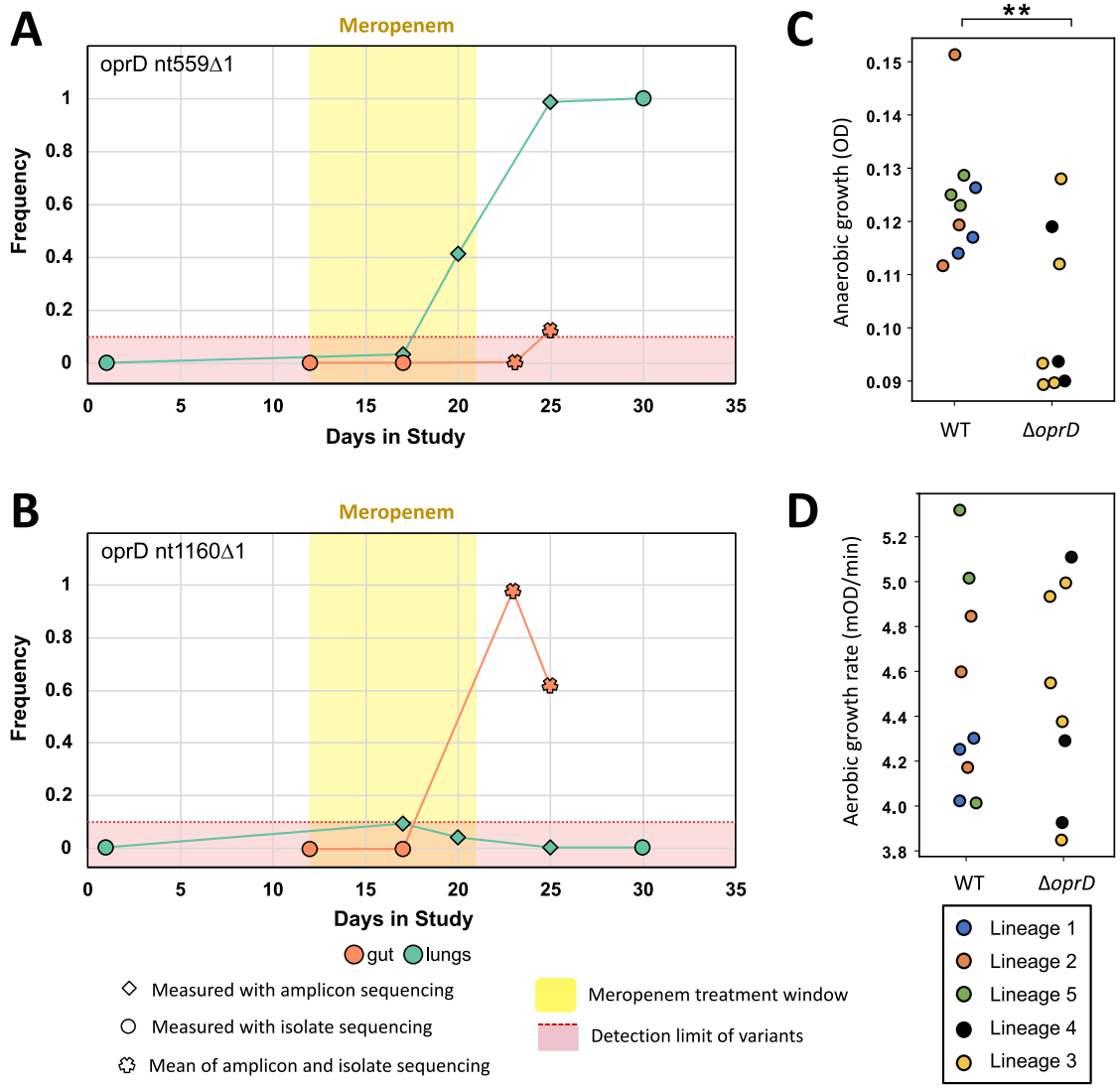

Fig. 4 | Evolution and transmission of meropenem resistance. A, B Dynamics of *oprD* variants, as determined by isolate (circle) and *oprD* amplicon (diamond) sequencing data. For the two sampling points (gut day 23 and gut day 25) where both amplicon and isolate sequencing was carried out, the mean of the two frequencies is shown (star). Measurements of SNP frequency from isolate sequencing and amplicon sequencing were strongly correlated ($R^2 = 0.9963$; Supplementary Fig. 3). The yellow area shows the window of meropenem treatment and the red area shows a conservative minimum detection limit of variants from amplicon sequencing due to the error rate of nanopore sequencing. **C**, **D** Growth of isolates with an o*prD* variant (Δ o*prD*) compared to isolates with the wild-type *oprD* background. Anaerobic growth (**C**) was measured as OD_{595} after 72 hours growth in anaerobic broth. Values plotted for each point (i.e. isolate) are calculated from $n = 3$ biologically independent replicates (Source Data), and at least three isolates from each lineage were measured. Aerobic growth (**D**) was measured as exponential growth rate in standard culture conditions. Isolates are colour coded according to phylogenetic lineage, as defined in Fig. 2. Values plotted for each point (i.e. isolate) are calculated from $n = 7$ biologically independent replicates (Source Data), and at least three isolates from each lineage were measured. *oprD* mutations were associated with impaired growth under anaerobic conditions ($P = 0.010$), but not aerobic conditions ($P = 0.950$), as judged by a nested ANOVA. Data from isolates from different lineages are shown together because fitness measures did not differ between lineages nested within *oprD* genotype ($P > 0.5$). All statistical tests for this analysis are two-tailed. Source data are provided as a Source Data file.

peri-anal swabs was less successful for reasons that are not clear, and we only obtained amplicon sequencing data from days 23 and 25. Importantly, the frequency of *oprD* variants measured by amplicon and isolate sequencing from samples that yielded both isolates and *Pseudomonas* DNA was essentially identical (Supplementary Fig. 3), validating the use of amplicon sequencing to measure changes in allele frequency.

The most common *oprD* mutation in sequenced isolates (nt1160Δ1) arose in the main clade of intestinal isolates (Fig. 2, lineage 4) and was never convincingly detected in lung samples (Fig. 4B). This combination of isolate and amplicon sequencing results provides strong evidence that this mutation arose in the gut and swept to near fixation under meropenem treatment. The frequency of this mutation ultimately declined in the gut due to the expansion of a carbapenem sensitive lineage (Fig. 2A – lineage 5). We speculate that this lineage may have survived carbapenem treatment by either colonising a region of the gut with low carbapenem toxicity or by forming persister cells.

Isolate sequencing revealed a second frameshift mutation in *oprD* (nt559Δ1) that was linked to a mutation in the *mexR* effux pump transcriptional regulator. Isolate sequencing revealed the presence of the *oprD* nt559Δ1/*mexR*T305C lineage in gut samples from day 25 and lung samples from day 30, providing strong evidence that this resistant lineage transmitted between colonisation sites. Amplicon

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sequencing first detected this mutation as a polymorphism (frequency approx. 20%) in the lung at day 20 (Fig. 4A), suggesting that this mutation arose in the lung following the transmission of lineage 3 from the gut. According to this model, the presence of rare *oprD* nt559Δ1 variants (in isolates and amplicons) at day 25 reflects secondary lung to gut transmission. However, we emphasise that it is not possible to completely exclude the possibility that *oprD* nt559Δ1 arose in the gut and then transmitted to the lung where it spread rapidly by positive selection.

Antibiotic concentrations vary between host tissues, and it is unclear to what extent pathogen populations adapt to local variation in selective pressures associated with antibiotic treatment. Meropenem achieves higher concentrations in lung tissues than in the gut[40], suggesting that selection for resistance is stronger in the lung than in the gut. Isolates from the lung-associated *oprD* nt559Δ1/*mexR*T305C lineage had higher meropenem resistance (MIC = 16 µg/mL, s.e. = 0 µg/mL, $n = 7$) than isolates from the gut-associated *oprD* nt1160Δ1 lineage (MIC = 4 µg/mL, s.e. = 0 µg/mL, $n = 19$; Fig. 2C), which is consistent with the idea that selection for meropenem resistance varies between organs.

A key challenge in evolutionary studies of AMR is to understand how resistance can be maintained in pathogen populations in the absence of continued antibiotic use[41,42]. In this case, *oprD* nt559Δ1 resistance remained stable in the lung following antibiotic treatment, but the frequency of *oprD* nt1160Δ1 declined in the gut. To test the role of selection in the stability of resistance, we measured the growth rate of isolates of all 5 major phylogenetic lineages in anaerobic culture medium (Fig. 4C) and aerobic culture medium (Fig. 4D), which recapitulates one of the physiological differences between the gut and lung. Meropenem resistant lineages (Δ *oprD*) were not associated with decreased growth rate under aerobic conditions, suggesting that the *oprD* and *mexR* mutations have little, if any, associated costs under these conditions (Fig. 4D; $F_{1,12} = 0.0032$, $P = 0.956$). In contrast, both meropenem lineages were associated with decreased growth under anaerobic conditions, suggesting that fitness costs associated with *oprD* mutations drove the loss of resistance in the gut (Fig. 4C; $F_{1,12} = 9.27$, $P = 0.010$).

Discussion

The goal of this project was to understand the link between gut and lung *Pseudomonas* colonisation in a single patient. By combining clinical and genomic data, we were able to demonstrate a clear cut case of gut to lung transmission while the patient was in ICU. Whilst it is difficult to generalise the findings of a single case study, these findings support the idea that gut to lung transmission may be a major driver of *P. aeruginosa* respiratory tract colonisation in critically ill patients[11,12,43].

Carbapenem antibiotics such as meropenem are key to the treatment of *P. aeruginosa* infections[23,44,45], and carbapenem-resistant *P. aeruginosa* has been identified as an important threat by the World Health Organisation and the Centers for Disease Control and Prevention. In this patient, meropenem treatment for a suspected urinary tract infection drove the repeated evolution of resistance, providing a poignant example of the importance of 'bystander selection' for AMR[46]. Ultimately, selection led to the emergence of a population of highly resistant bacteria that persisted in the lung in the absence of antibiotic treatment, suggesting that the respiratory tract may act as a source of carbapenem resistant *Pseudomonas* that can transmit to other body sites and potentially to other patients. Isolate sequencing, amplicon sequencing and immunological profiling all support the idea that gut to lung translocation coincided with meropenem treatment. This association may have arisen due to chance, but it is also possible that antibiotic treatment facilitated gut to lung transmission, for example by eliminating commensal lung bacteria that protected against *Pseudomonas* colonisation.

Migration increases genetic variation[24], suggesting that within-host translocation may accelerate bacterial adaptation to antibiotics[1–3,47–50]. In this case, resistance was driven by the spread of independent lineages in the gut and lung that were adapted to local differences in antibiotic concentration. We found some evidence of translocation of resistant lineages, but the impact of within-host migration on resistance was weak compared to selection, leading to the emergence of a highly structured meropenem resistant pathogen population[1–3,49]. We speculate that the high in vivo mutation rate of *Pseudomonas* was key to shaping local adaptation to antibiotic selection across tissues, and that within-host transmission is likely to provide a more important source of resistance at smaller spatial scales[2], or when mutation rate is low.

Hospital acquired infections caused by epidemically successful MDR and XDR strains of *P. aeruginosa* have become a serious problem worldwide[19], and there is an urgent need to develop new antibiotics to treat infections caused by these strains. At the same time, the incredible ability of *Pseudomonas* to evolve resistance to antibiotic treatment[18,21,23,47] highlights the need to develop novel approaches to prevent or treat *Pseudomonas* infections. Our study suggests that preventing gut colonisation or gut to lung transmission may be an effective strategy for preventing *Pseudomonas* infection in critically ill patients[51–54].

Methods

Clinical timeline

The patient was admitted to the intensive care unit (ICU) of Hospital Universitari Germans Trias i Pujol in Badalona, Spain with a primary diagnosis of seizure. This patient was recruited as part of an observational multicenter European epidemiological cohort study (ASPIRE-ICU[25]), which was conducted according to the principles of the Declaration of Helsinki, in accordance with the Medical Research Involving Human Subjects Act and local guidelines in the participating countries. The study protocol was approved by the Research Ethics Committee of the Germans Trias i Pujol University Hospital and participants gave written informed consent.

At the time of ICU admission, the patient did not suffer from pneumonia or any other active *P. aeruginosa* infection (APACHE-II score = 22 and Glasgow Coma scale = 3). No antibiotic use was reported in the two weeks prior to hospital admission. After 48 h of ICU admission, informed consent was obtained and the patient was enrolled in the ASPIRE-ICU study (day 1)[25]. Mechanical ventilation was started on ICU admission and was continued for a total duration of 39 days. Amoxicillin/clavulanic acid (1000 mg IV q8h for 8 days) was started on ICU admission for bronchoaspiration, the suspected inhalation of oropharyngeal or gastric contents into the lower respiratory tract. Meropenem (1000 mg IV q8h for 10 days) was started on day 12 to treat a suspected urinary tract infection. Patient endotracheal aspirate (ETA) and peri-anal swab samples were collected and screened for *P. aeruginosa* isolates via selective plating until day 30[23]. Patient ETA samples were first blended (30,000 rpm, probe size 8 mm, steps of 10 s, max 60 s in total), diluted 1:1 v/v with Lysomucil (10% Acetylcysteine solution) (Zambon S.A, Belgium) and incubated for 30 min at 37 °C with 10 s vortexing every 15 min. Selective plating to screen for *P. aeruginosa* was carried out using CHROMID *P. aeruginosa* Agar (BioMérieux, France) and blood agar (BBL®Columbia II Agar Base (BD Diagnostics, USA) supplemented with 5% defibrinated horse blood (TCS Bioscience, UK)). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to identify up to 12 *P. aeruginosa* colonies per sample, which were stored at −80 °C until further use. This resulted in a total of 52 P. aeruginosa isolates collected from endotracheal aspirate samples ($n = 12$) and peri-anal swabs ($n = 40$). In addition, patient blood cultures from day 2, day 11 and day 21 were screened at the local laboratory, all reported no growth (negative for

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P. aeruginosa). The patient was discharged from the ICU and transferred to a general medical ward on day 41.

Resistance phenotyping

All isolates were grown from glycerol stocks on Luria-Bertani (LB) Miller Agar plates overnight at 37 °C. Single colonies were then inoculated into LB Miller broth for 18–20 h overnight growth at 37 °C with shaking at 225 rpm. Overnight suspensions were serial diluted to ~5×10^5 CFU/mL. Resistance phenotyping to meropenem was carried out as minimum inhibitory concentration (MIC) testing via broth microdilution as defined by EUCAST recommendations[55,56], with the alteration of LB Miller broth for growth media and the use of *P. aeruginosa* PAO1 as a reference strain. Resistance to meropenem was assayed along a 2-fold dilution series between 0.25–64 µg/mL. We defined growth inhibition as $OD_{595} < 0.200$ and we calculated the MIC of each isolate as the median MIC score from three biologically independent assays of each isolate (Source Data).

Growth assays

P. aeruginosa isolates were grown from glycerol stocks on LB Miller Agar plates overnight at 37 °C. Single colonies were then inoculated into LB Miller broth for 18–20 h overnight growth at 37 °C with shaking at 225 rpm. Overnight suspensions were serially diluted to an OD_{595} of ~0.05 within the inner 60 wells of a 96-well plate equipped with a lid. To assess growth rate under standard aerobic conditions, isolates were then grown in LB Miller broth at 37 °C and optical density (OD595 nm) measurements were taken at 10-min intervals in a BioTek Synergy 2 microplate reader set to moderate continuous shaking. Growth rate (Vmax; mOD/min) was calculated as the maximum slope of OD versus time over an interval of ten consecutive readings, and we visually inspected plots to confirm that this captured log-phase growth rate. We measured the growth rate of all 52 gut and lung isolates with a minimum of seven biological replicates to assess the relationship between meropenem resistance and fitness (Source Data). Throughout growth assays a media control (to control for contamination) and a PAO1 control (to control for replicate plates) were included. We measured anaerobic growth using an anaerobic jar (Thermo Scientific™ Oxoid™ AnaeroJar™ base jar) system with anaerobic gas generating sachets (Thermo Scientific™ Oxoid™ AnaeroGen™ sachets). An Oxoid Resazurin indicator strip was placed in the jar as an indicator to confirm generation of an anaerobic environment. For growth measurements, single colonies were inoculated into LB Miller broth in the wells of a 96-well plate and placed in the anaerobic jar for 72 h, after which plates were removed and OD_{595} was measured. For the comparison of o*prD* variant (Δ o*prD*) isolates to wild-type *oprD* background (WT *oprD*) isolates, growth measurements were taken for a minimum of three isolates (and a minimum of three biological replicates) selected as representatives from each phylogeny group to generate a mean growth measurement for each Δ o*prD* and WT *oprD* group. To test for an association between *oprD* mutations and impaired growth we used a nested ANOVA that included main effects of *oprD* (ie either WT or Δ o*prD*, 1 df) and phylogenetic lineage nested within *oprD* (5 lineages shown in Figs. 2 and 3 df).

Illumina sequencing

All isolates were sequenced in the MiSeq or NextSeq illumina platforms yielding a sequencing coverage of 21X–142X. Raw reads were quality controlled with the ILLUMINACLIP (2:30:10) and SLIDINGWINDOW (4:15) in trimmomatic v. 0.39[57]. Quality controlled reads were assembled for each isolate with SPAdes v. 3.13.1[58] with default parameters. These assemblies were further polished using pilon v. 1.23[59] with minimum number of flank bases of 10, gap margin of 100,000, and kmer size of 47. Resulting contigs were annotated based on the P. aeruginosa strain UCBPP-PA14[60] in prokka v. 1.14.0[61]. Each isolate was typed using the Pseudomonas aeruginosa multilocus sequence typing (MLST) scheme from PubMLST (last accessed on 11.06.2021)[62].

Long-read sequencing

Two isolates (EP717 (day 1 lungs) and EP623 (day 17 gut)) were sequenced using the Oxford nanopore MinION platform with a FLO-MIN106 flow-cell and SQK-LSK109 sequencing kit. EP717 had sequencing coverage of 141X and EP623 of 233X. Raw reads were basecalled using guppy v. 0.0.0 + 7969d57 and reads were demultiplexed using qcat v. 1.1.0 (https://github.com/nanoporetech/qcat). Resulting sequencing reads were assembled using unicycler v. 0.4.8[63], which used SAMtools v. 1.9[64], pilon v. 1.23[59], and bowtie2 v. 2.3.5.1[65], in hybrid mode with respective illumina reads. The EP717 assembly had a N50 of 1,797,327 for a total of 6,217,789 bases distributed in 11 contigs. The EP623 assembly had a N50 of 6,133,283 for a total of 6,330,243 bases distributed in 5 contigs.

Variant calling

To identify mutations and gene gain/loss during the infection, short-length sequencing reads from each isolate were mapped to each of the long-read de novo assemblies with BWA v. 0.7.17[66] using the BWA-MEM algorithm. Preliminary SNPs were identified with SAMtools and BCFtools v. 1.9. Low-quality SNPs were filtered out using a two-step SNP calling pipeline, which first identified potential SNPs using the following criteria: (1) Variant Phred quality score of 30 or higher, (2) At least 150 bases away from contig edge or indel, and (3) 20 or more sequencing reads covering the potential SNP position. In the second step, each preliminary SNP was reviewed for evidence of support for the reference or the variant base; at least 80% of reads of Phred quality score of 25 or higher were required to support the final call. An ambiguous call was defined as one with not enough support for the reference or the variant, and, in total, only one non-phylogenetically informative SNP position had ambiguous calls. Indels were identified by the overlap between the HaplotypeCaller of GATK v. 4.1.3.0[67] and breseq v. 0.34.0[68]. The maximum parsimony phylogeny was constructed based on high-confidence SNPs. To construct a dated genealogy of isolates, we dated the internal nodes of this tree using bactdate in BactDating v1.1.0[26] (updateRoot = TRUE, minbralen=0.1) after first converting multifurcating nodes to binary nodes. Phylogenies were plotted with ggtree v3.0.4[69].

The variable genome was surveyed using GenAPI v. 1.098[70] based on the prokka annotation of the short-read de novo assemblies. The presence or absence of genes in the potential variable genome was reviewed by mapping the sequencing reads to the respective genes with BWA v.0.7.17[66].

Amplicon Sequencing of oprD

Amplicon sequencing of the *oprD* gene was carried out to quantify the presence of the two key *oprD* variants observed in the isolate sequencing in whole gDNA samples that were available from the lung and gut of this patient. DNA was extracted from the ETA and peri-anal swab samples using a ZymoBIOMICS DNA Miniprep Kit (Zymo Research, CA USA).

On these extracted gDNA samples, a PCR amplification strategy using barcoded primers to amplify the *oprD* gene (1489 bp product length) and add sample specific DNA barcodes was followed[71]. This was performed with PCR in Q5 High-Fidelity Master Mix (New England BioLabs) and using a universal reverse primer and sample specific forwards primers containing 12 nt barcodes listed in Supplementary Table 2[71]. The temperature profile was 98 °C for 30 s, followed by 30 cycles of 98 °C for 10 s, 70 °C for 30 s, 72 °C for 40 s, followed by a final extension of 72 °C for 2 min. The barcoded *oprD* PCR products were pooled and sequenced on an Oxford nanopore MinION platform using a FLO-MIN106 flow-cell and the SQK-LSK109 Ligation Sequencing kit.

Amplicon sequencing raw reads were basecalled using guppy v. 0.0.0 + 7969d57. This yielded 163,766 reads with an estimated read length N50 of 2.64 kb. The data was demultiplexed allowing 2/12 sequencing errors in the barcode sequence and a maximum of 1 error in the downstream and upstream 4-mer. To identify the genotype of each read, we searched for the 11-mer sequence including the variant base and 5 bases downstream and upstream from this position. Using this conservative approach, we recovered 32–43% of the reads (Supplementary Table 3).

Cytokine measurements

After ETA was blended, 0.5 g of the sample was diluted 1:1 with Sputolysin (Merck, Overijse, Belgium), vortexed and incubated at room temperature for 15 min. Samples were then centrifuged for 5 min at 2000×*g* at room temperature. Supernatant was stored at −80 °C until further processing. Levels of interleukin (IL-)4, IL-33, IL-22, IL-8 and fractalkine were measured with the Mesoscale Discovery platform (Rockville, MD, USA) following the manufacturer's instructions. In brief, the plate was coated with capturing antibodies for 1 h with shaking incubation at room temperature followed by washing off the plate. Samples were loaded and incubated for 1 h, after which the plate was washed and incubated with detection antibodies. A final wash was performed and MSD reading buffer 2x was applied before reading the plate in the QuickPlex SQ 120 (Rockville, MD, USA).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All clinical data analyzed for this patient as part of the study are included in this article. Isolates can be obtained from the corresponding author for research use via an MTA subject to permission from the ASPIRE research committee. The source data are provided with this paper and have been deposited in the Oxford Research Archive for Data ("DOI: 10.5287/bodleian:r1ekRa9wE" [https://doi.org/10.5287/bodleian:r1ekRa9wE]).

All sequencing data generated in this study has been deposited in the NCBI short-read archive ("PRJNA802704") and all data on isolates can be found at: "SRR17868883", "SRR17868884", "SRR17868885", "SRR17868886", "SRR17868887", "SRR17868888", "SRR17868889", "SRR17868890", "SRR17868891, "SRR17868892", "SRR17868893", "SRR17868894", "SRR17868895", "SRR17868896", "SRR17868897", "SRR17868898", "SRR17868899", "SRR17868900", "SRR17868901", "SRR17868902", "SRR17868903", "SRR17868904", "SRR17868905", "SRR17868906", "SRR17868907", "SRR17868908", "SRR17868909", "SRR17868910", "SRR17868911", "SRR17868912", "SRR17868913", "SRR17868914", "SRR17868915", "SRR17868916", "SRR17868917", "SRR17868918", "SRR17868919", "SRR17868920", "SRR17868921", "SRR17868922", "SRR17868923", "SRR17868924", "SRR17868925", "SRR17868926", "SRR17868927", "SRR17868928", "SRR17868929", "SRR17868930", "SRR17868931", "SRR17868932", "SRR17868933", "SRR17868934" Source data are provided with this paper.

Code availability

Code used for analyses are publicly available via Github ["https://github.com/juliofdiaz/Wheatley_DiazCaballero_etal"].

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Author contributions

R.M.W., J.D.C., T.E.vdS., F.H.R.D.W., L.P.S., N.K., C.R., L.T. and A.L. contributed to data acquisition and analysis. J.K., M.E., C.P.A., A.O., S.K.S., S.M.K. and R.C.M. contributed to project conception and study design. R.M.W., J.D.C., S.K.S., S.M.K. and R.C.M. wrote and revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Antimicrobial Resistance & Infection Control

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An evaluation of a *Stenotrophomonas maltophilia* outbreak due to commercial arterial blood gas collection kit

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Abstract

Background *Stenotrophomonas maltophilia* is a gram-negative bacterium that can cause hospital infections and outbreaks within hospitals. This study aimed to evaluate an outbreak of *Stenotrophomonas maltophilia*, caused by ready-to-use commercial syringes containing liquid lithium and heparin for arterial blood gas collection in a university hospital.

Methods Upon detecting an increase in *Stenotrophomonas maltophilia* growth in blood cultures between 15.09.2021 and 19.11.2021, an outbreak analysis and a case-control study (52 patients for the case group, 56 patients for the control group) were performed considering risk factors for bacteremia. Samples from possible foci for bacteremia were also cultured. Growing bacteria were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The genetic linkage and clonal relationship isolates were investigated with pulsed-field gel electrophoresis (PFGE) in the reference laboratory.

Results In the case-control study, the odds ratio for the central venous catheter [3.38 (95% confidence interval [CI]: 1.444, 8.705 ; $p=0.006$)], for surgery [3.387 (95% confidence interval [CI]: 1.370, 8.373 ; $p=0.008$)] and for arterial blood gas collection history [18.584 (95% confidence interval [CI]:4.086, 84.197; $p<0.001$)] were identified as significant risk factors. *Stenotrophomonas maltophilia* growth was found in ready-to-use commercial syringes used for arterial blood gas collection. Molecular analysis showed that the growths in the samples taken from commercial syringes and the growths from blood cultures were the same. It was decided that the epidemic occurred because the method for sterilization of heparinized liquid preparations were not suitable. After discontinuing the use of the kits with this lot number, the outbreak was brought under control.

Conclusions According to our results, disposable or sterile medical equipment should be included as a risk factor in outbreak analyses. The method by which injectors containing liquids, such as heparin, are sterilized should be reviewed. Our study also revealed the importance of the cooperation of the infection control team with the microbiology laboratory.

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Keywords Arterial blood gas collection kit, Hospital infections, Outbreak, Outbreak analysis, *Stenotrophomonas maltophilia*

Background

Stenotrophomonas maltophilia is a gram-negative bacterium found in various environments, such as soil, plant roots, watery or moist surfaces, and plumbing systems [1]. Although it is accepted as a colonized or contaminant bacteria, it may cause lower respiratory tract and bloodstream infections. It can also be seen as a causative agent in various infections [2]. *S. maltophilia*, which is among the multi-drug resistant pathogens worldwide, can cause hospital infections and outbreaks. When examining hospital-acquired infections, they can be mortal, especially in immunocompromised patients or those who have undergone invasive procedures [3]. This study aims to evaluate an outbreak caused by *S. maltophilia* detected in hospitalized patients in a university hospital.

Materials and methods

The observed hospital is a 900-bed tertiary hospital for adults and children with different clinics and eight intensive care units. In the hospital, consultations regarding infectious diseases are carried out by infectious disease and clinical microbiology specialists, and the infection control team nurses monitor the growth and infection control measures with active surveillance conducted throughout the hospital. During these consultations and during follow-ups, an increase in *S. maltophilia* growth in the clinical samples was noted. Therefore, growth was reviewed and detected in various cultures of 81 children and adult patients from different clinics between 15.09.2021 and 19.11.2021. Because this increase was significant when compared to all instances *S. maltophilia* growth in 2019 and 2020–2021, it was decided that an analysis be conducted.

First, patients with *S. maltophilia* growth between 15.09.2021 and 19.11.2021 in various clinical samples were examined. It was determined that blood culture growth was predominant among all types of growth. The case description was determined as 'clinical and intensive care patients with at least one blood culture were positive for *S. maltophilia* between 15.09.2021–19.11.2021.' The outbreak analysis and case-control study were performed considering risk factors for bacteremia, such as catheter type, presence of IV solutions, drugs, blood and blood products. The control group included 'patients with similar characteristics (age, clinic, etc.) but with no *S. maltophilia* growth in their blood culture over the same period.' In the comparison of *S. maltophilia* growth between the case and control groups, when the effect size was determined to be 20%, the minimum number of patients required to be included in the groups was determined to be 50 for 5% significance and 80% power.

The patients' data were obtained from the hospital data processing system. The analysis included characteristics of samples with growth, growth dates, general characteristics of patients, prognosis, underlying diseases, clinics where the patients were hospitalized, whether the patients used antibiotics, surgery and haemodialysis, intubation history, whether interventional procedures were performed, bronchoscopy, enteral nutrition, history of total parenteral nutrition, blood product collection and history of potassium replacement use. Relevant procedure dates were also sourced from the patients.

Epidemiological surveillance

Samples from possible foci (distilled water, sink water from patients' rooms, heparinized flush solutions, treatment solutions [saline, dextrose, ringer lactate, etc.], alcohol, povidone-iodine, chlorhexidine and any syringes used on the patient) and unopened syringes within the same batch were cultured.

Arterial catheter: blood gas sampling

Arterial blood gas tests were performed on patients with arterial catheters. Arterial catheters consisting of a standard arterial flush system were placed directly in the systemic arteries. The flush solution contained 2 U/mL heparin with 0.9% sodium chloride. Flush solution and arterial line connected with vascular three-way stopcock. First, the three-way stop cock was disinfected with 2% chlorhexidine, and then the nurse aspirated 2 mL of blood with sterile syringes. This blood sample was thrown into a medical waste container. Next, 1 mL of blood was aspirated with heparinized syringes using a three-way stopcock.

Microbiologic identification and molecular analysis

Blood cultures were monitored using the BACTEC™ 9240 (Becton Dickinson, Sparks, MD, USA) fully automated blood culture device for 5 days or until bacterial growth was identified. Gram staining was prepared from the samples showing growth signals. The samples were inoculated on 5% sheep blood agar and eosin methylene blue (EMB) agar and incubated at 37 °C for 24–48 h. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany) was used to identify microorganisms reproduced in the media. The antibiotic susceptibility of these causative microorganisms was studied with the Phoenix™ 100 fully automated system and evaluated in

accordance with the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4].

The solutions, drugs and equipment thought to be a source for the outbreak (blood products, blood collection and component separation bags, liquid containing 0.9% NaCl, 5% dextrose, ready alcohol used in the fields, 2% chlorhexidine, hand antiseptic and alcohol prepared in the warehouse [70% diluted alcohol, tap water], the outer surface of multidose heparin vials taken from intensive care areas in which cases were common and heparin inside of the vials, and commercial kits consisting of syringes (containing liquid lithium and heparin used to collect arterial blood gas) were cultured. The samples were inoculated on 5% sheep blood agar and thioglycolate liquid and incubated at 37 °C for 24–48 h. MALDI-TOF was used to identify microorganisms with growth in the media.

On detecting the blood gas collection kit as a possible source of the outbreak, the outbreak analysis was repeated with this risk factor. After detecting the significant OR value in the second analysis, the blood culture samples and sterile blood gas collection kit samples were randomly selected and sent to a reference laboratory (National Antimicrobial Resistance Surveillance Laboratory) to investigate genetic linkage and clonal relationships. Bacterial identification of the 20 submitted strains (14 patients' blood culture samples and 6 blood gas collection kits) was first confirmed by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). The source/epidemiological analysis of the 20 strains isolated from patients and medical equipment was performed with the PFGE method using the CHEF-DR III system (Bio-RadLaboratories-Belgium). In this test, bacterial cells were embedded in 1% low-melting-point agarose plugs (SeaKem® Gold Agarose, Lonza Rockland, USA) and lysed with lysozyme and proteinase K. The digestion of chromosomal DNA was carried out using SpeI (Thermo Scientific-Fermantas Corporation, Vilnius, Lithuania). DNA fragments were separated on 1% pulsed-field certified agarose (Lonza Rockland, USA) using a CHEF-DR III system (Bio-Rad Lab, Nazareth, Belgium) at 6 V/cm^2 for 18 h at 14 °C and the pulse time was changed from 5 s to 70 s. Band profiles were analysed with BioNumerics version 7.5 software (Applied Maths, Sint-Martens-Latem, Belgium), and a similarity analysis was conducted using a Dice coefficient with a tolerance of 1.5% and an optimization of 1%. A dendrogram was constructed using the unweighted-pair group method with arithmetic mean (UPGMA). The clonal relationship among isolates was interpreted and evaluated according to the 1997 criteria developed by Tenover et al. [5]. Isolates with identical patterns were regarded as genotypically indistinguishable. Those with 1–3 band differences were considered closely related, those with 4–6 band differences were possibly related, and those with 7 or more band differences were considered unrelated or different.

Table 1 Distribution of samples with *Stenotrophomonas maltophilia* growth in their cultures between 15.09.-19.11.2021

Sample	Growth number (*n*)	%
Blood	72	69.2
EAS*	9	8.6
Sputum	7	6.7
Drain fluid	9	8.6
Peritoneal fluid	1	1
Bile	3	2.9
Pus	1	1
Conjunctiva	1	1
Urine	1	1

*EAS : Endotracheal aspirate samples

Patient follow-up

We conducted follow-ups for patients with potential colonizations or infections with contaminated arterial gas kits between September and December 2021. All inpatients who used heparinized syringes for blood gas measurement were monitored and followed to the last discharged patient. Furthermore, after the use of arterial blood gas collection kits was stopped, cultures with *S. maltophilia* growth were closely monitored for two months.

Statistical methods

The results are presented as frequencies and percentages. Binary logistic regression was performed, and the crude odds ratios (OR) and their 95% confidence intervals (CIs) were used to test confounding variables associated with risk factors and reported. $p<0.05$ was considered as the significance level. Statistical analyses were performed using IBM SPSS ver. 28.0 (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp.).

Results

Examination of the 104 clinical samples with *S. maltophilia* growth between 15.09.2021 and 19.11.2021 in various clinical samples showed that most were seen between 1.10.2021 and 31.10.2021. Of those samples with growth, 72 (69%) were in the blood culture, 9 (8.6%) in the deep tracheal aspirate and 9 (8.6%) fluid samples were taken from the drain. Other samples are shown in Table 1.

Based on the fact that 72 samples from 52 of the patients had *S. maltophilia* growth in the blood culture, all these patients fit the case description. The demographic and clinical characteristics of these patients and their underlying diseases are indicated in Table 2.

Of the blood cultures, 45% were taken from the catheter and 55% from the peripheral veins. In the case-control

Table 2 The clinical characteristics of the blood culture positive patients for *Stenotrophomonas maltophilia*

	n **(%)**
Age Children (≤ 18 years)	16 (30.7%)
Adult (> 18 years)	36 (69.2%)
(median year, average range)	39 (1 months – 87 years)
Gender (Female)	30 (57%)
ICU	32 (61.5%)
Baseline diseases	
Hypertension	9 (17%)
Malignancy	9 (17%)
Leukemia	3 (5.7%)
Diabetes mellitus	8 (15.3%)
Hearth diseases	13 (25%)
Chronic renal failure	9 (17%)
Acute renal failure	2 (3.8%)
Liver disease	1 (1.9%)
COVID 19	10 (19.2%)
Pulmonary disease	10 (19.2%)
Sepsis	2 (3.8%)
Acute gastroenteritis	1 (1.9%)
Intracranial abscess	1 (1.9%)
Catheter	46 (88.4%)
Central venous catheter	43 (83%)
Previous antibiotic usage	40 (76.9%)
Cephalosporins	6 (11.5%)
Carbapenem	30 (57.6%)
Piperacillin-tazobactam	5 (9.6%)
Sulbactam-ampisilin	2 (3.8%)
Aminoglycosides	2 (3.8%)
Glycopeptides	16 (30.7%)
Bronchoscopy	5 (9.6%)
Total parenteral nutrition	10 (19.2%)
Operation	21 (40.38%)
Interventional radiology	2 (3.8%)

study (52 patients in the case group and 56 patients in the control group), the odds ratio for the central venous catheter [3.38 (95% confidence interval [CI]: 1.444, 8.705 ; $p=0.006$)], for surgery [3.387 (95% confidence interval [CI]: 1.370, 8.373 ; $p=0.008$)] and for blood gas collection history [18.584 (95% confidence interval [CI]: 4.086, 84.197; $p<0.001$)] were identified as significant risk factors, as shown in Table 3.

Among the cultured medical instruments that were thought to be a source for the break, such as liquids containing 0.9% NaCl and 5% dextrose, sample taken from tap water, the alcohol sample, heparin liquid, swab sample taken from the outer surface seal of the heparin vial, none of the samples showed *S. maltophilia* growth.

Microbiological analysis was performed from 11 randomly selected, ready-to-use commercial syringes containing liquid lithium and heparin for arterial blood gas collection. The 24-, 48- and 72-hour evaluations of cultures showed growth; *S. maltophilia* was found in 6 samples cultured in solid and liquid media. These microbiological findings were consistent with case control study results, as shown in Table 3.

The clonal relationships of 20 randomly selected isolates (14 patients' blood culture samples and 6 sterile blood gas collection kits) were investigated with PFGE in the reference laboratory. 7 Different PFGE groups were identified, of which only 5 contained 1 strain. The other two PFGE groups contained cluster-forming strains, and the cluster width varied between 2 and 13. The clustering rate was determined to be 65%. Considering the similarity rate of ≥ 85% among PFGE profiles, 5 out of 20 strains showed specific profiles, while the remaining 15 strains were classified into 2 different epidemiologically related groups. A clonal relationship was found between isolates with plate numbers 24, 25, 26 and 28 (injector) and plate numbers 11, 12, 13, 14, 16, 17, 19, 20 and 22 (patients) included in the cluster of 13 strains. The isolates with plate numbers 27 and 29 (injectors) were found to be the same but were not associated with other isolates. The isolates with plate numbers 10, 15, 18, 21 and 23 (belonging to patients) were considered specific profiles and were not associated with any isolate. The dendrogram that shows the clonal relationships of the strains is shown in Fig. 1. A clonal association was determined between the isolates of commercial blood gas collection kits and blood culture samples.

Based on these results, the ready-to-use blood gas collection kits, which were determined as the source, were discontinued. These syringes were withdrawn from all units and returned to the manufacturer. The infection control team trained the entire hospital staff and explained the rules to be followed regarding blood gas sampling.

A report was immediately sent to the Ministry of Health on December 2021. After that, the Ministry of Health sent a definitive letter to all hospitals to stop using these commercial blood gas collection kits. In the following days, patients with *S. maltophilia* growth in their blood cultures were followed. It was observed that growth stopped in the blood culture after the kits were discontinued. The epidemic curve, which includes the distribution of *S. maltophilia* isolates grown in blood culture and the number of patients with *Stenotrophomonas maltophilia* bacteremia by months, is shown in Fig. 2. This significant regression in growth is also shown by the outbreak curve in this figure.

The outbreak was controlled by stopping the usage and sending back the heparinized blood gas syringes belonging to the same manufacturing batch with the same serial code.

Table 3 Univariate logistic regression results of case-control study and associated risk factors among blood culture positive patients for *Stenotrophomonas maltophilia*

Variables	*Cases ($n=52$)		**Control Group ($n=56$)		p	OR	OR (95% CI)	
Age	16 (≤ 18 years) 36 (≥ 18 years)		18 (≤ 18 years) 38 (≥ 18 years)		0.500	0.996	0.983	1.008
Sex	28 (Female)		23 (Female)		0.247	1.572	0.730	3.386
Ward	32		30		0.824	1.091	0.506	2.353
	Yes n(%)	**No n(%)**	**Yes n(%)**	**No n(%)**				
CVC***	43 (83%)	9 (17%)	31 (55%)	25 (45%)	**0.006**	**3.545**	**1.444**	**8.705**
Whole blood transfusion	0 (0%)	52 (100%)	0 (0%)	56 (100%)	0.979	1.039	0.063	17.061
Erythrocyte transfusion	7 (13%)	45 (87%)	3 (5%)	53 (95%)	0.177	2.644	0.645	10.839
Thrombocyte transfusion	3 (6%)	49 (94%)	2 (4%)	54 (96%)	0.619	1.592	0.255	9.936
Fresh frozen plasma transfusion	3 (6%)	49 (94%)	5 (9%)	51 (91%)	0.500	0.600	0.136	2.649
Surgery	21 (40%)	31 (60%)	9 (16%)	47 (84%)	**0.008**	**3.387**	**1.370**	**8.373**
Interventional radiology	2 (4%)	50 (96%)	7 (13%)	49 (87%)	0.112	0.269	0.053	1.359
Blood gas collection history	50 (96%)	2 (4%)	31 (55%)	25 (45%)	**< 0.001**	**18.548**	**4.086**	**84.197**

*Patients with *Stenotrophomonas maltophilia* bacteremia

** Patients without *Stenotrophomonas maltophilia* bacteremia

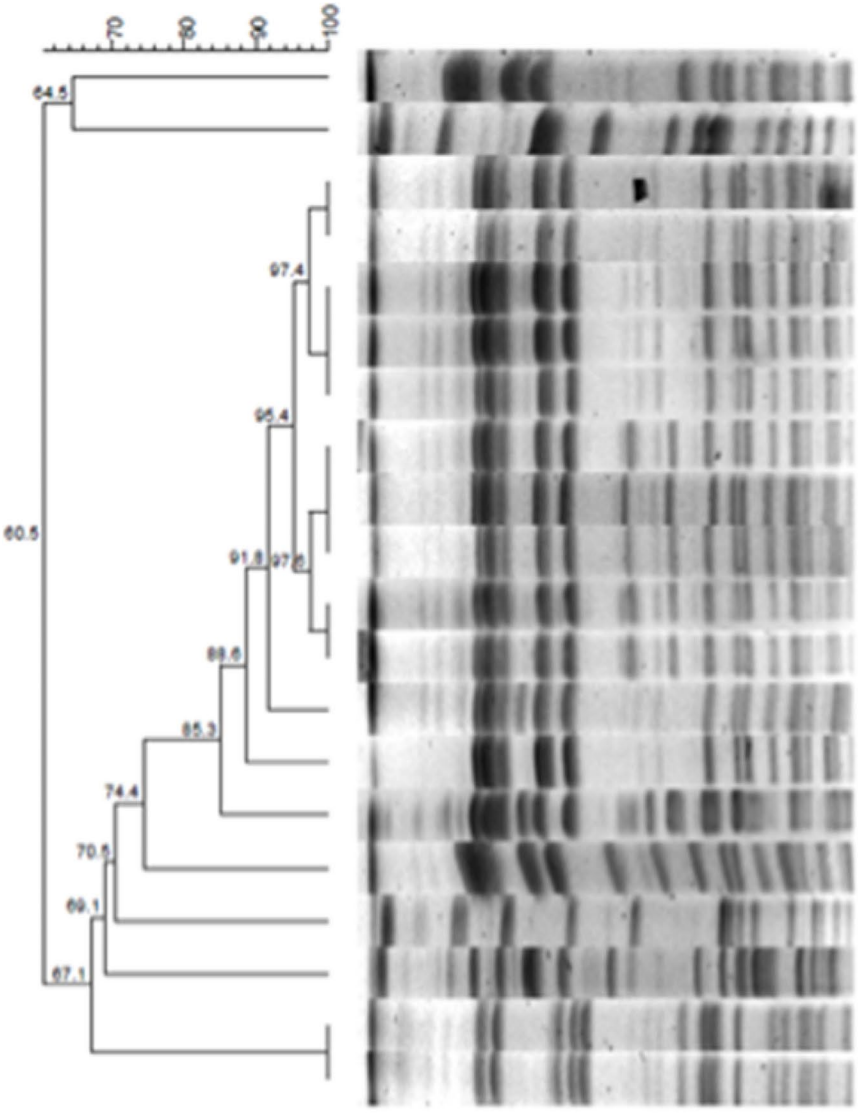
*** CVC: Central venous catheter

Discussion

S. maltophilia are mostly found in aquatic environments and soil. Bacteria that are generally considered contaminant bacteria can be found on environmental surfaces and medical instruments within the hospital [6]. As an agent that can settle on many surfaces, including sterile areas, such as catheters and prostheses, and form a biofilm, *S. maltophilia* can cause disease in patients who have undergone invasive procedures and or have compromised immune systems. Bacteremia, which often affects the respiratory tract, is common in patients hospitalized for a long time who use broad-spectrum antibiotics such as carbapenem or are immunosuppressed. This can lead to endocarditis and infections of the central nervous system and urinary system [7]. The resistant bacteria can also cause outbreaks in hospitals [8, 9]. Long-term hospitalization and a history of antibiotic use, as well as invasive procedures such as mechanical ventilation, were determined to be the most important risk factors for the development of infection [9].

Although this bacterium is generally reported to be of low virulence, *S. maltophilia* bacteremia may be fatal in some patients [10]. Between 2002 and 2016, 140 patients with nosocomial *Stenotrophomonas maltophilia* bacteremia were examined in a university hospital, and the mortality rate on the 14th day was reported to be 32.9%. It has been shown that starting antibiotics as soon as possible and withdrawing the central venous catheter is essential for patients' survival [11].

Outbreaks with this bacterium caused by disinfectants or different sterile solutions have been reported for a very long time [12]. When examining outbreaks due to *S. maltophilia* reported in the literature, it was found that water and medical equipment were identified as possible sources in the foreground. However, outbreaks can be seen in different forms, such as in the contamination of dialysates used in dialysis centres or acute endophthalmitis outbreaks detected in eye centers where intravitreal injections are applied [13–15]. A study conducted in Mexico examined the blood and urine cultures of pediatric patients in the emergency room together with 21 *S. maltophilia* isolates taken from the taps of the same unit and found a total of 7 PFGE clones with 52% genetic variation [16]. Similarly, 7 different PFGE groups were identified in the current study. Only 5 of them contained 1 strain, and the similarity rate between PFGE profiles was considered as ≥85%. While 5 out of 20 strains showed specific profiles, the remaining 15 strains were classified into 2 different epidemiologically related groups. The use of multiple-dose vials can also be a source of these bacteria. In fact, as a result of the use of a vial containing contaminated bevacizumab solution in different patients



Resource	Plague
Patient	P19
Patient	P20
Syringe 1	P24
Syringe 4	P26
Patient	P11
Patient	P12
Patient	P14
Patient	P16
Patient	P22
Syringe 2	P25
Patient	P13
Patient	P17
Syringe 10	P28
Patient	P15
Patient	P10
Patient	P18
Patient	P21
Patient	P23
Syringe 5	P27
Syringe 3	P29

Fig. 1 The dendrogram showing the clonal relationship of the strains

in India, acute endophthalmitis developed in 28 patients within 3 days [17]. However, contaminated, ready-made and disposable preparations can also cause outbreaks. It was reported in the USA that between 2004 and 2005, 36 patients from different states developed infections due to a heparin-saline flush solution that was contaminated with *Pseudomonas fluorescens* during production [18]. Dias et al. evaluated 154 patients in their hospital who they thought had been given a ready-made heparin catheter lock solution thought to be contaminated, 32 individuals (out of 48 who had a central venous catheter) had *Pseudomonas putida* growth in their blood cultures, and 9 patients had *S. maltophilia* growth in their blood cultures. A molecular analysis also revealed that growth in solutions thought to be contaminants was similar to that of *S. maltophilia* growth in blood cultures [19].

Our study began with an observation of the increased blood culture growth over a two month period. On the genetic similarity of the growths in the samples taken from the disposable, ready-to-use blood gas collection kit with the growths in the patient blood cultures, the use of kits with this lot number was stopped, and the epidemic was brought under control. We think that the heparinized syringe and the three-way stop cock come into contact for a while, and possible contact contamination occurs. Afterwards, in order to keep the arterial tract open, 5 ml of heparinized solution was drawn with a new and clean syringe and given back to the arterial line. In possible contamination of the three-way stop cock, this contaminant was transferred to the arterial line. Menekse et al. [20] reported a commercial needle blood gas injector as the source of a *S. maltophilia* outbreak over the same period as the present study. They also reported that

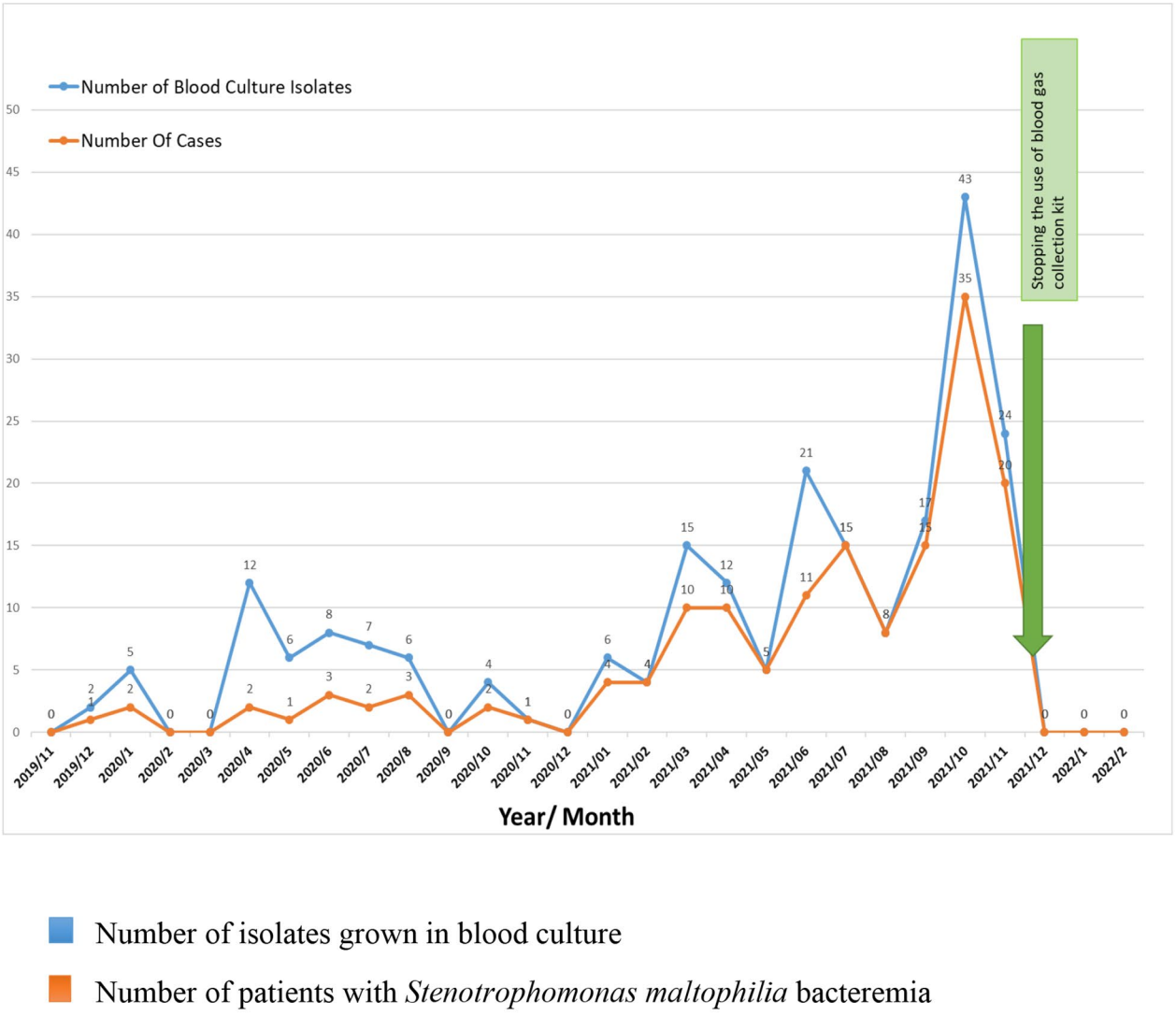


Fig. 2 The outbreak curve of *Stenotrophomonas maltophilia*

their investigation for blood gas kits started after the letter from the Ministry of Health was released.

The aseptic manufacturing process is essential to producing sterile products and minimising the contamination risk for patients. It involves multiple steps under sterile conditions for the handling of materials and medical equipment [21]. Gas sterilization with ethylene oxide is still a common method used for sterilizing some medical devices; however, it was found that this method is not sufficient for the sterilization of liquid products due to limitations of penetration depth [22]. Similarly, gamma irradiation is used for various disposable product sterilizations [23]. However, this method bears the risk of changing the composition of the heparin. Other methods should be investigated for heparinized solutions.

Limitations of the study

In our study, all strains of *S. maltophilia* isolated from blood cultures were not subject to molecular investigation. However, a link between the possible source and blood culture isolates was demonstrated in the samples studied.

Conclusions

Disposable or sterile medical equipment should be included in outbreak analyses as a potential source for such outbreaks, especially for bacteria that can be found in liquid media. The appropriate methods for sterilization of such heparinized liquid preparations are still unclear and this issue should be discussed. This study also shows the importance of the work of infection control teams and their cooperation with microbiology laboratories, the review and identification of resources, including

disposable, ready-made preparations, and taking appropriate precautions in preventing nosocomial infections.

Abbreviations

EUCAST	European Committee on Antimicrobial Susceptibility Testing
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
PFGE	Pulsed-field gel electrophoresis
UPGMA	Unweighted-pair group method with arithmetic mean

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Author contributions
The first author is corresponding author. F.A.,G.C participated in the data acquisition. Data analysis and interpretation were performed by H.A., C.O, M.H., E.Y., S.C., E.K., F.A., G.C., S.C., Y.H, U.O. Material preparation, bacterial studies, analysis, data interpretation were performed by U.T. and C.O. Moleculer studies, analysis, data interpretation were performed by H.S, Z.B., Y.C. The manuscript was written by E.K., U.O, H.A. All authors commented on previous versions of the manuscript. Review and editing were performed by E.K., H.A. All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Approval for this study was obtained from Bursa Uludag University Clinical Research Ethics Committee (2011-KAEK-26/726). The trial was conducted according to the requirements of the Declaration of Helsinki.
This outbreak analysis complies with Outbreak Reports and Intervention Studies of Nosocomial Infection (ORION) guidelines [24].

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Outbreak of cephalosporin resistant *Enterobacter cloacae* infection in a neonatal intensive care unit

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SUMMARY *Enterobacter cloacae* resistant to third generation cephalosporins emerged rapidly during an outbreak of serious infections due to this organism in a neonatal intensive care unit where ampicillin and gentamicin were used as first line antibiotic treatment. Organisms resistant to cephalosporins were isolated from 12 infants, six of whom developed systemic infection. Two infants died. Isolates of *E. cloacae* from four of five infants treated with cefotaxime showed a loss of sensitivity to this antibiotic during treatment, but in the three infants who survived sensitive organisms were again isolated after treatment had stopped. Stopping treatment with the cephalosporins, closure of the unit to new admissions, and strict cohorting of colonised infants resulted in a prompt end to the outbreak.

This outbreak suggests that the routine use of third generation cephalosporins for suspected sepsis may be inappropriate in the presence of a large reservoir of organisms with the potential for rapidly developing resistance. Routine bacteriological surveillance, however, might permit their use on a rotational basis.

Infection is common in babies in neonatal intensive care units. It may be overwhelming but early signs are largely non-specific and babies from such a population often receive broad range antibiotics before the results of cultures are available. The combination of an aminoglycoside and a penicillin is widely used.[1] Recently, the emergence of gentamicin resistant organisms and the potential problems of aminoglycoside ototoxicity, nephrotoxicity, and poor penetration into cerebrospinal fluid (CSF) has led to third generation cephalosporin monotherapy being advocated for routine first line treatment.[2] We describe our experience of third generation cephalosporins in a unit that has continued to use ampicillin and gentamicin as first line treatment. We suggest that the routine use of third generation cephalosporins as first line treatment for suspected infection in neonatal intensive care units may be inappropriate.

Background

The regional neonatal intensive care unit, Liverpool Maternity Hospital, admits between 450 and 500 babies each year. Roughly 30% weigh less than 1500 g at birth and about 30% are outborn.

Handwashing with a chlorhexidine solution followed by application of an alcohol rub is carried out before every infant contact. Gloves are worn during nursing procedures and napkin changes. Routine infection surveillance includes culture and sensitivity testing on daily specimens of tracheal aspirate from intubated babies and weekly rectal and superficial swabs from all babies. A full infection screen, consisting of superficial swabbing and urine, CSF, and blood cultures, is carried out on all outborn babies on admission.

The combination of ampicillin and gentamicin has been used as the standard initial treatment of suspected infection for five years. Over 95% of babies receive broad range antibiotics at least once during their admission.

Laboratory methods

Standard methods and media were used for isolation of organisms.[3] Species identification of isolates was carried out on the basis of biochemical reactions, using the API 20E system (API Products, Basingstoke). Sensitivities to selected antibiotics were determined by the breakpoint method, using Oxoid DST agar and a multipoint inoculator that produced

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an inoculum of 10^5 colony forming units. To incorporate antibiotics of required concentrations into the DST agar, Adatabs (Mast House, Bootle) were used. Breakpoints were 1 mg/l (low) and 4 mg/l (high) for gentamicin and 2 mg/l (low) and 8 mg/l (high) for cephalosporins. The plates were incubated at 37°C overnight. Isolates were considered resistant to gentamicin or to the tested cephalosporin if growth was noticed on plates containing 4 mg/l or 8 mg/l of respective antibiotic.

Clinical observations

The pattern of colonisation in this unit is one of fluctuating numbers of infants colonised with *Staphylococcus epidermidis,* yeasts, and strains of *Pseudomonas, Klebsiella, Serratia,* and *Enterobacter.*

From March 1985 increasing numbers of isolates of gentamicin resistant *Staph. epidermidis* resulted in the use of cefuroxime for infections caused by this organism. Infection with *Staph. epidermidis* accounts for most blood cultures that produce positive results and cefuroxime therefore became the third most commonly used antibiotic in the unit. Concurrent with this increase in use of cefuroxime routine bacteriological surveillance revealed an increasing proportion of babies colonised with *Enterobacter cloacae,* most of whom were resistant to cefuroxime. Up to the end of June 1985 all isolates of *E. cloacae* were sensitive to the third generation cephalosporins cefotaxime and ceftazidime. During July strains resistant to all cephalosporins tested first occurred.

Results of weekly bacteriological surveillance from 4 June 1985 to 17 September 1985 are presented in the Figure. Screening swabs were obtained from each baby on the unit every Tuesday. The total number of babies in the unit is shown with the proportion colonised with *E. cloacae* and the proportion harbouring strains resistant to cefotaxime. Resistant organisms were recovered from 12 infants on 21 occasions over an eight week period. Seven of these infants had not been on treatment with a cephalosporin at the time of isolation of the resistant strain.

During this time a cluster of serious infections with *E. cloacae* arose, with two episodes of ventriculitis and septicaemia (one episode of ventriculitis prolonged at 19 days) and four more cases of isolated septicaemia. Two infants died and two required treatment with the new quinolone ciprofloxacin. The isolates of *E. cloacae* from five of these six infants with systemic infection were initially sensitive to cefotaxime, and these five were thus treated with cefotaxime: isolates of *E. cloacae*

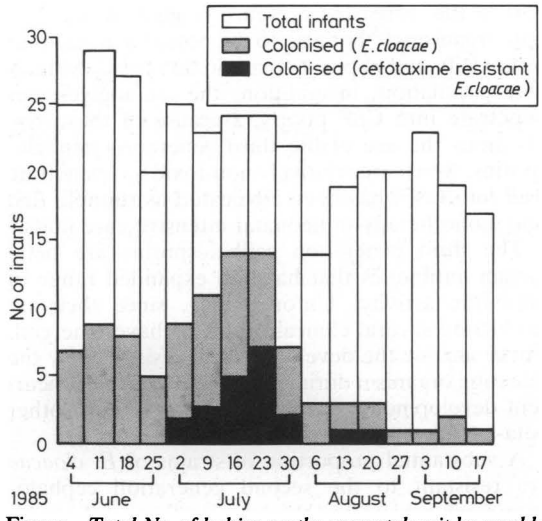

Figure *Total No of babies on the neonatal unit by weekly intervals on the days that infection screening was undertaken, showing the proportion colonised with* Enterobacter cloacae *and with cefotaxime resistant strains.*

Table *Details of infants with systemic* Enterobacter cloacae *infection initially sensitive to cefotaxime but who developed resistance after treatment*

Case No	*Birth weight (g)*	*Gestational age (weeks)*	*Infection*	*Days**
1	1840	30	BCs	7
2	1210	28	BCs, CSF	7
3	1090	28	BCs	2
4	1320	27	BCs, CSF	3

*Days after start of treatment that resistant organisms isolated.
BCs=Blood cultures; CSF=Cerebrospinal fluid.

from four revealed a loss of sensitivity to cefotaxime and to other third generation cephalosporins during treatment (Table). In the three of these four infants who survived *E. cloacae* sensitive to the third generation cephalosporins was once again isolated after exposure to cefotaxime had ceased.

Infection control measures were begun and included strict cohorting of infants colonised with *E. cloacae,* stopping treatment with all cephalosporins, and closure of the unit to new admissions. Third generation resistant strains of *E. cloacae* disappeared promptly and the level of colonisation with this organism fell rapidly.

Discussion

Concern has been voiced regarding the use of gentamicin in neonatal intensive care units due in

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part to the increasing presence of gentamicin resistant organisms[4 5] but also to the potential hazards of ototoxicity and nephrotoxicity in an already vulnerable population; in addition, the aminoglycosides penetrate into CSF poorly. Because of these reservations the use of the third generation cephalosporins, which are relatively non-toxic and penetrate well into CSF, has been advocated as suitable first line monotherapy in neonatal intensive care units.[2]

The third generation cephalosporins are beta-lactam antibiotics that have an expanded range of antibiotic activity. Unfortunately, since their introduction several clinical problems have emerged. These include the development of resistance by the infecting organism during treatment and the concurrent development of cross resistance to most other beta-lactam antibiotics.[6 7]

A substantial proportion of strains of *E. cloacae* are resistant to the second generation cephalosporin cefuroxime and the increased use of this antibiotic for *Staph. epidermidis* infections was undoubtedly responsible for the selection of such resistant strains, with a consequent increase in numbers of infants colonised with *E. cloacae*. This enlarging reservoir of *E. cloacae* thus consisted of organisms resistant to cefuroxime but sensitive to the third generation cephalosporins cefotaxime and ceftazidime. Concurrent with the increasing number of infants colonised with *E. cloacae*, however, strains resistant to cefotaxime and ceftazidime occurred.

The possibility of introduction of strains of *E. cloacae* resistant to the third generation cephalosporins from outside the unit was unlikely as all outborn babies underwent a full infection screen on admission. Furthermore, the biotypes of the isolates were identical.

Production of beta-lactamase activity may be either plasmid or chromosomally mediated; the oxime cephalosporins, cefotaxime and ceftazidime, show a high degree of stability to plasmid encoded beta-lactamase activities and are also relatively resistant to the normal amounts of chromosomally mediated cephalosporinase activities found in most wild type Enterobacteriacae.[8] If these amounts are increased, however, either by exposure of the wild type to a beta-lactamase inducer (reversible derepression) or mutation to the stably derepressed state, most third generation cephalosporins may be inactivated.[9 10] Both second and third generation cephalosporins are inducers of cephalosporinase activities in the genera *Pseudomonas, Serratia,* and *Enterobacter,* which possess chromosomally mediated inducible beta-lactamase activities (type I[11]).[7]

Both mechanisms of increased production of beta-lactamase activity may have played a part in the outbreak of infection described. *E. cloacae* resistant to the third generation cephalosporins were isolated from seven babies on routine screening swabs. At the time of isolation none had been on treatment with a cephalosporin. This observation suggests nosocomial transmission, although this cannot be proved as no information regarding serotypes was available. The biotypes of the isolates were, however, identical. As induction requires the continuing presence of the inducer, with induction ceasing when the inducer is removed, induction of beta-lactamase activity was probably not involved here; the passage of a derepressed mutant is a possibility.

In four of the five infants with systemic infection treated with cefotaxime *E. cloacae* resistant to cefotaxime and cefotazidime were first isolated after the start of treatment (Table). Once again, though the possibility of multiple strains exists, reversible induction of production of beta-lactamase activity was probably responsible for the occurrence of resistance here. This is supported by the observation that in the three infants who survived *E. cloacae* isolated after exposure to cefotaxime had ceased was again sensitive to the third generation cephalosporins.

The emergence of cefotaxime resistance during treatment with this antibiotic is recognised[5 7 12] but has been described only once before in a neonatal intensive care unit.[5] This problem is compounded because multiple drug resistance may arise simultaneously. Emergence of beta-lactam resistance during treatment with a combination of a third generation cephalosporin and an aminoglycoside has also been reported.[13]

Strains possessing inducible lactamase activities from the genera *Pseudomonas, Serratia,* and *Enterobacter* are commonly responsible for colonisation of infants in neonatal intensive care units. *E. cloacae* has been shown to have a high mutation rate.[10] The common need for treatment with antibiotics, the predilection of these infants to nosocomial infection, and the rapid emergence of resistance to the third generation cephalosporins, such resistance additionally occurring during treatment, suggests that the routine use of these agents is inappropriate in the presence of a large reservoir of infants colonised with the above organisms. First line monotherapy with a third generation cephalosporin may well, however, be appropriate in the absence of such a pool of organisms. The value of the third generation cephalosporins for isolated, proven infection is undisputed. The epidemiological considerations governing the frequent use of an antibiotic blind as first line treatment for suspected sepsis must be clearly distinguished, however, from the use of an

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antibiotic relatively infrequently for proven sepsis. An antibiotic suitable in one context may not be suitable in the other.

Our observations once again illustrate the importance of routine bacteriological surveillance in a neonatal intensive care unit.[14] Routine surveillance also permits the introduction of alternative first line treatment regimens on a rotational basis.

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ARTICLE

The epidemiology of the first described carbapenem-resistant *Klebsiella pneumoniae* outbreak in a tertiary care hospital in Saudi Arabia: how far do we go?

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Abstract The purpose of this investigation was to describe the first documented carbapenem-resistant *Klebsiella pneumoniae* (CRKP) outbreak in a tertiary care facility in Saudi Arabia. We initiated a prospective study to follow all cases of CRKP as well as the active surveillance of patients in areas where cases were identified. We also conducted a retrospective review of the microbiology database for any missed cases of CRKP. Pulsed field gel electrophoresis (PFGE) was conducted for the available CRKP isolates. During March 2010, a cluster of eight CRKPs was detected primarily in the adult intensive care unit (ICU). Patients with CRKPs were put under strict contact isolation, along with appropriate infection control measures. A retrospective review of *K. pneumoniae* isolates over the previous 6 months revealed two more CRKPs. The PFGE results during the outbreak period showed that the majority of strains were genetically indistinguishable or closely related. The majority of patients had prolonged hospital stay (91%), indwelling devices (81%), surgical procedures (74%), carbapenem use (62%), and colonization/infection with other multiple drug-resistant organisms (MDROs) (57%). Two-fifths of patients with CRKP had clinical infection and 38% died during the current hospitalization. Contact isolation, hand hygiene, environmental cleaning, and staff education may control CRKP outbreak in the acute care setting, but did not prevent endemicity.

Background

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is a global concern, leading to healthcare-associated infections

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worldwide. The most important mechanism of resistance is the production of a carbapenemase enzyme that has the ability to hydrolyze carbapenems [1]. Since its first description in North Carolina in 1996 [2], carbapenemase-producing *K. pneumoniae* (KPC) has been identified in 24 states across the US [3]. CRKP is currently seen all over the world [4–6]. There have been no described outbreaks with CRKP reported from Saudi Arabia or Gulf Cooperation Council (GCC) States.

Healthcare-associated infections (HAIs) caused by CRKP may be linked to increased mortality, length of hospitalization, and increased cost [7]. Data from the National Healthcare Safety Network (NHSN; Centers for Disease Control and Prevention [CDC], Atlanta GA, USA) showed that 8.7% of *Klebsiella* isolates causing HAIs in 2006–2007 were carbapenem-resistant compared to just less than 1% in 2000 [8, 9]. Since carbapenems are considered as an effective last line of defense in the treatment of severe infections caused by antimicrobial-resistant *Enterobacteriaceae*, CRKP represent a global threat to the control of HAIs, particularly in the presence of very limited therapeutic options [3]. Moreover, the difficulty of CRKP detection using routine antibiotic susceptibility testing adds to the complexity of the CRKP challenge in the healthcare setting [10]. The CDC recommends aggressive infection control measures to control the CRKP spread in acute care (inpatient) facilities that include early detection and active surveillance [9]. We sought to describe our experience in detecting and containing the first documented CRKP outbreak in Saudi Arabia.

Methods

Setting

The current study was conducted at King Abdulaziz Medical City (KAMC) in Riyadh, Saudi Arabia. KAMC is an approximately 900-bed tertiary care facility that provides healthcare services to about 600,000 Saudi National Guard soldiers, employees, and their families. The care provided ranges from primary and preventive care to tertiary care. KAMC has eight different intensive care units (ICUs) and four step-down units.

Study design

This a retrospective/prospective surveillance study describing the outbreak investigation and containment.

Surveillance and isolation methods

The outbreak was declared after reviewing the first three CRKP isolates at adult ICUs (between March 5th and 13th, 2010). Patients were then placed under strict contact isolation, along with intensified infection control measures and hand hygiene observation. The emerging cluster of CRKP patients were examined prospectively for microbiological and clinical data until discharge or death. The contacts of all clinical cases were screened, minimally through a peri-rectal swab; however, those who were ventilated were also screened through an endotracheal culture and placed under contact isolation until the culture results were available. Included in our study are patients with the first isolate of CRKP related to either a clinical infection or colonization. Patients who were positive by screening surveillance initially and then had invasive disease due to CRKP were reported according to the first positive specimen. The possibility of previously unnoticed CRKP cases was explored through a retrospective review of the microbiology database for any suspected cases of carbapenem resistance among the multiple drug-resistant *K. pneumonia* isolates over the previous 6 months. Commencing March 24th, weekly active surveillance cultures for CRKP were obtained from all ICU patients using rectal swabs and respiratory specimens. In addition, any new admission to the ICU was screened via peri-rectal swab and placed in contact isolation until the results were available. Weekly active surveillance was replaced by monthly active surveillance after declaring the end of the outbreak. Patients who became ready for transfer from the ICU were maintained on contact isolation in the receiving ward until the results became available. Those who were found to be colonized received weekly surveillance cultures until they became negative or until they were discharged.

Data collection

A standard data collection form was developed and discussed with infection control staff who were responsible for data collection. Index data was defined as the first date of CRKP isolation from surveillance or clinical cultures. Data collection involved using current and previous patient clinical records, microbiology laboratory records, and, sometimes, contact with the treating physician. Data collected included demographic data, location and duration of hospital stay, clinical presentation (clinical healthcare-associated infections or colonization), clinical severity score, comorbid medical conditions, current antimicrobial therapy, potential risk factors for multiple drug resistance, and outcome data (discharge or death) (Table 1 and 2). Potential risk factors at the index date were clearly defined (as shown in Table 3) before the start of data collection. Death was defined as death occurring during the current hospitalization (up to August 31), irrespective of hospital location at the time of death.

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Table 1 Demographic and clinical characteristics of confirmed carbapenem-resistant *Klebsiella pneumoniae* (CRKP) cases at King Abdulaziz Medical City (Riyadh, Saudi Arabia) between September 2009 and August 2010

ID	Age	Sex	PFGE strain	Primary diagnosis on admission*	Hospital transfer	Hospital admission date	Initial admission location*	Index date (first positive)	Admission location at index date	Stay in index location before index date*	Hospital days before index date	Days of current ICU stay	Source of first positive isolate	Presentation of infection	Recent carbapenem intake	History of MDRO	Outcome	Days to outcome
1	76	Male	A1	Acute coronary syndrome	No	02-Sep-09	Ward	01-Dec-09	Ward	Yes	90	12	Wound	Wound infection	Yes	Yes	Death	216
2	68	Female		Liver failure and stroke	No	30-Jan-10	Emergency	23-Feb-10	Adult ICU	Yes	24	29	Respiratory	Colonization	No	No	Death	8
3	40	Female	D	Liver failure and intestinal obstruction	No	12-Dec-09	Emergency	5-Mar-10	Adult ICU	Yes	83	68	Urine	Colonization	Yes	Yes	Death	33
4	22	Male		MVA	Yes	22-Feb-10	Adult ICU	9-Mar-10	Adult ICU	Yes	15	30	Respiratory	Colonization	Yes	Yes	Still in hospital	175
5	28	Male	B1	MVA	No	8-Mar-10	Adult ICU	13-Mar-10	Adult ICU	Yes	5	17	Wound	Wound infection	Yes	Yes	Discharged home	38
6	77	Female	A1	Gastric outlet obstruction	No	21-Jan-10	Emergency	24-Mar-10	ICU step-down	Yes	62	77	Rectal	Colonization	Yes	Yes	Death	31
7	66	Male	A3	Liver failure and hepatitis C	No	11-Feb-10	Adult ICU	27-Mar-10	Liver step-down	Yes	44	63	Blood	BSI	No	Yes	Death	103
8	19	Female	A1	Liver failure and biliary atresia	No	16-Mar-10	Liver step-down	28-Mar-10	Adult ICU	Yes	12	15	Respiratory	Colonization	Yes	Yes	Death	4
9	27	Male	A1	Patellal fracture	No	31.3.2010	Ward	31-Mar-10	Ward	No	0	NA	Wound	Colonization	No	No	Discharged home	4
10	81	Male	A1	UTI and renal impairment	Yes	31-Mar-10	Emergency	6-Apr-10	Adult ICU	Yes	6	5	Rectal	Colonization	No	No	Discharged home	41
11	35	Female		Renal failure	No	30-Mar-10	Emergency	8-May-10	Ward	Yes	39	24	Wound	Wound infection	Yes	No	Discharged home	49
12	43	Male		Calcaneal fracture	Yes	22-Apr-10	Emergency	1-Jun-10	Ward	No	40	NA	Wound	Colonization	Yes	Yes	Discharged home	89
13	72	Female		Shortness of breath	No	18-May-10	Emergency	4-Jun-10	Adult ICU	Yes	17	55	Respiratory	Colonization	Yes	Yes	Death	52
14	72	Female	A1	Infected wound	No	21-Apr-10	Emergency	16-Jun-10	Ward	Yes	56	14	Wound	Infected wound	No	Yes	Discharged home	38
15	32	Female	B1	Epilepsy	No	11-Apr-10	Emergency	7-Jul-10	Ward	No	87	NA	Wound	Abscess	No	No	Still in hospital	55
16	34	Female	A2	MVA	No	5-Jul-10	Adult ICU	18-Jul-10	Adult ICU	Yes	13	32	Respiratory	Colonization	Yes	No	Still in hospital	44
17	60	Female	A2	Heart failure	No	16-Jun-10	Emergency	22-Jul-10	ICU step-down	Yes	36	30	Urine	Colonization	No	No	Death	7
18	31	Female	A1	Abdominal pain during pregnancy	No	15-Jul-10	Adult ICU	15-Aug-10	ICU step-down	Yes	31	46	Catheter tip	BSI	Yes	No	Still in hospital	16
19	52	Male	C	Post-amputation gangrene	No	6-Aug-10	Adult ICU	21-Aug-10	Adult ICU	Yes	15	25	Wound	Gangrene	Yes	Yes	Still in hospital	10
20	81	Male	B2	Parkinsonism	No	22-Aug-10	Emergency	22-Aug-10	Emergency	No	0	1	Urine	Colonization	No	No	Still in hospital	9

PFGE, pulsed field gel electrophoresis; multiple drug-resistant organism (MDRO); ICU, intensive care unit; MVA, motor vehicle accident; index location, adult ICU or ICU step-down; UTI, urinary tract infection

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Table 2 Demographic and clinical characteristics of patients with CRKP at King Abdulaziz Medical City (Riyadh, Saudi Arabia) between September 2009 and August 2010

Age (years)	
Mean±SD	50.8±21.8
≤18	0 (0%)
19–59	12 (60%)
≥60	8 (40%)
Sex	
Male	9 (45%)
Female	11 (55%)
Primary diagnosis	
Trauma (MVA or fractures)	5 (25%)
Liver or kidney failure	5 (25%)
Neurological disease	3 (15%)
Gastrointestinal obstruction	2 (10%)
Sepsis	1 (5%)
Other	6 (30%)
Comorbidity	
Surgery	10 (50%)
Hypertension	10 (50%)
Diabetes	9 (45%)
Trauma	6 (30%)
Gastrointestinal disease	5 (25%)
Liver disease	5 (25%)
Heart disease	5 (25%)
Renal disease	4 (20%)
Immunosuppression	3 (15%)
Lung disease	3 (15%)
Stroke	3 (15%)
APACHE III score (median and IQR, days)	67.0 (44.8–95.0)
First admission location	
Emergency rooms	11 (55%)
Adult ICU	6 (30%)
Step-down unit or wards	3 (15%)
Current intensive care admission	17 (85%)
Hospital and ICU stay (median and IQR, days)	
Stay before index date	27.5 (12.3–53.0)
Total ICU stay	29.0 (14.5–50.5)
Total hospital stay	63.0 (34.8–125.8)
Infection presentation	
Colonization	12 (60%)
Clinical infection	8 (40%)
Infected wound	4 (50%)
Bloodstream infection (BSI)	2 (25%)
Other	2 (25%)

SD, standard deviation; IQR, inter-quartile range; APACHE, acute physiology and chronic health evaluation

Table 3 Potential multiple drug-resistant organism (MDRO) risk factors among patients with CRKP at King Abdulaziz Medical City (Riyadh, Saudi Arabia) between September 2009 and August 2010

Potential risk factors at index date*	
Prolonged hospital stay (≥5 days)	18 (90%)
Recent antimicrobial therapy (last month)	17 (85%)
Recent indwelling device	16 (80%)
Recent admission to ICU (last month)	15 (75%)
Recent surgical procedure	13 (72%)
Recent mechanical ventilation	14 (70%)
Previous hospitalization (last 3 months)	12 (63%)
Previous MDRO (last month)	12 (60%)
Immunosuppression	8 (44%)
Recent endoscope	5 (26%)
Recent trauma	4 (21%)
Transferred from another hospital	3 (15%)
CRKP patient in an adjacent room	1 (5%)
CRKP patient in the same room	0 (0%)
Recent burns	0 (0%)
Prior antimicrobial use**	
Carbapenem	12 (60%)
Antianaerobic	10 (50%)
Colistin	9 (45%)
Fluoroquinolones	7 (35%)
Vancomycin	7 (35%)
Tigecycline	3 (15%)
Cephalosporins III	2 (10%)
Aminoglycoside	1 (5%)
Tazocin	1 (5%)
Previous MDRO	
Acinetobacter	4 (20%)
Pseudomonas	3 (15%)
MRSA	2 (10%)
VRE	1 (5%)
Outcome	
Death	8 (40%)
Discharged home	6 (30%)
Still in hospital	6 (30%)
Cause of death	
Septic shock	5 (71%)
Liver/kidney failure	4 (57%)
Cardiopulmonary arrest	4 (57%)
Days before death	
Mean±SD***	56.8±72.1
Median and IQR***	32.0 (7.3-90.3)

*Risk factors presence over the last 2 weeks, unless mentioned otherwise

**For at least 2 days over the last month

***Abbreviations as per Table 2

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Microbiological examination and clonal analysis

K. pneumoniae isolates were identified using the standard laboratory methods of the Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. The identification of *K. pneumoniae* to the species level was done using the MicroScan WalkAway system (Siemens) and then confirmed using API 20E. Antimicrobial susceptibility testing was determined using the MicroScan WalkAway system (Siemens) and confirmed using the disk diffusion method and E-test (AB Biodisk). Minimum inhibitory concentration (MIC) breakpoints of carbapenems (meropenem and or imipenem) were defined according to the 2009 CLSI guidelines [11]. *K. pneumoniae* isolates found to have elevated MICs of carbapenems or with reduced disk diffusion zone sizes were tested for the presence of carbapenemases by use of the modified Hodge test (MHT) [12]. Carbapenemase production was detected by using meropenem disks; when the test isolate produced the enzyme and allowed the growth of a carbapenem-susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk, it was considered as positive. Pulsed field gel electrophoresis (PFGE) was done for the genotyping of *K. pneumoniae* isolates as previously described [13, 14].

Data analysis

The distribution of *K. pneumoniae* isolates by carbapenem resistance was plotted over time. Categorical data were presented as a percentage, while continuous data were presented as mean and standard deviation or median and interquartile range, as appropriate. SPSS software (release 18.0, SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

Results

In the year between September 2009 and August 2010, 20 (1.17%) out of 1,706 *K. pneumoniae* isolates detected at KAMC (Riyadh) were CRKP (Fig. 1). The epidemic curve showed a clear outbreak peak that did not resolve completely, creating an endemic pattern (Fig. 1). CRKP isolates were detected in samples from wound (40%), respiratory tract (25%), urine (15%), and other sites, including blood and the rectum (20%).

During March 2010, staff of the infection control and microbiology laboratory noticed the emergence of a cluster of CRKP isolates ($n=7$) in mainly adult ICU patients. Patients with CRKP were put under strict contact isolation, along with appropriate infection control measures for staff and visitors. These include intensifying hand hygiene compliance, starting CRKP active surveillance, activating within hospital transfer precautions, enhancing concurrent and terminal cleaning of patients' rooms, and staff education. Whenever feasible, respiratory therapists, nursing staff, and nursing aids were also cohorted during their shifts and on a rotating basis, to care exclusively for CRKP patients under contact isolation. Careful review of *K. pneumoniae* isolates over the previous 6 months revealed two previously unnoticed CRKPs; one in late February 2010 and another one in early December 2009. Admission and periodic active surveillance cultures showed a decrease but persistence of CRKP isolates in following months. Reviewing patients' records during the outbreak period through May 2010 showed that all patients with CRKP but one had stayed for some time in adult ICUs before the index date (Fig. 2). All the study isolates were tested with the MHT and were positive (showing cloverleaf-like indentation). PFGE results were available for 15 (75%) out of 20 isolates. Review of the PFGE results during the outbreak period showed that the majority of outbreak strains were genetically indistinguishable or closely related (Fig. 3). Isolates 1, 6, 8, 9, 10, 14, and 18 are 100% identical and closely related to 16 and 17 (similarity 97.15%), with isolate 7 having 90.91% similarity. Isolates 5 and 15 are 100% identical and closely related to 20 (similarity 97.15%). Isolate 19 is 77% similar to 5, 15, and 20. Isolate 3 is a distant similarity of 67% with the other 14 isolates.

The majority of patients were initially admitted to emergency rooms (55%) and adult ICUs (30%). About 15% of the patients were transfers from other hospitals. The admitted patients were 55% females and 45% males. The majority of patients (60%) were aged 19 to 59 years (mean±standard deviation [SD] 50.8±21.8). Their primary admission diagnoses were trauma including motor vehicle accident (25%) and liver or kidney failure (25%). The median APACHE III score was 67. The main comorbid conditions were surgery (50%), hypertension (50%), diabetes (45%), trauma (30%), and gastrointestinal disease, liver disease, and heart disease (25% each). CRKP patients required, on average, 63 days of hospital stay, 27.5 of them before the index date. The majority (85%) of patients required ICU admission during their hospital stay, with a median of 29 days of ICU stay. Two-fifths (40%) of patients presented with clinical infection. Out of those patients who presented with clinical infection, 50% had wound infections and 25% had bloodstream infection.

Many of the potential risk factors for developing multiple drug-resistant organisms (MDROs) were observed in CRKP patients (Table 3). The majority (90%) of CRKP patients spent at least 5 days in the hospital (the majority of which were in the ICU) prior to the index date. The majority (85%) of CRKP patients received antimicrobial therapy (for at least 2 days) during the month prior to the index date. Carbapenem (60%), antianaerobics (50%), colistin (45%), fluoroquinolones (35%), and vancomycin (35%) were the

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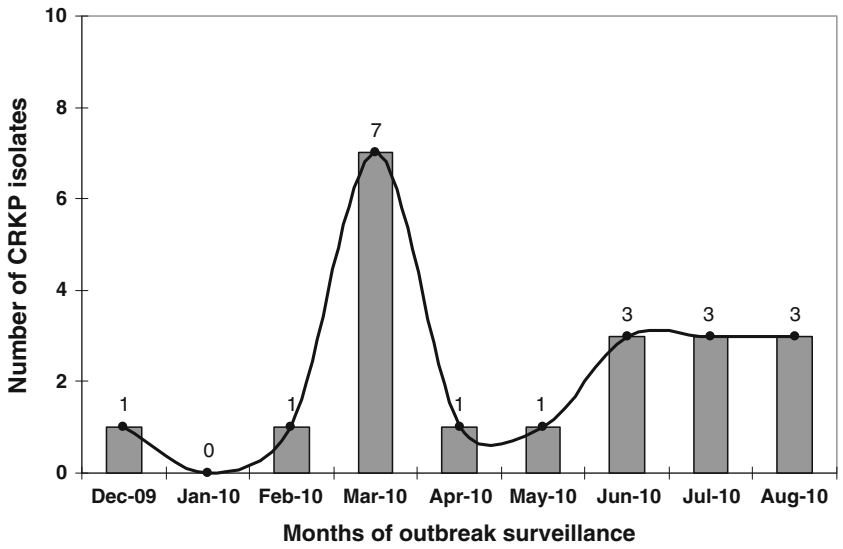

Fig. 1 Number and distribution of confirmed carbapenem-resistant *Klebsiella pneumonia* (CRKP) cases at King Abdulaziz Medical City (Riyadh, Saudi Arabia)

frequently used medications by CRKP patients. Half (50%) of the CRKP patients had previous colonization/infection with MDROs during the month prior to the index date, with *Acinetobacter* and *Pseudomonas* being the most frequently encountered MDROs. In addition, the majority of CRKP patients had recent history of indwelling devices, surgical procedures, and mechanical ventilation.

In addition to complete resistance to carbapenems, CRKP isolates had 90–100% resistance to cephalosporins, fluoroquinolones, and aminoglycosides. They were still generally susceptible to colistin (79%) and glycylcyclines (67%). The most frequently used mediations at the index date were carbapenem (55%), colistin (50%), fluoroquinolones (40%), tigecycline (40%), and tazocin (25%). CRKP

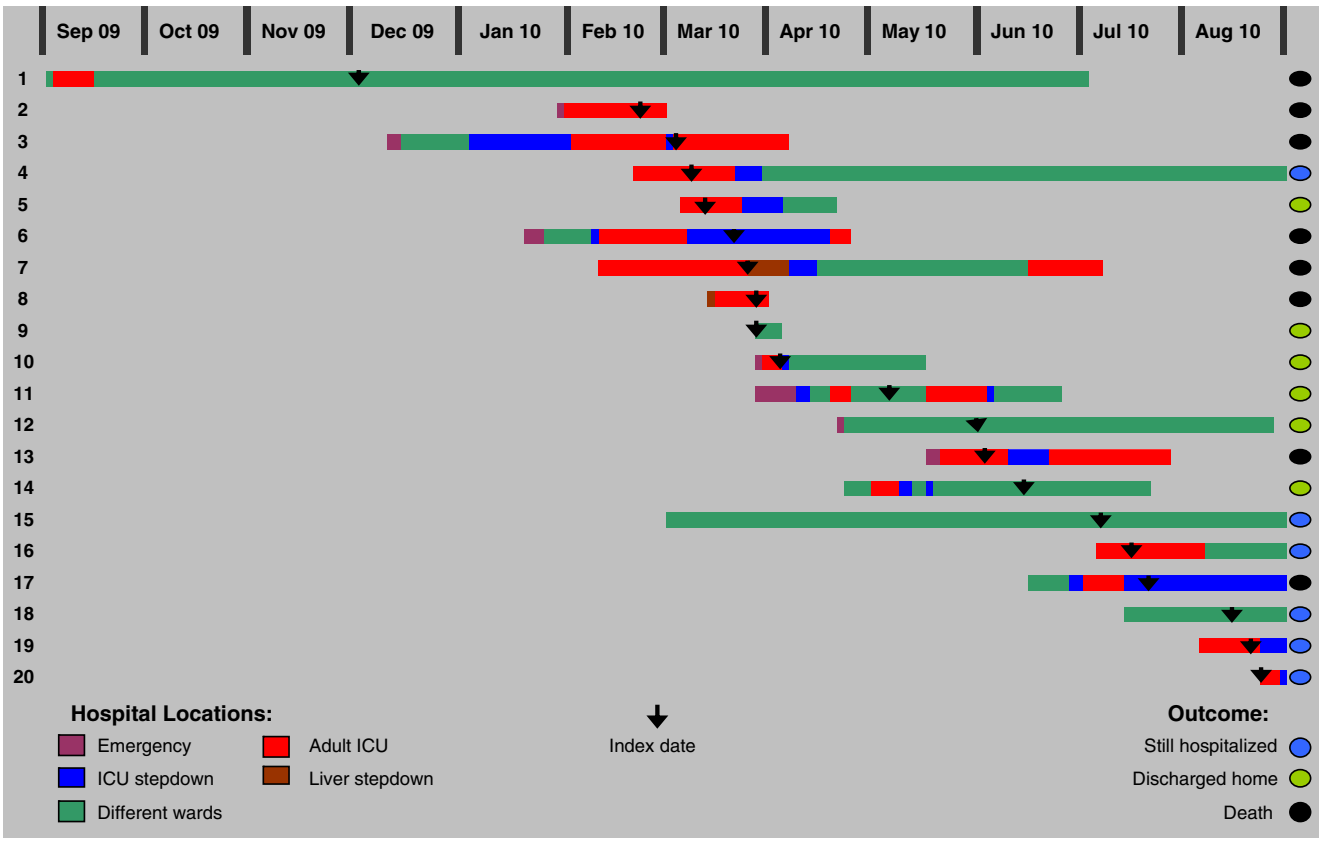

Fig. 2 Location and duration of hospitalization and outcome of CRKP at King Abdulaziz Medical City (Riyadh, Saudi Arabia) between September 2009 and August 2010

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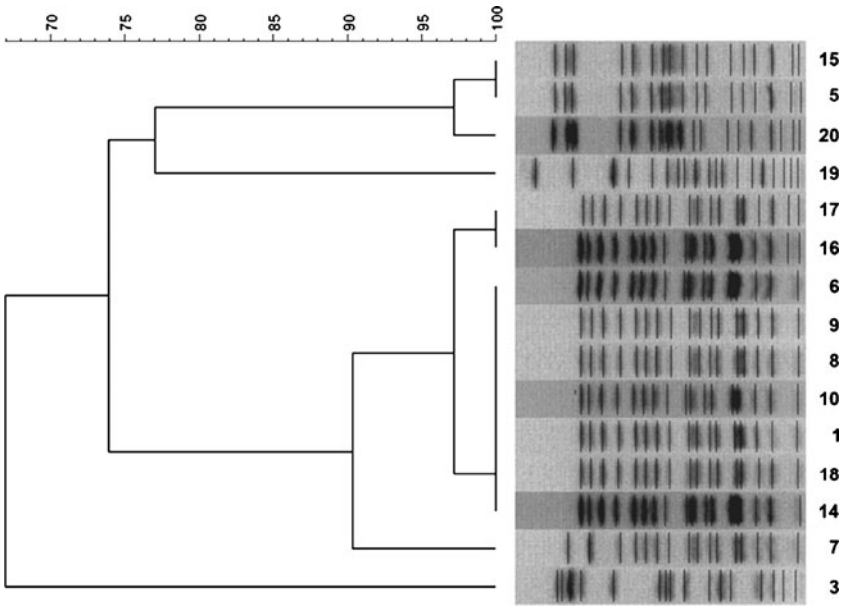

Fig. 3 Dendrogram based on pulsed-field gel electrophoresis (PFGE) of DNA restriction fragments of representative CRKP outbreak isolates (n=15). The dendrogram is shown on the left, with the percentage homology score indicated on top (0–100%). Actual pulsed field gel electrophoresis (PFGE) fingerprints are given on the immediate right of the dendrogram. Computer analysis of *Xba* I-digested isolates was performed using BioNumerics software (Applied Maths BVBA, Keistraat 120, 9830 Sint-Martens-Laten, Belgium)

patients required, on average, 24 days of antimicrobial therapy. By the end of August 2010, 8 (40%) of the CRKP patients died, 6 (30%) were discharged home, and 6 (30%) were still hospitalized (Table 3). The majority of deaths (75%) happened during the outbreak period through May 2010 and only 3 (38%) of the deaths happened within 4 weeks from the index date. Death happened at a median of 32 days from the index date. The most frequent causes of death were septic shock (71%), liver or kidney failure (57%), and cardiopulmonary arrest (57%).

Discussion

In this paper, we described our experience in detecting and containing the first documented CRKP outbreak in Saudi Arabia. As per CDC guidelines [9], we retrospectively reviewed the microbiological and clinical records of all *K. pneumoniae* isolates 6 months prior to the outbreak, as well as started active surveillance cultures. Active surveillance was shown to be very effective in controlling CRKP outbreak [15]. To early detect new CRKP isolates and to limit within-hospital spread, we have decided to keep CRKP active surveillance cultures to the routine workup done for all new ICU admissions. Unlike the outbreak setting, the effectiveness of active surveillance to limit the spread of CRKP and other multidrug-resistant *Enterobacteriaceae* in the non-outbreak setting is controversial [16–18]. Considering the cost benefit, we stopped monthly active surveillance cultures 3 months after declaring the end of the outbreak.

As shown in other studies [17, 19], reinforcing infection control measures together with contact isolation of patients colonized or infected with CRKP were successful in controlling the CRKP outbreak in our hospital. Staff education focused on the resistant nature of the CRKP organism and the absolute importance of staff adherence to infection control recommendations. Hand hygiene was an essential element of these measures, and we were able to increase its compliance at adult ICUs from 79.5% in March 2010 to 85–95% during the following months (data not shown). Nevertheless, infection control staff had to deal with some cultural and logistic challenges. Convincing the patient and his/her visitors with standard infection control measures is not always easy, especially when the patient is colonized. Cohorting respiratory therapists, nursing staff, and nursing aids was not always possible due to logistic reasons. Keeping the same level of infection control measures compliance at weekends is another challenge.

CRKP in the current study had 90–100% resistance to most of the commonly used antimicrobial agents, including carbapenems, cephalosporins, and fluoroquinolones, leaving very limited therapeutic options to treat severe hospital infections. Our isolates were, however, still generally sensitive to colistin and, to a lesser extent, to glycylcyclines [20, 21]. Recent reports showing the emergence of colistin-resistant CRKP in some parts of the world are alarming [21–23]. Although it is nephrotoxic, colistin is considered, by far, to be the only therapeutic choice currently available, as glycylcyclines have relatively low peak serum concentration to treat certain severe hospital infections, such as bloodstream infections [24]. Judicious use of antimicrobial agents may be seen as a long-term measure to reduce CRKP and other Gram-negative MDROs. The establishment of a comprehensive antimicrobial stewardship program, on the other hand, is a project worthy of leadership attention and support. As a consequence of CRKP outbreak, we initiated an antimicrobial use and resistance database in our ICUs to monitor carbapenem and other broad-spectrum antimicrobial use.

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Quick and adequate detection of CRKP is critical for the success of infection control measures. The detection of CRKP-producing bacteria based only on susceptibility testing is not easy, due to the heterogeneous expression of β-lactam resistance [10]. As per CDC recommendations [9], we used the MHT to confirm suspicious isolates based on the MICs breakpoints of the 2009 CLSI guidelines. Since the test is time-consuming and required reading expertise, securing results at the right time from an infection control point of view was sometimes challenging. The 2010 CLSI guidelines have overcome this problem by lowering the MICs for carbapenems, eliminating the need to perform the confirmatory MHT. The down side to this, however, is the large number of isolates that would mandate a larger number of patients to be placed on contact isolation according to an MDRO isolation policy. Therefore, we decided to continue to use the 2009 CLSI guidelines with all suspicious isolates to be reviewed by the director of the microbiology laboratory in order to decide on the need for the MHT.

The reported mortality in patients with CRKP is striking. It ranged between 40% and 70% [7, 25, 26]. Mortality in the current study was 38%. This crude mortality is probably overestimating the impact of CRKP and is confounded by multiple comorbidities. Only 38% of our CRKPs presented as HAI. It is estimated that about one-third to one-half of crude mortality associated with CRKP were actually attributable to CRKP infection [25, 26]. The high frequency of potential risk factors of MDROs in our CRKP patients is in agreement with previous reports describing CRKP risk factors, including prolonged hospital stay, prior hospitalization, prior antimicrobial intake, current stay in the ICU, recent surgery, and the use of a ventilator or indwelling device [7, 26, 27].

Conclusion

Reinforcing infection control measures together with contact isolation of patients colonized or infected with carbapenem-resistant *Klebsiella pneumoniae* (CRKP) were successful in controlling the CRKP outbreak, but did not prevent endemicity. Since CRKP has limited therapeutic options and is associated with high mortality, we have decided to focus on limiting the spread by continuing to carry out CRKP active surveillance cultures for all new intensive care unit (ICU) admissions. The need for new effective antibiotics against pan-resistant, Gram-negative, multiple drug-resistant organisms (MDRO) is now more critical than ever before.

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Author participation H.H.B., S.M.A.J., C.F., Y.A., A.A., and M.S. were actively involved in the outbreak management, collecting, and revising the data. A.E.-S. was involved in the data analysis and preparation of the manuscript. A.A.A.-Q., M.N.A.-A., and H.T.A. were involved in running the PFGE and the analysis. H.H.B. and A.E.-S. prepared and edited the final manuscript. All authors approved the final version of the manuscript.

Ethical approval This work was conducted after obtaining Institutional Review Board (IRB) approval from King Abdullah International Medical Research Center (KAIMRC), King Saud University for Health Sciences, Riyadh, Saudi Arabia.

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MAJOR ARTICLE

The Effect of Antibiotic Restriction Programs on Prevalence of Antimicrobial Resistance: A Systematic Review and Meta-Analysis

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Background. In hospital settings, restriction of selected classes of antibiotics is usually believed to contribute to containment of resistance development. We performed a systematic review and meta-analysis to assess the effect of restricting the use of specific antibiotic classes on the prevalence of resistant bacterial pathogens.

Methods. We conducted a systematic literature search in Embase and PubMed/OVID MEDLINE. We included studies until June 4, 2020 in which a restrictive antibiotic policy was applied and prevalence of resistance and use of antibiotics were reported. We calculated the overall effect of antimicrobial resistance between postintervention versus preintervention periods using pooled odds ratios (ORs) from a mixed-effects model. We stratified meta-analysis by antibiotic-pathogen combinations. We assessed heterogeneity between studies using the I^2 statistic and sources of heterogeneity using meta-regression.

Results. We included 15 individual studies with an overall low quality of evidence. In meta-analysis, significant reductions in resistance were only observed with nonfermenters after restricting fluoroquinolones (OR = 0.77, 95% confidence interval [CI] = 0.62–0.97) and piperacillin-tazobactam (OR = 0.81, 95% CI = 0.72–0.92). High degrees of heterogeneity were observed with studies restricting carbapenem (Enterobacterales, I^2 = 70.8%; nonfermenters, I^2 = 81.9%), third-generation cephalosporins (nonfermenters, I^2 = 63.3%), and fluoroquiolones (nonfermenters, I^2 = 64.0%). Results were comparable when excluding studies with fewer than 50 bacteria. There was no evidence of publication bias for any of the antibiotic-pathogen combinations.

Conclusions. We could not confirm that restricting carbapenems or third-generation cephalosporins leads to decrease in prevalence of antibiotic resistance among Enterobacterales, nonfermenters, or Gram-positive bacteria in hospitalized patients. Nevertheless, reducing fluoroquinolone and piperacilline-tazobactam use may decrease resistance in nonfermenters.

Keywords. antibiotic restriction; antibiotic stewardship; antimicrobial resistance.

The misuse of antibiotics is known to drive the development of antimicrobial resistance [1, 2]. To counter the growing problem of antimicrobial resistance, most hospitals have adopted antimicrobial stewardship programs, whereby the goal is to optimize the beneficial effects of antibiotics, while at the same time keeping its negative consequences for both individual patients and the community to a minimum.

Many researchers have pointed out the evident relationship between use of antimicrobials and rates of resistance, and thus a decrease of overall use is generally desirable to curb resistance [1]. Indeed, a decrease in antibiotic use can be considered beneficial if there is evidence that antibiotics are being used inappropriately. However, if antimicrobial use is already optimal in a specific setting, decrease of antibiotic use might give way to undertreatment. Furthermore, a decrease in the use of one antibiotic can result in increased use of an alternative antibiotic, possibly diminishing any intended overall effect on resistance. Taken together, it is important to realize that the quantity of antimicrobial use is difficult to interpret if it is given as a sole outcome measure. The spectrum of the used antibiotics must also be taken into account [3–5].

We previously performed a systematic review and meta-analysis in which we assessed whether 14 antimicrobial stewardship objectives, such as pathogen-directed therapy or switching from intravenous to oral therapy, had tangible effects in hospitals and long-term care facilities [6]. We could establish an effect on resistance rates for only 1 stewardship objective, namely, using a list of restricted antibiotics. It is unfortunate that, due to the broad scope of that study, we were unable to

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[a]Our friend and colleague Johan Mouton regretfully passed away on July 9, 2019.

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further elaborate on other results obtained from the included studies. For that reason, in the present systematic review, we took a more in-depth look and also performed an update of the initial literature search, with the objective of systematically determining whether and which antibiotics can be restricted to help reverse antimicrobial resistance rates. More specifically, we aimed to analyze the specific effect of restricting particular antibiotic classes on the resistance rates of various bacterial pathogens. We additionally intended to assess whether other accompanying variables could influence the effect of restricted antibiotic use on resistance prevalence.

METHODS

Search Strategy

We had previously reviewed the evidence published until April 11, 2014 of the influence of 14 hospital antimicrobial stewardship objectives on 4 patient outcomes (ie, clinical outcomes, adverse events, costs, and bacterial resistance prevalence) [6]. This study encompassed individual systematic reviews for these 14 objectives, one of which involved using lists of restricted antibiotics. With the help of a clinical librarian, we performed an updated search of studies that investigated the effect of using a list of restricted antimicrobials published in Embase and PubMed/OVID MEDLINE from April 11, 2014 until June 4, 2020. Detailed search terms are presented in Supplementary A Search strategy.

All titles and abstracts were reviewed by one author (E.C.S.), and a second author (J.M.P. or J.W.M.) independently screened a random selection of 10% of all titles and abstracts. Discussion was used to resolve any discordances on inclusion or exclusion of studies, when necessary with a third reviewer. Both reviewers then assessed the eligibility of all articles initially identified as potentially relevant by reading the full text. We searched for additional studies suitable for inclusion using the reference list of the articles that were viewed in full text.

Inclusion and Exclusion Criteria

We looked for studies that assessed the effect of restrictive antimicrobial prescribing. By restrictive prescribing, we mean removal of specific antibiotics from the formulary, restriction of use by requiring preauthorization from a specialist (infectious diseases or medical microbiology), or allowing use for only 24–72 hours with mandatory approval for further use. The main antibiotic classes of interest were carbapenems, fluoroquinolones, and third-generation cephalosporins. Papers in which none of these 3 antibiotics were restricted were excluded.

Prespecified study types that were eligible for inclusion were randomized controlled trials, nonrandomized controlled trials (controlled before-after studies), interrupted time series, observational studies (including cohort, cross-sectional, and case-control studies), and systematic reviews. Excluded designs were case reports, narrative reviews, discussion papers, conference papers, letters to the editor, and editorials. We included studies in English, German, and Dutch.

We excluded studies that did not report data on both resistance prevalence and antimicrobial use. The number of isolates tested for each bacterial pathogen, with their resistance prevalence, had to be reported or calculable. If a study reported resistance percentages without the number of bacterial isolates tested, the effect size was unable to be estimated and hence the study was excluded. We did not restrict our search to a specific infection; however, study outcomes needed to relate to antibiotic treatment and be performed in hospitals or long-term care facilities. We included research in adults (18 years or older) and not in children or adolescents. We excluded studies addressing the outpatient or general practitioner setting. Studies in an outbreak setting were excluded, because these often contain multiple interventions used for infection control. Studies on methicillin-resistant *Staphylococcus aureus* or Clostridium *difficile* were not included in this study.

Data Extraction

The following parameters were extracted from the included papers: the year of publication, first author's name, study setting (intensive care unit [ICU], hospital, mixed), details and duration of the intervention and control conditions, number of individuals and number of individuals with antimicrobial-resistant bacteria in the preintervention and postintervention periods, quantity of antibiotics used in the preintervention and postintervention periods, postintervention duration, and type of infection studied (nosocomial, community-acquired, both). If bacteria were not clearly reported as nosocomial or community-acquired, we considered colonization and infection from studies conducted in the ICU department as nosocomial.

We extracted data (1) from the period before the start of the intervention and (2) at the end of the intervention or the last reported measurement. The prevalence of resistance during the preintervention and postintervention periods had to be reported for Enterobacterales, nonfermenters, or Gram-positive bacteria. If the number of susceptible bacteria were only given, we assumed that the remaining bacteria had resistance. We also extracted the use of nonrestricted antibiotics, if reported, to assess whether there was any carryover effect of restrictive use to other antibiotics, which could then influence resistance prevalence. We limited these antibiotics to cotrimoxazole, piperacillin-tazobactam, and first- and second-generation cephalosporins.

We extracted data from either the manuscript text and/or tables. If necessary, we extracted data by estimating the approximate number from a graph. When 2 or more antibiotics with comparable resistance prevalence were reported within a class, we used the mean number of isolates with resistance across antibiotics divided by the total number of isolates tested

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to calculate the resistance prevalence for that class. We included all measures for use of antibiotics, such as defined daily doses or the amount of antibiotics in grams, and reported the difference in use between preintervention and postintervention periods as percentages. We did not extract data on costs.

The information from all included studies was extracted by one reviewer (E.C.S.) and fully checked for accuracy by a second reviewer (J.M.P.). Missing data were not requested from study authors, and if the predefined data were not present, incomplete, or not interpretable, the study was excluded.

Quality Assessment

The quality of each included study was assessed independently by 2 reviewers (E.C.S. and J.M.P.) using the Cochrane Risk of Bias tool for randomized controlled trials and the Newcastle-Ottawa Quality Assessment Scale for nonrandomized studies [7, 8]. Disagreement between the reviewers was resolved by discussion, with the involvement of a third reviewer if necessary.

Statistical Analysis

We carried out all analysis using STATA (version 15.1; College Station, TX) and R (version 3.6.1; Vienna, Austria). For each study, we calculated odds ratios (ORs) comparing the odds of having antimicrobial resistance during the preintervention versus postintervention periods. We conducted meta-analyses of the calculated ORs using the "metan" command in STATA. We stratified meta-analysis by all combinations of antibiotic targeted (carbapenem, fluoroquinolones, third-generation cephalosporins, cotrimoxazole, piperacillin-tazobactam, and first- and second-cephalosporins) and bacteria (Enterobacterales, nonfermenters, or Gram-positive bacteria). We used a random-effects model to calculate DerSimonian and Laird pooled ORs. We also conducted sensitivity analyses in which studies with fewer than 50 participants in either the preintervention or postintervention period were excluded.

We assessed heterogeneity between studies using the I^2 statistic with an I^2 of >75% indicating substantial heterogeneity [9]. We explored sources of heterogeneity with meta-regression using the "metareg" command in STATA. The unit of measure analyzed was antibiotic-pathogen combination, and we stratified meta-regression by antibiotic targeted where there were more than 5 antibiotic-pathogen combinations available. The covariates in the meta-regression included difference in antibiotic usage in the postintervention versus preintervention period (relative change in percentage), type of bacteria (Enterobacterales, Gram-positive, or nonfermenters), prevalence of antimicrobial resistance in the preintervention period, duration of the postintervention period (in years), type of infection (nonspecified or nosocomial), and setting (hospital or ICU). We assessed the influence of outlying studies within antibiotic-pathogen combinations, provided there were more than 3 studies within a subgroup, using the method developed by Viechtbauer and Cheung [10] in the "metafor" package in R. We removed studies identified as outlying in a sensitivity analysis. We also assessed for publication bias using the Egger test of bias [11] with $P < .05$ indicating significant publication bias.

RESULTS

Search Results

Figure 1 summarizes the selection of studies for this updated systematic review. In our initial systematic review [6], we included 26 studies reporting data on resistance prevalence after applying a restrictive antibiotic policy. After excluding 16 studies that did not meet inclusion criteria for this analysis, 10 studies remained.

During the updated search (June 4, 2020), we found 911 new citations after removing duplicates: 447 in Embase and 464 in PubMed/MEDLINE. After screening titles and abstracts, we selected 44 potentially relevant studies for full-text screening. Less than 2.5% of the papers screened by the second author were missed as eligible by the first author. We identified 2 relevant papers after reviewing reference lists. Five studies were eligible for final inclusion (Figure 1, flowchart of systematic search; Supplementary A, search strategy). The main reasons for excluding studies were as follows: lack of data on predefined outcomes, study design and publication format, written in a language other than those of the search parameters, or missing data. Ten studies were excluded for not providing the number of isolates tested for each bacterial pathogen [12–21].

Combined with the 10 studies from the previous systematic review, 15 articles were included in the present analysis (Figure 1, flowchart of systematic search). The 15 studies originated from 11 countries, covering 5 continents (Supplementary B, characteristics). The duration of restrictive policies differed between studies, varying from 6 months to 10 years. Most studies [8] reported data from hospital wards, 6 from the ICU exclusively and 1 study from both settings. Only 1 study was performed in more than 1 hospital, whereas the remainder were single-center studies set at university [1], general care [5], and tertiary care hospitals [8]. No studies were performed in long-term care facilities. All studies were observational with a serious risk of qualitative bias, and thus the quality of research was judged to be poor (Supplementary B, characteristics of included studies).

Studies were grouped based on the restriction of the 3 main antibiotic classes: carbapenems [6, 22–27], fluoroquinolones [8, 22, 23, 28–33], and third-generation cephalosporins [9, 22–24, 29, 31, 32, 34–36]. In addition, we found studies on use and resistance prevalence of nonrestricted antibiotics for piperacillin-tazobactam [3, 22, 24, 36] and first- and second-generation cephalosporins [3, 22, 23, 31], but not for cotrimoxazole. For both restricted and nonrestricted antibiotics, most studies reported

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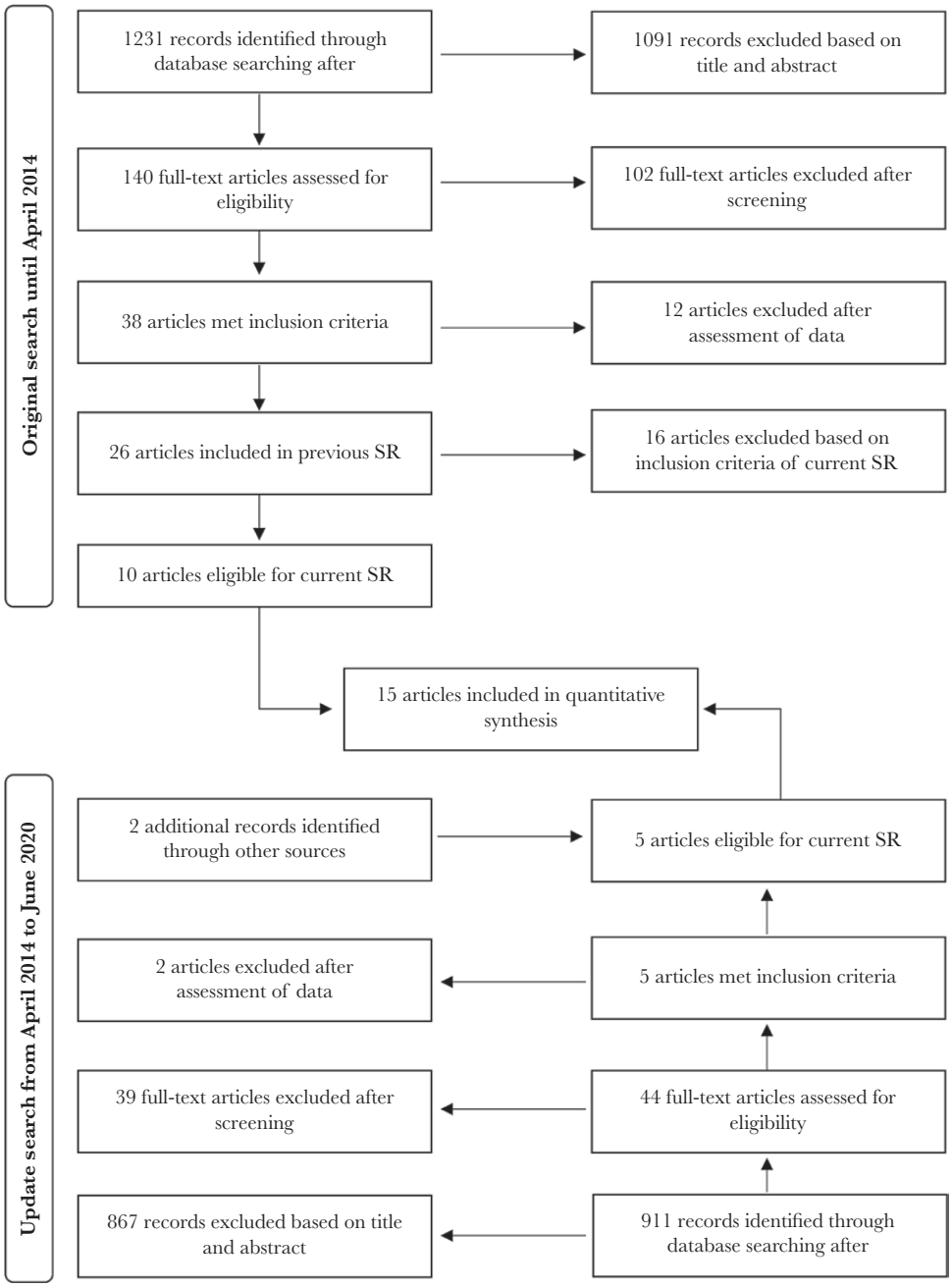

Figure 1. Flowchart of systematic search. SR, systematic review.

resistance prevalence in Enterobacterales and nonfermenters, whereas only 2 studies reported resistance prevalence in Gram-positive bacteria [23, 31]. Antibiotic use during preintervention and postintervention periods are provided according to study in Supplementary C, antibiotic use per study.

Effect of a Restrictive Intervention

Figure 2 displays a forest plot with individual ORs, indicating the effect sizes of each study. We observed a high degree of heterogeneity (I^2) across studies for most antibiotic-pathogen combinations, indicating large between-study variability. Exceptions were piperacillin-tazobactam/nonfermenters (I^2 = 0.0%), piperacillin-tazobactam/Enterobacterales (I^2 = 0.0%), and fluoroquinolones/Enterobacterales (I^2 = 0.0%). We did observe a significant effect for several antibiotic-pathogen combinations in individual studies. For example, data from Zhang et al [27] showed a significant 41% decrease in the odds of resistance of nonfermenters to carbapenems during the postintervention versus preintervention period (OR = 0.59, 95% confidence interval [CI] = 0.44–0.78). However, the pooled OR for this antibiotic-pathogen combination was not significant (OR = 0.87, 95% CI = 0.70–1.08). For 2 antibiotic-pathogen

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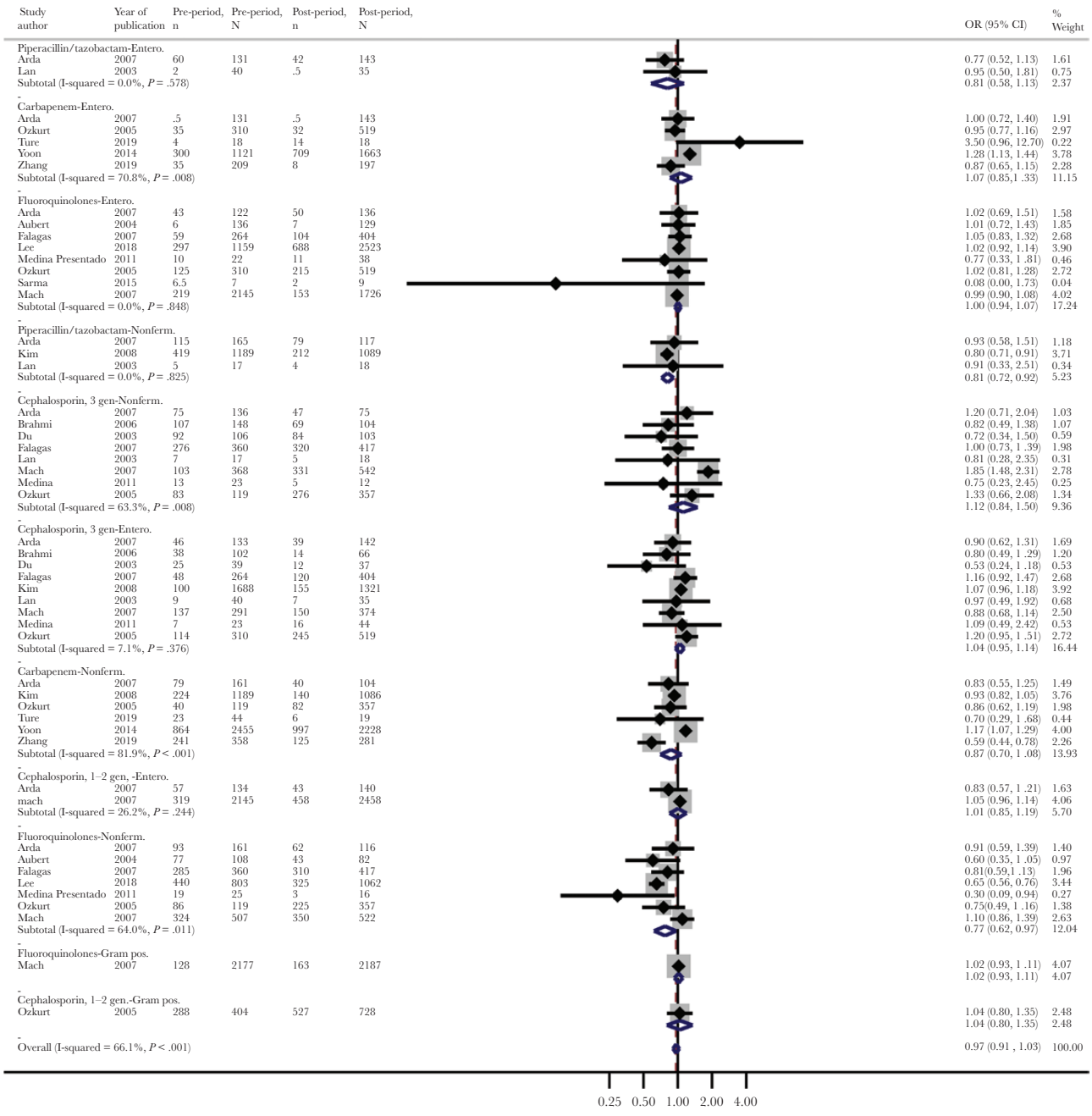

Figure 2. Effect sizes (odds ratios) comparing the prevalence of antibiotic resistance before and after implementing interventions to restrict antibiotic usage, analyzed in subgroups of antibiotic pathogen.

combinations, we did find a modest effect of restriction when comparing the pooled odds of having resistance during the postintervention versus preintervention periods: a 23% decrease in resistance to nonfermenters after restricted use of fluoroquinolones (OR = 0.77, 95% CI = 0.62–0.97) and a 19% decrease in resistance to nonfermenters after restricted use of piperacillin-tazobactam (OR = 0.81, 95% CI = 0.72–0.92). It is notable that, in these studies, piperacillin-tazobactam was not a part of the restricted antibiotics during the postintervention period. Due to a limited number of studies, a possible carryover effect could not be investigated for the remaining nonrestricted antibiotics (first- and second-generation cephalosporins and cotrimoxazole).

Results were comparable when excluding studies with fewer than 50 bacteria in either the preintervention or postintervention period (Supplementary D, forest plot of studies with ≥50 individuals in both periods). We identified 5 outlying studies for the bug-drug combinations that could be evaluated. When excluding these studies, we obtained comparable results, yet heterogeneity overall was reduced, and a significant 20% reduction

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in resistance to nonfermenters was observed after restricted use of carbapenems (Supplementary E, influential studies removed). There was no evidence of publication bias for any of the antibiotic-pathogen combinations (Supplementary F, funnel plots).

In Table 1, we summarized the results of the meta-regression stratified on specific classes of antibiotics. Studies performed in the ICU versus other settings had significantly higher increases in resistance after restricting carbapenems (exp(β) = 2.78, 95% CI = 1.01–7.65, *P* = .04), whereas this was the opposite after restricting cephalosporins (exp(β) = 0.41, 95% CI = 0.20–0.84). The preintervention prevalence of antimicrobial resistance did not significantly change the effect on resistance after restricting carbapenems or third-generation cephalosporins (Figure 3A and B, respectively). However, higher preintervention prevalence of resistance did lead to stronger reductions in resistance after restricting cephalosporins (exp(β) percent = 0.98, 95% CI = 0.97–0.99) and fluoroquinolones (exp(β) percent = 0.98, 95% CI = 0.97–0.99), yet the observation with fluoroquinolones seems to be driven by 2 smaller studies with high preintervention prevalence (Figure 3C). No other factors, such as nosocomial infections, postintervention antibiotic use, or intervention duration, were found to explain the heterogeneity between studies within the same class of antibiotics.

DISCUSSION

Our systematic review and meta-analysis of 15 studies indicates that applying restrictive antibiotic interventions has a significant effect on the resistance of nonfermenters to fluoroquinolones. Reducing piperacillin-tazobactam use, although not through a restrictive intervention, also showed to have a significant effect on resistance of nonfermenters. For the other antibiotic-pathogen combinations, restriction did not result in a reduction of resistance. After excluding outlying studies in a sensitivity analysis, we obtained comparable results, while heterogeneity overall was reduced and a significant 20% reduction in resistance to nonfermenters was also observed after restricted use of carbapenems. In an exploratory analysis, no consistent factors were identified explaining the heterogeneity between studies within the same class of antibiotics.

Our findings are in disagreement with current in-hospital practice and with individual studies. It is therefore important to consider the substantial inconsistencies in the direction of effect sizes between included studies, which led to a high degree of heterogeneity, and the generally low quality of evidence. Sensitivity analyses excluding small studies yielded comparable results, and they did not indicate the presence of publication bias. The potential bias of analyzing aggregated data instead of individual-patient data could have also influenced the lack of observed relationships between antibiotic use and resistance [37]. Based on the present comprehensive overview of the available evidence, it is therefore perhaps too straightforward to conclude that applying a restrictive antibiotic policy is in general ineffective. However, high-quality research is lacking and is clearly needed before concluding otherwise.

This study has several strengths. First, we designed a broad search strategy and did not limit the search to specific antibiotics or bacterial species. Second, the risk of bias was assessed for each study by 2 independent authors, with a scale that was most suitable to the study design. Third, we looked for evidence for possible carryover effects of restriction by examining whether restrictive use of one antibiotic led to an increase in the use of other, nonrestricted antibiotics, with resulting increased resistance prevalence to these antibiotics. Finally, many of these

Table 1. Univariate Meta-Regression for the Effects of Antibiotic Restriction on Prevalence of Antimicrobial Resistance[a]

	Carbapenem			Cephalosporins			Fluoroquinolones		
	N	exp(β) (95% CI)	*P*	N	exp(β) (95% CI)	*P*	N	exp(β) (95% CI)	*P*
Relative difference in antibiotic usage (%)	11	0.99 (0.97–1.01)	.17	9	1.00 (0.99–1.02)	.60	14	1.01 (1.00–1.02)	.07
Bacteria									
Enterobacterales	5	Ref		9	Ref		8	Ref	
Nonfermenters	6	0.63 (0.16–2.51)	.47	8	1.19 (0.57–2.50)	.62	7	0.63 (0.34–1.16)	.13
Gram-positive	0	--		0	--		1	1.34 (0.44–4.05)	.58
Prevalence antimicrobial resistance preintervention (%)	11	0.99 (0.94–1.04)	.73	17	0.98 (0.97–0.99)	.04	14	0.98 (0.97–0.99)	.04
Duration of intervention (years)	11	0.86 (0.66–1.14)	.26	14	1.34 (0.83–2.17)	.21	16	0.98 (0.88–1.09)	.69
Type of Infection									
Nonspecified	5	Ref		8	Ref		13	Ref	
Nosocomial	6	1.57 (0.33–7.39)	.53	6	0.60 (0.26–1.38)	.21	3	1.03 (0.38–2.76)	.95
Setting									
Hospital	7	Ref		7	Ref		10	Ref	
ICU	4	2.78 (1.01–7.65)	.04	7	0.41 (0.20–0.84)	.02	6	0.74 (0.35–1.55)	.40

Abbreviations: CI, confidence interval; ICU, intensive care unit; N, number of antibiotic-pathogen combinations included; Ref, Reference variable.

[a]The reduction in resistance prevalence after antibiotic restriction was significantly stronger because the exponentiated regression coefficient (β) was significantly less than one and vice versa. "—," no studies pertained to these categories and hence the parameter estimate could not be calculated. Analysis was not performed for first- and second-generation cephalosporins and piperacillin-tazobactam due to limited numbers of antibiotic-pathogen combinations.

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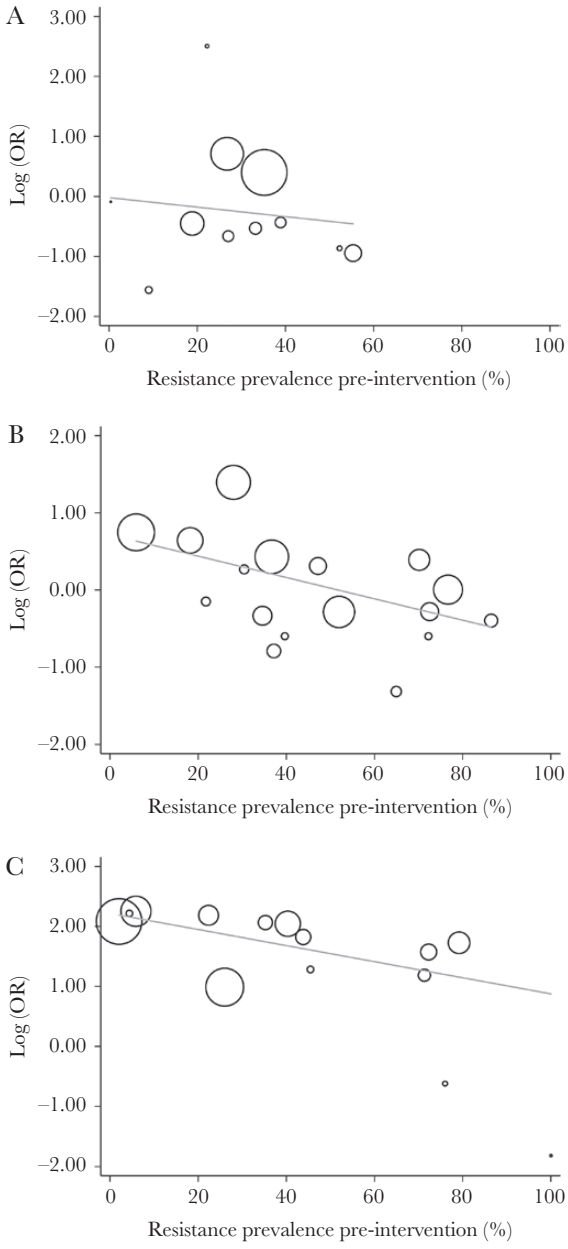

Figure 3. Effect size in relation to resistance prevalence preintervention for carbapenems (A), third-generation cephalosporins (B), and fluoroquinolones (C). OR, odds ratios.

studies included low numbers of isolates and might not have been sufficiently powered to describe the effect of a restrictive intervention on a given antibiotic-pathogen combination. By pooling these studies together, we are able to offer a more robust picture of the effect that these interventions have on resistance.

However, we should be aware that 4 studies did not clearly report whether bacteria were nosocomial or community-acquired [23, 29–31]. Second, only a limited number of studies were included per antibiotic group, and, although we carefully assessed factors influencing resistance rates, it is possible that certain variables affecting antimicrobial resistance were left out of the meta-regression. Third, we did not perform a search of gray literature and restricted our search to Embase and PubMed/OVID MEDLINE. This may have resulted in some relevant studies being missed. We did not apply a filter for publication date in our search, and the final analysis included studies published between 1985 and 2020. The resistance rates and antibiotics used have changed considerably over this period. Finally, we only focused on 3 restricted antibiotic classes in this systematic review, and thus we are unable to draw any conclusions on the effect of restrictive antibiotic policies for other antibiotics.

It has been commonly understood that increased antibiotic use is correlated with the development of antimicrobial resistance at the population level [38]. Furthermore, restricting overall antibiotic use has been shown to decrease antibiotic resistance in livestock, in outpatients, and during infection outbreaks [39–44]. In our previous systematic review[6], we reported that "Restrictive antibiotic policies were associated with reduced resistance rates in most of the studies we assessed, but inconsistent relations between antibiotic use and resistance rates were also found." A recent qualitative systematic review also provided support towards a beneficial effect of these policies for several antibiotic-pathogen combinations [45]. In our current, in-depth analysis, which only included studies reporting the number of isolates tested, we conclude that, in contrast to interventions in livestock and in the community, restrictive antibiotic policies as a single intervention might not be an effective tool to achieve decreases in the prevalence of resistance in hospitalized adults. Considering that most studies reported nosocomial infections or were conducted in an ICU setting, resistance in these patients was probably acquired or selected during admission.

A systematic review and meta-analysis by Baur et al [46] has shown a significant reduction in the incidence of infections and colonization with antibiotic-resistant bacteria through implementation of antibiotic stewardship programs. This indicates a beneficial effect of a multifactorial intervention. However, the direct effect of a single stewardship improvement strategy, such as restriction, could not be assessed in their study.

CONCLUSIONS

The currently available evidence for the selected antibiotic-pathogen combinations in hospitalized patients is insufficient to conclude that applying a short-term restrictive antibiotic prescribing policy has the generally presumed effect of decreasing bacterial resistance. A restrictive intervention might be useful when specifically targeting a decrease in resistance of nonfermenters. Long-term effects cannot be excluded, because the time window of these interventions was often relatively short. Given the poor quality of studies with regard to methods used and reporting of data, we feel that there is a strong need for high-quality studies to address this question and to explore alternative interventions that could bring way to reduced prevalence of antimicrobial resistance.

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Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Therapeutic Advances in Drug Safety Review

Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem

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***Abstract*:** Antimicrobial resistance is a global public health challenge, which has accelerated by the overuse of antibiotics worldwide. Increased antimicrobial resistance is the cause of severe infections, complications, longer hospital stays and increased mortality. Overprescribing of antibiotics is associated with an increased risk of adverse effects, more frequent re-attendance and increased medicalization of self-limiting conditions. Antibiotic overprescribing is a particular problem in primary care, where viruses cause most infections. About 90% of all antibiotic prescriptions are issued by general practitioners, and respiratory tract infections are the leading reason for prescribing. Multifaceted interventions to reduce overuse of antibiotics have been found to be effective and better than single initiatives. Interventions should encompass the enforcement of the policy of prohibiting the over-the-counter sale of antibiotics, the use of antimicrobial stewardship programmes, the active participation of clinicians in audits, the utilization of valid rapid point-of-care tests, the promotion of delayed antibiotic prescribing strategies, the enhancement of communication skills with patients with the aid of information brochures and the performance of more pragmatic studies in primary care with outcomes that are of clinicians' interest, such as complications and clinical outcomes.

Keywords: Antibiotic resistance, primary care, point-of-care tests, rational use of antibiotics, strategies

Introduction

Antimicrobial resistance (AMR) is recognized as one of the greatest threats to human health worldwide. Just one organism, methicillin-resistant *Staphylococcus aureus* (MRSA), kills more Americans every year than emphysema, HIV/AIDS, Parkinson's disease and homicide combined [Infectious Diseases Society of America *et al.* 2011]. Globally, 3.7% of new cases and 20% of previously treated cases of tuberculosis are estimated to be caused by strains that are resistant to isoniazid and rifampicin. For decades, these antituberculosis agents have been effective against tuberculosis, but today the effect is insufficient. Nowadays, only one-half of multidrug-resistant tuberculosis is effectively treated with the existing drugs [World Health Organization, 2014]. Extensively drug-resistant tuberculosis (defined as multidrug-resistant tuberculosis plus resistance to any fluoroquinolone and any second-line injectable drug) has been identified in 84 countries globally [World Health Organization, 2013]. Carbapenem-resistant Enterobacteriaceae spp. and extended-spectrum beta-lactamase-producing Enterobacteriaceae have been isolated in recent years [Nordmann *et al.* 2009; Ho *et al.* 2010; Oteo *et al.* 2010; Society for Healthcare Epidemiology of America, Infectious Diseases Society of America, and Pediatric Infectious Diseases Society, 2012]. There is a striking lack of development of new drugs active against these multidrug-resistant Gram-negative bacteria, particularly those producing carbapenemases [Boucher *et al.* 2013], and none of the antibiotics currently available are now effective [Falagas *et al.* 2008; Chen *et al.* 2009; Society for Healthcare Epidemiology of America, Infectious Diseases Society of America, and Pediatric Infectious Diseases Society, 2012].

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While antibiotic resistance has predominantly been a clinical problem in hospital settings, recent data show resistant organisms have also been detected in patients in primary care [National Collaborating Centre for Infectious Diseases, 2010]. A recent report from the World Health Organization (WHO) clearly states that this is not a phenomenon occurring in just poor or developing countries; the problem of AMR is now found throughout the world [World Health Organization, 2014]. Diseases associated with AMR in primary care include tuberculosis, gonorrhoea (specifically *Neisseria gonorrhoeae*), typhoid fever and Group B streptococcus [Centers for Disease Control and Prevention, 2012]. Community-acquired AMR is of particular concern, as these infections can be common and easily transmitted. The most recent data from the European Antibiotic Surveillance Reports found that antibiotic resistance rates of *Escherichia coli* and/or *Klebsiella pneumoniae* vary markedly between countries. Rates of resistant *E. coli* varied 18-fold between Sweden (1.0%) and Greece (18.2%) and for *K. pneumoniae* the differences were even more pronounced, ranging from 0.7% in Sweden to 64.1% in Greece [European Centre for Disease Prevention and Control, 2011]. However, antibiotic resistance of *E. coli* and *Klebsiella* spp. is highest in Asia (≥60%), with rates of 10–30% in Southern Europe, and 5–10% in Northern Europe, Australasia and North America [Livermore, 2012]. European data from 2011 demonstrate an alarming increase in the resistance of these organisms, with around a third of European countries showing a rise in combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides over the previous 4 years [European Centre for Disease Prevention and Control, 2011]. Some of these types of antibiotics are considered by the WHO as 'critically important antimicrobials' in medicine [World Health Organization, 2009], and these broad-spectrum antibiotics should be avoided when narrow-spectrum antibiotics remain effective, as they also increase the risk of *Clostridium difficile* infection, MRSA and resistant urinary tract infections [Public Health England, 2013]. The problem of resistance not only involves the community, it also affects the individual. A recent review describing patients with bacterial urinary tract and respiratory tract infections treated with antibiotics reported that individual resistance may persist for up to 12 months post-treatment, thereby creating situations with the need of requiring second-line antibiotics [Costelloe *et al.* 2010].

Infection with antibiotic-resistant bacteria may cause severe illness, increased mortality rates, and an increased risk of complications and admission to hospital [Kollef, 2008; Paul *et al.* 2010; Livermore, 2012]. According to the European Centre for Disease Prevention and Control, 25,000 people in Europe die each year as a direct result of resistant infection [European Centre for Disease Prevention and Control, 2011]. Antibiotic resistance leads to an increased amount of healthcare costs. It is estimated that complications associated with antibiotic resistance cost €9 billion annually in Europe [Oxford and Kozlov, 2013]. A recent review demonstrated that the additional cost of resistance could be of £20,000 per patient episode in hospital [Smith and Coast, 2013].

In the context of few innovative or new antibiotics in the drug development pipeline, the WHO describes a future of a post-antibiotic world and warns that not only will this eliminate the advances in healthcare made over the past 100 years, which have ensured longer life in most parts of the developed and developing worlds, but it may also result in simple infections becoming unmanageable and potentially fatal [World Health Organization, 2012a, 2012b]. The United Kingdom Chief Medical Officer has highlighted the need for clinicians to preserve the effectiveness of antibiotics by giving clear evidence-based guidance on their appropriate use [Department of Health, 2012] and has stated that we are losing the battle against infectious diseases, and antibiotics may no longer be effective in the long term [Davies *et al.* 2013].

Most of the antibiotics used in medicine are prescribed by general practitioners (GP). In fact, primary care accounts for 80–90% of all antibiotic prescriptions in Europe and most antibiotics are prescribed for respiratory tract infections [European Centre for Disease Prevention and Control, 2014]. Utilization of antibiotics is also very important in other sectors; for instance, approximately 80% of antibiotics in the United States are consumed in agriculture, farming and aquaculture [Hollis and Ahmed, 2013]. Data show a direct correlation between the use of antibiotics and resistance. Countries with a higher consumption of antibiotics show higher rates of resistance [Goossens et al. 2005; Riedel *et al.* 2007]. Antibiotic prescribing differs profoundly from one European country to the next, although

Box 1. Risks that have been shown to be associated with overuse of antibiotics.

- Increase of antimicrobial resistance
- Increase of more severe diseases
- Increase of the length of disease
- Increase of the risk of complications
- Increase of the mortality rate
- Increase of healthcare costs
- Increase of the risk of adverse effects, some being life-threatening
- Increase of re-attendance due to infectious diseases
- Increased medicalization of self-limiting infectious conditions

there is no evidence of differences in the prevalence of infectious diseases. On average, the European consumption rate of antibiotics is 18.3 defined as daily doses/1000 inhabitants/day (DID) in 2010, with the highest rate in Greece with 39.4 DID and the lowest in two Baltic countries with 11.1 DID or 11.2 DID of Holland [European Centre for Disease Prevention and Control, 2010]. A recent study has shown that the consumption of antibiotics is even greater in the new southern and eastern European countries, with an antibiotic use of 42.3 DID in Turkey [Versporten *et al.* 2014].

Apart from spreading resistance, antibiotic overprescribing is also associated with other problems (Box 1). Consumption of antibiotics puts patients at risk of adverse effects. Antibiotics account for approximately 20% of all drug-related emergency department visits in the United States. Although nearly 80% of these visits are attributable to allergic reactions, certain commonly prescribed antibiotics contribute to conditions that range from gastrointestinal to neurologic and psychiatric disorders [Lode, 2010]. Most of these adverse effects are mild, but some life-threatening adverse effects have been reported, such as hepatotoxicity due to amoxicillin and clavulanate [Chang and Schiano, 2007]. Antibiotic overprescribing has been shown to increase patient re-attendance as it medicalizes conditions, which are self-limiting [Little *et al.* 1997]. And more attendance means more prescription of antibiotics.

Why are there such differences in antibiotic consumption in Europe? These differences cannot be explained by a different pattern of infectious diseases across countries. It is clear that the main concern is to avoid under-treatment [Kumar *et al.* 2003]. None of us wants to be seen to have withheld treatment from a patient who subsequently deteriorates, especially if the patient is hospitalized. Although rare, it might damage doctor–patient relationships and lead to complaints and medical–legal consequences. However, most of the respiratory tract infections attended by GPs are self-limiting. In Europe, upper respiratory tract infections account for 57% of the antibiotics used, with a further 30% for lower respiratory tract infections; in contrast, the next most common condition is urinary tract infections at 7% [van der Velden *et al.* 2013]. Moreover, respiratory tract infections are the most commonly treated acute problem in primary care [Francis *et al.* 2009], with most caused by a virus, to which antibiotics have shown to have a limited effect on symptoms. In a recent controlled clinical trial with placebo, with data from the GRACE project (http://www.grace-lrti.org) in 12 European countries including more than 3000 adults with acute cough (≤28 days) and without suspicion of pneumonia based on clinical grounds, the percentage of patients with new or worsening symptoms was slightly less frequently observed among those treated with amoxicillin 3 g daily compared with controls (16% *versus* 19%, number needed to treat = 30) but the prevalence of nausea, diarrhoea, or rash occurred more frequently in the former (number needed to harm = 23) [Little *et al.* 2013a].

The benefits of antibiotic therapy for most respiratory tract infections are modest in the best-case scenario [Kenealy and Arroll, 2013; Spinks *et al.* 2013; Venekamp *et al.* 2013; Smith *et al.* 2014]. If respiratory tract infections are mostly self-limiting, why do we treat between 52% and 100% of the cases, with a median of 88%, with antibiotics? [Ashworth *et al.* 2005]. As mentioned recently by Hay and Tilling, how is it possible that

88% of patients with acute bronchitis are special [Hay and Tilling, 2014]?

In general, prescribing has been shown to be influenced by several factors, including cultural aspects related to the country background, socio-cultural and socio-economic factors, and the cultural beliefs of the patient and the prescriber, patient demand, and clinical autonomy [Butler *et al.* 1998; Moore and McNulty, 2012]. In different countries, people hold different ideas about health, causes of disease, labelling of illness, coping strategies, and treatment modalities [Hulscher *et al.* 2010]. Diagnostic uncertainty plays an important role in antibiotic overprescribing [Harbart and Samore, 2005]. In a Dutch study, the use of antibiotics was strongly associated with uncertainty avoidance (unwillingness to accept uncertainty and risks). The authors also observed that hierarchical societies such as those in Southern Europe consume more antibiotics than mainly egalitarian societies such as Scandinavian countries, the UK or the Netherlands [Deschepper and Vander Stichele, 2001]. Socioeconomic factors have also been associated with variability of antibiotic prescription. Aspects such as the way in which healthcare is funded or reimbursed, the percentage of generic drugs in the market, the economic incentives or the pressure of pharmaceutical industries can affect antibiotic prescription by clinicians [Hulscher *et al.* 2010]. Inequalities might also explain the variability of antimicrobial use. Moreover, Kirby and Herbert observed a moderate correlation between AMR and income inequality with the utilization of data from 15 large European countries [Kirby and Herbert, 2013].

Other factors related to the professional care-delivery system of antibiotics are probably important, such as care coordination, professional's collaboration, communication teamwork, clinician's knowledge about management of infectious diseases and doctor–patient relationship. Misconceptions and uncertainties regarding the role of antibiotics also exist among patients [Altiner *et al.* 2007]. For example, a European study reported that around half of the patients believed antibiotics were effective in treating viruses, cold and flu, with considerable differences across countries [European Commission, 2010]. There also appears to be a dissonance between physician and patient expectations during a consultation of a respiratory tract infection [Coenen *et al.* 2013]. Clinicians' attitudes on antibiotics also play an important role in antibiotic overprescribing. A recent survey including more than 1000 GPs carried out in the UK found that 55% felt under pressure, mainly from patients, to prescribe antibiotics, even if they were not sure that they were necessary, and 44% admitted that they had prescribed antibiotics to get a patient to leave the surgery [Cole, 2014].

Prescribing fewer antibiotics is needed

A reduction in antibiotic consumption leads to a reduction of resistance. The classical Finnish study focusing on macrolide resistant *Streptococcus pyogenes* clearly showed how a reduction in macrolide use could lead to a reduction in AMR. Antibiotic resistance dropped from 9.2% in 1997 to 7.4% in 2000, with a statistically significant association between regional macrolide resistance and consumption rates [Bergman *et al.* 2004].

What can we do to prescribe fewer antibiotics? Our goal is not just to reduce the amount of antibiotics. It is also to promote a rational use of antibiotics by prescribing antibiotics only to patients who are expected to benefit from the treatment. Many studies have been performed to determine the effectiveness of different types of intervention in promoting a more rational use of antibiotics. According to the last Cochrane review on interventions to improve antibiotic prescribing, multifaceted interventions combining physician, patient and public education in a variety of venues and formats were the most successful. Interactive educational meetings were more effective than didactic lectures, but levels of improvement were limited. Inappropriate antibiotic prescribing was reduced by less than 20% across a broad range of study populations [Arnold and Straus, 2005; Gonzales *et al.* 2013]. In a recently published paper, van der Velden and colleagues assessed the effectiveness of physician-targeted interventions aiming to improve antibiotic prescribing for respiratory tract infections in primary care. The authors included 58 studies and found that overall antibiotic prescribing was reduced by 11.6%. Within the 59 interventions aiming to decrease overall antibiotic prescribing, it was found that concurrently performed interventions (multiple interventions) were more effective than single interventions focusing on only one issue. Multifaceted interventions that included educational materials for physicians were the most effective strategies. The authors observed that communication skills in training and near-patient

testing achieved the largest intervention effects [van der Velden *et al.* 2012]. The Cochrane review showed that interventions aimed at reducing overall antibiotic prescribing were less effective than interventions focusing on adherence to first choice antibiotics [Arnold and Straus, 2005]. However, other reviews have reported the opposite [van der Velden *et al.* 2012]. In general, multifaceted interventions were associated with an average increased prescribing of first-choice antibiotics of approximately 10% [Steinman *et al.* 2006]. The strategies for primary care that have been observed to be most successful are presented in this review.

Enforcement of governmental laws prohibiting over-the-counter sale of antibiotics

Self-medication with antibiotics is common in many parts of the world. In several countries, antibiotics are sold, illegally, without a prescription [Morgan *et al.* 2011]. This is particularly common in many countries in Asia, Africa, South and Central America, and even in Southern European countries, such as Italy, Spain, Greece and Malta [Borg and Sciclunca, 2002; Väänänen *et al.* 2006; Carrasco-Garrido *et al.* 2008; Plachouras *et al.* 2010]. In some countries, antibiotics are also available on the free market, i.e. outside pharmacies. Law enforcement to prohibit the illegal over-the-counter sale of antibiotics at pharmacies and the sale of antibiotics for humans and animals on the free market should be promoted worldwide.

Antimicrobial stewardship programmes, campaigns and audits

In many countries, there have been educational campaigns that aim to change healthcare professional and patient behaviour in antibiotic consumption. Interventions include the publication of guidelines, educational sessions on appropriate prescribing of antibiotics, educational sessions on the diagnosis and management of infectious diseases, review of prescribing data for practices, local interviews by pharmacists, messages included on TV, radio and other mass media, etc. Although the effects of these public campaigns and primary-care projects are positive, they are not sufficient to reduce the problem of AMR. An analysis of 22 national- or regional-level campaigns in high-income countries from 1990 to 2007 did find a reduction in antibiotic use. However, as all but one campaign targeted the patient and healthcare professional simultaneously [Huttner *et al.* 2010], it cannot be concluded whether patient education and awareness alone is an effective intervention to decrease antibiotic use. However, some campaigns have been particularly successful. Interventions targeting doctors and patients in primary care with the active participation of GPs in audits with discussion of results obtained have been found to be effective in achieving a reduction of antibiotics prescribed, as was found in the European-funded project Happy Audit [Bjerrum *et al.* 2011]. The Scottish Antimicrobial Prescribing Group, established by the Scottish Government in 2008, led a national initiative to actively address antimicrobial stewardship with the development of prescribing indicators for hospital and primary care, and observed reductions in antibiotic prescribing with no adverse effect on mortality or AMR patterns [Nathwani *et al.* 2012]. In Sweden, the Strategic Programme for the Rational Use of Antimicrobial Agents (STRAMA) and Surveillance of Resistance antimicrobial stewardship initiative reported a reduction in antibiotic use and lowered AMR rates over 10 years, without measurable negative consequences [Mölstad *et al.* 2008].

More evidence from pragmatic studies carried out in primary care is required

GPs often state that the patients attended are special and they need antibiotics; in fact, clinicians think their use is associated with a more rapid recovery or because they have an underlying comorbid condition, such as asthma, chronic lung disease, or they are smokers or have purulent sputum, and because of these features they need antibiotics. These are the so-called 'special situations', and many GPs claim that published clinical trials published usually do not take these patients into account. However, in recent years some good-quality papers have been published considering these special situations, including patients with outcomes that are of interest to GPs, such as clinical outcomes with length of symptoms and incidence of complications [McNulty *et al.* 2013]. The publication of these pragmatic studies can help GPs believe the results obtained. One example is the recent study on the low number of complications found in patients with sore throat (quinsy, otitis media, sinusitis, impetigo or cellulitis), observed in 1.3% of the cases, regardless of whether they were treated with antibiotics [Little *et al.* 2013b].

Promoting the use of valid point-of-care tests

If you visit a primary-care consultation in a Scandinavian country and compare it with a similar consultation in a Southern European country,

you soon realize that the most important difference is the number of diagnostic tools available in Scandinavia. GPs in Northern countries usually use rapid antigen detection testing for the diagnosis of streptococcal pharyngitis, C-reactive protein (CRP) devices for ruling out serious respiratory tract infections, equipment capable of determining the number and type of leukocytes and agar plates for urine culture and susceptibility testing of bacteria (e.g. Flexicult plates, Petri plates that give clinicians knowledge about the bacterial aetiology of a urinary tract infection and the susceptibility pattern of the involved microorganisms in less than 24 hours).

The major contribution of point-of-care tests seems to decrease doctors' uncertainty, adding useful information that helps to identify who to treat or not to treat with antibiotics. However, not all of the rapid tests are useful in primary care; only those that are accurate, precise, easy to use and interpret, fast and affordable for a primary care setting are acceptable [Cals and van Weert, 2013]; but above all, point-of-care tests should be able to predict prognosis and expected response to antibiotic treatment. In primary care, as opposed to the hospital, knowledge about the aetiological agent of an infection is not the most important thing; it is more important to be able to predict the expected evolution of the infectious disease and thereby consider the potential effect of an antibiotic treatment [Llor and Butler, 2014]. Several point-of-care tests have been shown to be effective in reducing the number of antibiotics prescribed. For example, CRP rapid testing, which gives the result in less than 3 minutes, has been shown to significantly reduce antibiotic prescribing in lower respiratory tract infections without compromising the clinical evolution of the patients [Huang *et al*. 2013]. In a recent trial, carried out in six European countries, Internet-based training on the CRP rapid test was associated with a significant reduction of antibiotics prescribed for acute lower respiratory tract infections [Little *et al*. 2013c]. Two Dutch studies showed significant decreases in antibiotic prescriptions when GPs used CRP testing to guide antibiotic management in lower respiratory tract infections, observing reductions from 53% to 31% in one study [Cals *et al*. 2009] and from 56.6% *versus* 43.4% in the other [Cals *et al*. 2010]. Furthermore, no differences in clinical outcomes were observed between patients treated and not treated with antibiotics.

Distinguishing pneumonia from acute bronchitis with only clinical findings is problematic in primary care, and the use of CRP rapid testing has been shown to perform better in predicting the diagnosis of pneumonia than any individual or combination of clinical symptoms and signs in lower respiratory tract infection [Hopstaken *et al*. 2003; Flanders *et al*. 2004; van Vugt *et al*. 2013]. The addition of the CRP level together with the signs and symptoms of a lower respiratory tract infection predicts the diagnosis of pneumonia better [van Vugt *et al*. 2013]. CRP tests have also proven to be a good predictor of clinical evolution in acute exacerbations of mild-to-moderate chronic obstructive pulmonary disease, being much better than the classic Anthonisen criteria (purulence of sputum, increased coughing or increase of dyspnoea) [Llor *et al*. 2012]. CRP has also been useful for achieving a reduction of antibiotics prescribed for acute sinusitis [Bjerrum *et al*. 2004]. The use of procalcitonin has been shown to be effective in reducing the amount of antibiotics in patients with lower respiratory tract infections [Tang *et al*. 2009]. However, it takes approximately 20 minutes to perform the test.

The utilization of rapid antigen-detection tests or Strep A has been associated with a lower prescribing of antibiotics for patients with sore throat. McIsaac and colleagues reported a 45% reduction in antibiotic prescribing in adults using rapid tests compared with empirical treatment [McIsaac *et al*. 2004]. Worrall and colleagues reported a proportion of antibiotic prescribing of 58% among physicians who did not use these rapid tests and 27% among those who did use this rapid test [Worrall *et al*. 2007]. Similarly, in another primary-care study carried out in Switzerland, the use of Strep A reduced antibiotic prescribing from 60% to 37% [Humair *et al*. 2006]. Curiously, in recent studies carried out in paediatrics, in which the incidence of streptococcal infection is higher, greater reductions were observed, with percentages of antibiotic prescription ranging from 22% to 28% among physicians assigned to rapid testing [Maltezou *et al*. 2008; Ayanruoh *et al*. 2009]. However, despite the fact that the use of these rapid antigen detections tests reduces the percentage of antibiotics prescribed, some recent papers cast doubts on the clinical benefit of their use. In a retrospective analysis of 726 patients, post-streptococcal complications were observed in a substantial number of patients with negative results, thereby limiting the usefulness of this rapid point-of-care test [Dingle *et al*. 2014]. We clearly need new rapid diagnostic instruments that allow clinicians to make a rapid decision on the basis of their results [Laxminarayan *et al*. 2013].

Promoting delayed prescribing of antibiotics

Delayed antibiotic prescribing means that the prescriber delivers an antibiotic prescription, but recommends the patient not to redeem it the same day. The prescription should only be redeemed if the patient feels worse within a few days. If symptoms reduce spontaneously, the prescription should be discarded. Delayed antibiotic prescribing is a widespread practice in the UK and its use is enforced by national guidelines [National Institute for Health and Clinical Excellence, 2008], but it has been difficult to implement in other countries. However, recent evidence from Norway also indicates that delayed prescribing may lead to a reduction in antibiotic use, mainly for sinusitis and otitis media [Høye *et al*. 2013].

A Cochrane review evaluated outcomes of delayed antibiotic prescribing compared with immediate antibiotic or no antibiotic prescribing in patients with respiratory tract infections. The study found that delayed prescribing was not superior to no prescribing in terms of symptom control, such as fever and cough, and complications [Spurling *et al*. 2011]. However, in a recently published study, researchers from Southampton showed a significant reduction in the consumption of antibiotics among patients assigned to delayed antibiotic strategies (less than 40%), without prejudice to the patients' outcome, compared with the strategy of immediate antibiotic therapy [Little *et al*. 2014a]. Moreover, the authors pointed out that giving these patients responsibility for their own treatment apparently helps them to consult less frequently in the future. This is a likely and interesting explanation. They concluded that discussing concerns and expectations of patients and informing them of the natural course of an uncomplicated lower respiratory tract infection might reduce reconsultation rates. This has particularly been studied in acute pharyngitis, in which the group of patients assigned to delayed prescribing presented a lower incidence of complications when compared to the group in which antibiotic therapy was withheld and was associated with a lesser re-attendance in subsequent episodes compared with immediate prescribing [Little *et al*. 2014b].

Enhancing communication skills with patients

Improved communication in primary care can help to bridge this gap between physician and patient expectations. This can be achieved using various approaches. In a pragmatic clinical trial carried out in the Netherlands the authors observed that GPs assigned to CRP testing prescribed fewer antibiotics than those in the control group (30.7% *versus* 35.7%) and those trained in communication skills treated 26.3% of all episodes of respiratory tract infection with antibiotics compared with 39.1% treated by family physicians without [Cals *et al*. 2013]. The STAR programme of five sessions of Web-based training in enhanced communication skills, with patient scenarios and an expert-led face-to-face seminar, achieved a 4.2% reduction in global antibiotic use with no significant changes in admissions to hospital, reconsultations or costs [Butler *et al*. 2012]. Francis and colleagues showed that the use of a brief Web-based training programme and an interactive booklet on respiratory tract infections in children with uncomplicated respiratory tract infections within primary-care consultations led to an important reduction in antibiotic prescribing, with an odds ratio of 0.29 and reduced intention to consult without reducing satisfaction with care [Francis *et al*. 2009]. Gonzales and colleagues conducted a three-group randomized study at 33 primary-care practices in the United States in acute bronchitis patients, evaluating the effectiveness of two interventions: in one-third of the practices, the intervention was printed decision support in which educational brochures were given by triage nurses to patients with cough as part of routine care, and in another third of the practices a computer-assisted decision support intervention was implemented so that when triage nurses entered 'cough' into the electronic health record, an alert would prompt the nurse to provide an educational brochure to the patient; the remaining practices were control sites. Compared with the baseline period, the percentage of subjects prescribed antibiotics for uncomplicated acute bronchitis during the intervention period decreased from 80% to 68.3% at the printed decision support intervention sites and from 74% to 60.7% at the computer-assisted decision support intervention sites [Gonzales *et al*. 2013].

Communicating the possible length of mainly bothersome complaints, such as cough, is important in acute bronchitis, since the mean duration of any cough is between 15 and 20 days. In a recent study, Ebell and colleagues performed a population-based survey in the United States to determine patients' expectations regarding the duration of acute cough, reporting a median duration of 5–7 days [Ebell *et al*. 2013]. The mismatch between patients' expectations and reality for the natural history of acute cough illness has

Box 2. Communication tips that can help with patients with self-limiting respiratory tract infections.

Discuss with the patient that antibiotics do not significantly reduce the duration of symptoms of self-limiting respiratory tract infections and that they may cause adverse effects and lead to antibiotic resistance.

Back up the information provided with a leaflet or brochure given to the patient highlighting the most important information.

Set realistic expectations for symptom duration, including the average total duration of symptoms (after seeing the doctor): 4 days for acute otitis media, 1 week for acute sore throat, 1½ weeks for common cold, 2½ weeks for acute rhinosinusitis and 3 weeks for acute cough/bronchitis.

Define the diagnosis as a viral respiratory infection, chest cold, or sore throat instead of using the medical terms 'acute bronchitis' and 'acute tonsillitis'.

Clearly explain the red-flag symptoms patients should know about infectious diseases.

Consider delayed prescription of antibiotics in those situations in which an aetiology cannot be clearly established.

Consider the use of rapid tests in cases of doubt, such as C-reactive protein rapid testing or rapid antigen detection tests, and discuss the results with the patient.

important implications for antibiotic prescribing. If a patient expects that an episode will last about 1 week, it makes sense that they might seek care for that episode and request an antibiotic after 5 or 6 days. Notwithstanding, GPs often fail to satisfactorily communicate the mean length of cough to patients with acute bronchitis [Cals *et al*. 2007]. As physicians, we must avoid sentences such as: 'With these pills you will feel a rapid remission of your cough;' and, as the cough will not remit the patient will be prone to reconsult again and will probably demand medicine that is perceived as stronger, such as antibiotics. Educating patients about the natural history of infectious diseases is therefore crucial. Patients need to know that antibiotics are probably not going to be beneficial in most self-limiting infections, and that treatment with antibiotics is associated with significant risks and side effects. They should also be told that it is normal to still be coughing 2 or even 3 weeks after onset, and that they should only seek care if they are worsening or if an alarm symptom, such as high fever, bloody or rusty-coloured sputum, or shortness of breath occurs (Box 2). Careful word selection for the infection is also important [Phillips and Hickner, 2005]. One survey showed that patients were less dissatisfied after not receiving antibiotics for a chest cold or 'viral upper respiratory infection' than they were for acute bronchitis [Phillips and Hickner, 2005].

The use of information brochures is of aid. In the last few years, many studies on the benefit of discussing the evolution of the infectious conditions with the patient have been published. It is important to talk about the expected duration of symptoms and to deliver written material that explains when the patient should contact again in case of a possible deterioration (Box 2). Some papers have shown that a patient's awareness of the red-flag signs reassures and helps to better comply with the treatment regimen and the use of fewer antibiotics [Butler *et al*. 2012]. In a recent clinical trial in which the effect of an online course on improving communication skills along with the use of leaflets was evaluated, the group of GPs assigned to this intervention prescribed 32% fewer antibiotics compared with the control group, and those who were trained in this strategy and at the same time had access to CRP rapid testing in their consultations, reduced antibiotic prescribing by 62% [Little *et al*. 2013a]. In addition, patients assigned to this strategy better understood why they had to take antibiotics. Communicative aspects have to contemplate what patients are unaware of, such as side effects of antimicrobial agents or their lack of effectiveness in infections that are self-limiting, even in many of these 'special situations' [Moore *et al*. 2014]. Box 2 describes some aspects that GPs should consider when communicating with patients with self-limiting respiratory tract infections.

Conclusion

There is no reason to explain why antibiotic prescribing in respiratory tract infections (the most frequent primary care) is so great. Doctors have to do well and not harm, while

respecting the ethical principles of autonomy and justice. However, in the case of ethical conflict, nonmaleficence and justice (at a public and obligatory level) take precedence. We know that we can reduce antibiotic prescribing in many of the infections that are currently unnecessarily treated without compromising our patients' health. By accomplishing this, we will do less harm. Moreover, we know that antibiotics can stop being effective in the short and medium term. The use of the strategies discussed in this paper will help GPs to reduce prescribing of antibiotics. Our duty is to prescribe antibiotics only when they are necessary, i.e. in less than 20% of the infectious seen in primary care.

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1. Introduction

Retaining specimens has two primary purposes: to ensure there is a physical audit trail, in case the integrity or identification of the specimen needs to be

established; and to allow additional testing to be done on the original specimen, if required. In some cases, the original specimen may be consumed, exhausted or be otherwise unsuitable for any additional testing; however, retention is still required for audit and traceability purposes.

The control of clinical material is an essential part of quality management and is addressed by ISO 15189 standard 5.7.2 which deals with the identification, indexing, storage, retention and disposal of clinical samples and standard 5.2.3 storage facilities

2. Purpose and Scope

This procedure outlines the procedure for the control of clinical material to satisfy the standard to allow recovery of clinical material for audit trail purposes and to ensure safe secure storage and disposal of clinical material. The procedure is audited as part of the quality management system (QMS) to demonstrate that it has been effectively established, implemented and maintained.

3. Responsibility

Responsibility for complying with this procedure lies with the Operational Manager for each site.

4. Definitions

Clinical material: Includes any primary specimens / samples and relevant preparations made in the course of examination and any microbial isolates which require to be retained.

Stored Cultures: Propagated microbiological agents that may be used at a later date either for quality control, clinical or research purposes.

Schedule 5: Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of organisms not to be stored.

5. Safety Considerations

Risk assessment	Reference	Hazard	Precautions
Safety Manual	See local Safety Manuals	General	Good lab practice
Handling and Culturing Specimens (excluding Enteric	R507	Biological	Good lab Practice Training PPE

and CL3)			
Culturing faeces	R501	Biological	Wear PPE
Handling ACDP Cat 3 organisms	R058	Biological	Work in exhaust cabinet

6. Related Documents

ED525 -RCPath The Retention and Storage of Pathological Records and Specimens 5th Edition, 2015
ED546-Caldicott, Confidentiality and Data Protection Policy
ED552 - Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of pathogens not to be stored.
Human Tissue (Scotland) Act 2016

7. Procedure

Identification & indexing

- Clinical material is labelled with a unique laboratory accession number (including year), this is used for identification and indexing of samples. From this identifier the computer report can be accessed for the patient details, source material, date of receipt etc.
- Specimens (labelled as above) are stored in numerical order
- Isolates from clinical material are similarly numbered when first stored.
- An index of retained isolates is kept in each Laboratory
    - For Clyde Micro this is a Freezer Broth Inventory Database
    - For North a hardcopy index is kept of all isolates. Separate record sheet for *N. gonorrhoea*
    - For South Blood culture isolates only indexed

Security

- The laboratories have restricted authorized access (Swipe card/keypad entry). At GRI the CL3 Suite has CCTV in the corridor area and there are CCTV cameras at the passenger lifts
- Containment level 3 laboratory (CL3) access is also secured by keypad &/or swipe card entry.
- Retained material and isolates are therefore stored in a secure area

Retention

- See Table 1 for details of the specimen type and retention times.

Storage & retrieval

- Storage is as described in Table 1. By unique labelling it is possible to retrieve samples stored.

Disposal

- Clinical Material is disposed of as clinical waste as outlined in the Departmental H&S Procedures and Local Clinical Waste Disposal Procedures
- Retained material is only discarded after the material has exceeded the retention time
- Retention time stated is the minimum time material shall be retained.

Table 1. Identification & Indexing

Source	Type of Sample	Identity	Storage	Indexing by	Retention Time (minimum)
Clinical Material					
RCPath 87	Serum	Lab number/PI	+4°C	In rack, in numerical order	7 days
RCPath 95 Body fluids, aspirates and swabs	CSF	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Sterile Fluids	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Blood Cultures - Negative	Lab number/PI	37°C on analyser	By Lab number	2 days
	Blood Cultures - Positive	Lab number/PI	Room temperature	In rack, in numerical order	7 days
	Routine Miscellaneous specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	MRSA specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	Urine Samples	Lab number/PI	Room temperature	In rack, numerical order by day of receipt	3 days
	Faeces	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt of receipt	7 days
	Sputum Samples	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
	Samples for TB investigation	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
RCPath 122	Tissue	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	5 weeks
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**

Clinical Material					
	CSA Specimens	Lab number/PI	-20°C (long term)	Indefinitely	Locked -20°C
Retention of Slides					
RCPath 130	Gram films from Specimens E.g. blood cultures, sterile fluids and CSF samples	Lab number	Room temperature	In slide trays labelled for each day	7 days
	Gram films from routine culture plates	Lab number	Room temperature	Discard immediately after use	N/A
	Cryptosporidum Slides	Lab number	Room temperature	In slide trays labelled for each day	7 days
RCPath 130	Positive Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	3 months
	Negative Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	7 days
Retention of Microbiology Cultures					
RCPath 142	Microbiological Cultures Index Plates	Lab number	Room Temperature	Plate racks, for each day	7 days
	Blood culture isolates	Lab number	-80°C	In labelled Thermo boxes: • For Clyde in Cryovials by Freezer Broth Number • For North & South in Cryovials by accession number corresponding to log sheet	Refer to LP509, section 10.3 for storage of blood culture isolates
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**
Retention of Microbiology Cultures					
	Group A streptococcus	Lab number	-80°C	In labelled Thermo	1 year

	Neisseria gonorrhoea Any CSF isolates Any unusual organisms any other organisms referred to reference laboratory with the exception, Schedule 5 organisms*			boxes: • For Clyde in Cryovials by Freezer Broth Number • For North & South in Cryovials by accession number corresponding to log sheet	
	*Salmonella/Shigella /E.coli 0157 isolates/Vibrio Chlorea	Lab number	-20°C	-20°C Freezer in CL3Lab	7 days after receipt of report from reference laboratory
	Faeces positive and equivocal for C.difficile	Lab number	-80°C	In freezer racks in cryovials for each day	3 months
	TB Cultures	Lab number	-20°C	QEUH By accession number GRI By accession number	Locked -20°C 7 days after receipt of report from reference laboratory

Microbiology GG&C	MP512v2
	Authorised by: Janet Young
Issued: 27/06/2018	Author: Pam Saunders

Control of Clinical Material	
LOCATION OF COPIES	Electronic only

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Introduction

Retaining specimens has two primary purposes: to ensure there is a physical audit trail, in case the integrity or identification of the specimen needs to be

established; and to allow additional testing to be done on the original specimen, if required. In some cases, the original specimen may be consumed, exhausted or be otherwise unsuitable for any additional testing; however, retention is still required for audit and traceability purposes.

The control of clinical material is an essential part of quality management and is addressed by ISO 15189 standard 5.7.2 which deals with the identification, indexing, storage, retention and disposal of clinical samples and standard 5.2.3 storage facilities

2. Purpose and Scope

This procedure outlines the procedure for the control of clinical material to satisfy the standard to allow recovery of clinical material for audit trail purposes and to ensure safe secure storage and disposal of clinical material. The procedure is audited as part of the quality management system (QMS) to demonstrate that it has been effectively established, implemented and maintained.

3. Responsibility

Responsibility for complying with this procedure lies with the Operational Manager for each site.

4. Definitions

Clinical material: Includes any primary specimens / samples and relevant preparations made in the course of examination and any microbial isolates which require to be retained.

Stored Cultures: Propagated microbiological agents that may be used at a later date either for quality control, clinical or research purposes.

Schedule 5: Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of organisms not to be stored.

5. Safety Considerations

Risk assessment	Reference	Hazard	Precautions
Safety Manual	See local Safety Manuals	General	Good lab practice
Handling and Culturing Specimens (excluding Enteric	R507	Biological	Good lab Practice Training PPE

and CL3)			
Culturing faeces	R501	Biological	Wear PPE
Handling ACDP Cat 3 organisms	R058	Biological	Work in exhaust cabinet

6. Related Documents

ED525 -RCPath The Retention and Storage of Pathological Records and Specimens 5th Edition, 2015
ED546-Caldicott, Confidentiality and Data Protection Policy
ED552 - Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of pathogens not to be stored.
Human Tissue (Scotland) Act 2016

7. Procedure

Identification & indexing

- Clinical material is labelled with a unique laboratory accession number (including year), this is used for identification and indexing of samples. From this identifier the computer report can be accessed for the patient details, source material, date of receipt etc.
- Specimens (labelled as above) are stored in numerical order
- Isolates from clinical material are similarly numbered when first stored.
- An index of retained isolates is kept in each Laboratory
    - For North a hardcopy index is kept of all isolates. Separate record sheet for *N. gonorrhoea*
    - For South a hardcopy index is kept of all isolates

Security

- The laboratories have restricted authorized access (Swipe card/keypad entry). At GRI the CL3 Suite has CCTV in the corridor area and there are CCTV cameras at the passenger lifts
- Containment level 3 laboratory (CL3) access is also secured by keypad &/or swipe card entry.
- Retained material and isolates are therefore stored in a secure area

Retention

- See Table 1 for details of the specimen type and retention times.

Storage & retrieval

- Storage is as described in Table 1. By unique labelling it is possible to retrieve samples stored.

Disposal

- Clinical Material is disposed of as clinical waste as outlined in the Departmental H&S Procedures and Local Clinical Waste Disposal Procedures
- Retained material is only discarded after the material has exceeded the retention time
- Retention time stated is the minimum time material shall be retained.

Table 1. Identification & Indexing

Source	Type of Sample	Identity	Storage	Indexing by	Retention Time (minimum)
Clinical Material					
RCPath 87	Serum	Lab number/PI	+4°C	In rack, in numerical order	7 days
RCPath 95 Body fluids, aspirates and swabs	CSF	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Sterile Fluids	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Blood Cultures - Negative	Lab number/PI	37°C on analyser	By Lab number	2 days
	Blood Cultures - Positive	Lab number/PI	Room temperature	In rack, in numerical order	7 days
	Routine Miscellaneous specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	MRSA specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	Urine Samples	Lab number/PI	Room temperature	In rack, numerical order by day of receipt	3 days
	Faeces	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt of receipt	7 days
	Sputum Samples	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
	Samples for TB investigation	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
RCPath 122	Tissue	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	5 weeks
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**

Clinical Material					
	CSA Specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	Negative Culture - as per routine sample type retention Positive culture - 7 days after receipt of report from reference laboratory
Retention of Slides					
RCPath 130	Gram films from Specimens e.g. blood cultures, sterile fluids and CSF samples	Lab number	Room temperature	In slide trays labelled for each day	7 days
	Gram films from routine culture plates	Lab number	Room temperature	Discard immediately after use	N/A
	Cryptosporidum Slides	Lab number	Room temperature	In slide trays labelled for each day	7 days
RCPath 130	Positive Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	3 months
	Negative Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	7 days
Retention of Microbiology Cultures					
RCPath 142	Microbiological Cultures Index Plates	Lab number	Room Temperature	Plate racks, for each day	7 days
	Blood culture	Lab number	-80°C	In labelled boxes: For North & South in Cryovials by accession number corresponding to log sheet	Refer to LP509, section 10.3 for storage of blood culture isolates
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**
Retention of Microbiology Cultures					
RCPath 142	All positive MRSA	Lab number	-80°C	In labelled Thermo	1 year

	Group A streptococcus Neisseria gonorrhoea Any unusual organisms any other organisms referred to reference laboratory with the exception, Schedule 5 organisms* All isolates from Tissue/CSF/Sterile fluid			boxes: • For North & South in Cryovials by accession number corresponding to log sheet	
	*Salmonella/Shigella /E.coli 0157 isolates/Vibrio Chlorea	Lab number	-20°C	Nutrient agar slope at room temperature – enteric lab/MSC CL3 or -20°C Freezer in CL3Lab	7 days after receipt of report from reference laboratory
	Faeces positive and equivocal for C.difficile	Lab number	-80°C	In freezer racks in original container or cryovials for each day	3 months
	TB Cultures	Lab number	-20°C	QEUH By accession number GRI By accession number	Locked -20°C 7 days after receipt of report from reference laboratory

Microbiology GG&C	MP512v3
	Authorised by: Sandra Higgins
Issued: 03/09/2019	Author: Sandra Higgins

Control of Clinical Material	
LOCATION OF COPIES	North Quality Office

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Introduction

Retaining specimens has two primary purposes: to ensure there is a physical audit trail, in case the integrity or identification of the specimen needs to be

established; and to allow additional testing to be done on the original specimen, if required. In some cases, the original specimen may be consumed, exhausted or be otherwise unsuitable for any additional testing; however, retention is still required for audit and traceability purposes.

The control of clinical material is an essential part of quality management and is addressed by ISO 15189 and ISO 17025 in terms of the identification, indexing, storage, retention and disposal of clinical samples and storage facilities.

2. Purpose and Scope

This procedure outlines the procedure for the control of clinical material to allow recovery of clinical material for audit trail purposes and to ensure safe secure storage and disposal of clinical material. The procedure is audited as part of the quality management system (QMS) to demonstrate that it has been effectively established, implemented and maintained.

3. Responsibility

Responsibility for complying with this procedure lies with the Operational Manager for each site.

4. Definitions

Clinical material: Includes any primary specimens / samples and relevant preparations made in the course of examination and any microbial isolates which require to be retained.

Stored Cultures: Propagated microbiological agents that may be used at a later date either for quality control, clinical or research purposes.

Schedule 5: Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of organisms not to be stored.

5. Safety Considerations

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Working with ACDP Group 2 and 3 Organisms	R531	Biological	Good lab Practice
Handling and Culturing Specimens (excluding enteric and CL3)	R507	Biological	Good lab practice, PPE
Processing of Enteric Samples	R501	Biological	Good lab practice, PPE
Processing respiratory Samples in CL3	R505	Biological	Good lab practice, PPE
Environmental Laboratory Risk Assessment	R537	General	Good Lab Practice & PPE

6. Related Documents

ED525 -RCPath The Retention and Storage of Pathological Records and Specimens 5th Edition, 2015
ED546-Caldicott, Confidentiality and Data Protection Policy
ED552 - Anti-Terrorism Crime and Security Act 2001 (modified 2007). List of pathogens not to be stored.
Human Tissue (Scotland) Act 2016

7. Procedure

Identification & indexing

- Clinical material is labelled with a unique laboratory accession number (including year), this is used for identification and indexing of samples. From this identifier the computer report can be accessed for the patient details, source material, date of receipt etc.
- Specimens (labelled as above) are stored in numerical order
- Isolates from clinical material are similarly numbered when first stored.
- An index of retained isolates is kept in each Laboratory and recorded on LF558

Security

- The laboratories have restricted authorised access (swipe card entry)
- CCTV cameras cover laboratory areas

- Containment level 3 laboratory (CL3) access is also secured by keypad &/or swipe card entry
- Retained material and isolates are therefore stored in a secure area

Retention

- See Table 1 for details of the specimen type and retention times.

Storage & retrieval

- Storage is as described in Table 1. By unique labelling it is possible to retrieve samples stored.

Disposal

- Clinical Material is disposed of as clinical waste as outlined in the Departmental H&S Procedures and Clinical Waste Disposal Procedures
- Retained material is only discarded after the material has exceeded the retention time
- Retention time stated is the minimum time material shall be retained.

Table 1. Identification & Indexing

Source	Type of Sample	Identity	Storage	Indexing by	Retention Time (minimum)
Clinical Material					
RCPath 87	Serum	Lab number/PI	+4°C	In rack, in numerical order	7 days
RCPath 95 Body fluids, aspirates and swabs	CSF	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Sterile Fluids	Lab number/PI	+4°C	In rack, in numerical order by day of receipt	7 days
	Blood Cultures - Negative	Lab number/PI	37°C on analyser	By Lab number	2 days
	Blood Cultures - Positive	Lab number/PI	Room temperature	In rack, in numerical order	7 days
	Routine Miscellaneous specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	MRSA specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	7 days
	Urine Samples	Lab number/PI	Room temperature	In rack, numerical order by day of receipt	3 days
	Faeces	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt of receipt	7 days
	Sputum Samples	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
	Samples for TB investigation	Lab number/PI	+4°C	In rack, numerical order by day of receipt	7 days
RCPath 122	Tissue	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	5 weeks
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**

Clinical Material					
	CSA Specimens	Lab number/PI	+4°C	In appropriate storage box/bag by day of receipt	Negative Culture - as per routine sample type retention Positive culture - 7 days after receipt of report from reference laboratory
Retention of Slides					
RCPath 130	Gram films from Specimens e.g. blood cultures, sterile fluids and CSF samples	Lab number	Room temperature	In slide trays labelled for each day	7 days
	Gram films from routine culture plates	Lab number	Room temperature	Discard immediately after use	N/A
	Cryptosporidium Slides	Lab number	Room temperature	In slide trays labelled for each day	7 days
RCPath 130	Positive Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	3 months
	Negative Auramine and ZN films	Lab number	Room Temperature	CL3 lab in slide trays labelled for each day	7 days
Retention of Microbiology Cultures					
RCPath 142	Microbiological Cultures Index Plates	Lab number	Room Temperature	Plate racks, for each day	7 days
	Blood culture	Lab number	-80°C	In labelled boxes: For North & South in Cryovials by accession number corresponding to log sheet	Refer to LP509, section 10.3 for storage of blood culture isolates
Source	**Type of Sample**	**Identity**	**Storage**	**Indexing by**	**Retention Time (minimum)**
Retention of Microbiology Cultures					
RCPath 142	All positive MRSA Group A streptococcus	Lab number	-80°C	In labelled Thermo boxes:	1 year

	Neisseria gonorrhoeae Any unusual organisms any other organisms referred to reference laboratory with the exception, Schedule 5 organisms* All isolates from Tissue/CSF/Sterile fluid. Isolates as directed by medical staff. Any isolate involved in an infection control issue Any Pseudomonas aeruginosa isolated from endoscopy water. Environmental isolates involved in outbreaks etc.			• For North & South in Cryovials by accession number corresponding to log sheet	
	*Salmonella/Shigella /E. coli O157 isolates/Vibrio. cholerae	Lab number	-20°C	Nutrient agar slope at room temperature – enteric lab/MSC CL3 or -20°C Freezer in CL3Lab	7 days after receipt of report from reference laboratory
	Faeces positive and equivocal for C.difficile	Lab number	-80°C	In freezer racks in original container or cryovials for each day	3 months
	TB Cultures	Lab number	-20°C	QEUH By accession number GRI By accession number	Locked -20°C 7 days after receipt of report from reference laboratory

Source	Type of Sample	Identity	Storage	Indexing by	Retention Time (minimum)
Environmental laboratory – for index plates and stored isolates, refer to above retention policy criteria					
Local	Water Samples (non-Legionella)	Lab number	+4°C	Cold room storage for each day processed	8 days

Local	Legionella Waters	Lab number	+4°C	Cold room storage for each day processed	10 days
Local	Legionella Direct Concentrates	Lab number	6°C (± 2°C) in the dark	Racks labelled with month and year	1 year

Post-Mortem Specimens/Isolates

- **Routine post-mortem samples/isolates are stored as per retention policy**
- **Fiscal post-mortem samples are stored for 1 year in the freezer -80°C**
- **Fiscal derived significant isolates are stored for a minimum of 5 years (adults) and indefinitely (children) at -80°C in the freezer**
- **Note that for any fiscal case, the coroner can instruct different retention periods which must be adhered to**

Microbiology GG&C	LP538
	Authorised by: Sandra Higgins
08/03/2017	Author: Pat Millar

Potable Water Analysis (Non Endoscopy)	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Statutory regulations define the performance criteria of methods for compliance purposes for the monitoring of drinking water supplies. Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values.
Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, cleaning records and control monitoring charts are part of the quality assurance.

2. Principle and method of the procedure

The microbiological analysis of water and associated materials involves the use of selective procedures and media to ensure that these water samples are of a suitable standard. In addition, the nature of the organisms being isolated and enumerated can present challenges to the analysts. It is therefore important that the media used, and the procedures conducted reflect the quality of the water examined, and that the data generated is reliable
Samples require a combination of TVC's, Coliform and Pseudomonas investigations and on occasion, Atypical Mycobacterium screening.
It should be noted that all RENAL water samples are processed using Ultrapure/Standard methods
The standard for drinking water states that total coliforms should always be absent from drinking water samples, although in practice, up to 5% of samples may contain low numbers of coliforms.
Early detection of problems and repeat sampling may be required and therefore if coliforms and/or E.coli are detected in a drinking water sample, these results must be telephoned to the client immediately.
E.coli must always be absent from potable water supplies

3. Type of Sample

- Pharmacy waters
- Raw untreated waters
- Estates waters
- Renal waters
- Heterotrophic count waters
- Pools

4. Required Equipment and Reagents

Sterile 47mm filter unit
Manifold
Vacuum pump
0.45µ metrical GN-6 membranes

5. Environmental and Safety Controls

Risk assessment	Reference	Hazard	Precautions
Safety Manual	Refer to local safety manual	Biological	General lab practice
Working in the water/environmental lab	R537	Biological, Physical,	General lab practice, PPE

Working with ACDP hazard group 2 Organisms	R529	Biological	Wear PPE – Lab Coat

6. Procedural Steps

6.1. Sample Receipt

Samples are received from private companies and GG&C hospitals

- Stamp the date and time of receipt of the samples on their request forms using the Seiko clock unit
- Sample is taken directly to environmental lab for testing/storage
    - If NOT processing immediately all samples must be refrigerated
    - **If OOH put ALL samples directly into fridge**

6.2. Booking In Procedure

For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- Ensure the sample details match the request form, and sufficient information is recorded on the form to issue a report
- Label samples using designated Potable Water laboratory numbers
- If investigations required are unclear, confirm by checking with Technical Manager, or contacting sender

If new request forms are required by the customer, provide them with blank forms. If required to assist in completing forms, ensure the form is checked and initialled by the customer prior to accepting the samples.

- All samples are stored in the Cold Room until final report is issued
- Sample requirements depend on location, routinely the following
    - Pharmacy Waters : TVC, Coliforms, Pseudomonas
    - Raw Untreated waters : TVC, Coliforms
    - Estates Waters : TVC, Coliforms
    - Renal Waters : TVC
    - Heterotrophic Waters : TVC, Coliforms, Pseudomonas
    - Pools : TVC, Coliforms, Pseudomonas
- Also, other tests in addition to those above may be requested e.g. Atypical Mycobacterium

6.3. Agar Preparation

Yeast Extract Agar (YEA) and R2 Agar is used, depending on sample type.
Both agars are used in the pour plate method of TVC enumeration.
Bottles containing these agars are placed in the boiling water bath until the agar has melted. These bottles are then transferred to a 50ºC holding bath, whose temperature is monitored and recorded daily, from which they are taken and used.
Any bottle of agar, opened yet not completely finished, should be discarded.
Unopened bottles of agar may be remelted the next day.

6.4. ROUTINE POTABLE WATERS

6.4.1. TVC (Total Viable Count) : Pour Plate Method

Setting Up Pour Plates with Yeast Extract Agar

Label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish

- Mix water sample well before testing
- If the sample bottle is full, pour off a little water to allow adequate mixing
- Pipette 1 ml of sample into each Petri dish
- Aseptically add 15 ml molten Yeast Extract Agar to the Petri dish
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow to set at room temperature
- Incubate two plates at 37°C for 24 Hours and two at 22°C for 3 days

Negative Controls

- 2 x agar plates with no sample, incubate at 37ºC for 24 Hours
- 2 x agar plates with no sample, incubate at 22ºC for 3 days

TVC Plate Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

Count colonies as soon as the plates are removed from the incubator
Spreading colonies can interfere with counts, and the following gives guidance on what should be counted as a single colony

- A chain of colonies caused by the disintegration of a clump of organisms
- A spreading growth that develops as a film at the bottom of a Petri dish
- A colony that forms in a film of water at the edge or over the surface of the agar

If the plates cannot be counted immediately, store at 4ºC

Colony Counting Method using Stuart Counter

Before beginning any count examine the plate to ensure that the colonies are spread evenly over the whole plate.
If the appearance of colonies shows no uniform spread, perhaps colonies only present on one side, this would indicate that the initial mixing of the sample was not performed correctly. Unless uniform spread then the count cannot be reliably reported. TVC's will have to be repeated.

- Switch on light and ensure that the tally counter is zeroed and free to move when the counting area is depressed
- Place the plate to be counted, without lid, on the grid
- With a fine felt tip pen mark each colony, applying sufficient pressure to cause the tally counter to move
- Record the number of colonies counted from each plate on the relevant Daily Worksheet
- Calculate the mean count and record on form

If the number of colonies to be counted is too great, the plate may be divided into a half, quartered or a reduced area counted and multiplied by the reciprocal of that area i.e.

- Count 5 large squares using counter grid which are ≅ 1/10 plate and multiply by 10 for final count/ml
- Count 1 small square using counter grid which is ≅ 1/450 plate and multiply by 9 to give one large square, then multiply by 50 for final count/ml
    - If there are more than 20 colonies in 1 small square record the count as greater than 10^4 organisms per ml

Reporting TVC Results

- The TVC reported is the mean of the colony count from the two duplicate plates from an undiluted sample
- Recorded on daily worksheet
- Reported on client request form as cfu/ml

There is no statutory standard for TVC, but a rise in TVC would indicate the need for investigation and potentially further sampling by our clients

Standard of acceptability: (Client acceptable limits)

< 10 cfu/ml at 37^0C
< 100 cfu/ml at 22^0C

Notifiable Counts (TVC): (Defined by Clients)

> 10 cfu/ml at 37°C
> 100 cfu/ml at 22°C
> 300cfu/ml Any DMA sample (37°C & 22ºC)

If Any Notifiable Count is recorded and if not at final day for reporting– Telephone customer with an interim result. Once all work is complete report as final
If a Notifiable Count is recorded on the final day from a TVC then report directly as a final report with no requirement for a telephone result

Interim Result

- Telephone customer with interim count and record on telephone log & report form

Final Result

- Once all work complete report to customer and record as above
- Pass completed request forms to office to issue final report
- Office staff photocopy, send PDF via generic email. Original form stored
- Worksheet filed in lab for 1 month, after this pass to office for filing

6.4.2. Enumeration by Membrane Filtration

At the beginning of the days processing, all filter units require sterilising in a boiling water bath for 30 minutes. Thereafter and between samples a minimum of 15 minutes sterilisation will suffice.

A. Enumeration of COLIFORMS & E. Coli by Membrane Filtration

- Invert water sample mixing well
- Ensure the filter unit is sterilized prior to sample processing
- Place membrane on filter unit – grid side up using blunt end forceps
- Filter 100ml of sample through this membrane. Once all water has passed through filter, place membrane onto an MLGA agar plate using sterile forceps for each membrane. (**forceps sterilized with methylated spirits between each use**)
- Incubate at **37°C for 24 hours**

IQC is performed daily using positive (E. coli & Pseudomonas aeruginosa) and negative controls. Refer to Section C for full details.

Coliform / E. coli Plate Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

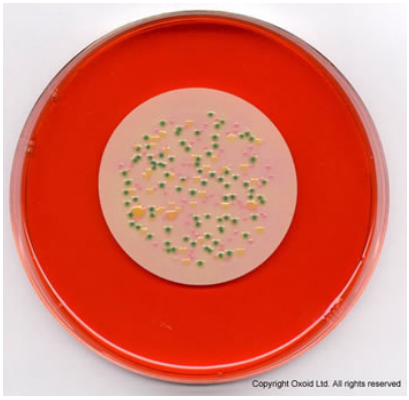
- Check for presence of Coliforms & E. coli on the MLGA plates
  - Coliforms are lactose positive & appear as YELLOW
  - E. Coli is both lactose positive and possesses glucuronidase & usually appear GREEN
  - **However, as E.coli can be a weak glucuronidase producer, all yellow isolates from an MLGA plate should still be fully identified**

- Perform a count on each colony type (e.g. 5 green, 2 yellow) and record on day sheet LF123/170 (sign & date)
- Have each colony type identified using Vitek/MS and record result on worksheet
- Report as number of coliforms/E.coli per 100ml
- Client MUST be informed of the presence of any suspect colonies

If insufficient individual colonies or too mixed

- Sub-culture one of each colony type, for purity, onto a Columbia Blood Agar plate
- Incubate 37ºC for 18-24 hrs
- Purity plates of these colonies can then be sent for ID. to the Identification section within the laboratory for Vitek ID by ID Sens bench

Example MLGA Plate



Reporting E. Coli / Coliforms

If isolate is a presumptive E.coli(i.e. before Vitek ID) :
Interim Result

- Telephone customer with interim count of presumptive E.coli and record on telephone log & report form

Final Result

- Once ID is confirmed – telephone customer and record as above. If awaiting further plates retain all paperwork.
- Once Final report is ready pass completed request form to office to issue final report
- Office staff photocopy, send PDF via generic email. Original form stored
- Worksheet filed in lab for 1 month, after this pass to office for filing

B. Enumeration of Pseudomonas Species by Membrane Filtration

- Invert water sample mixing well
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on Pseudomonas Agar plate
- Incubate at **37ºC 48 Hours**

IQC is performed daily using positive (E. coli & Pseudomonas aeruginosa) and negative controls. Refer to Section C for full details.

Pseudomonas Plate Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

- Check for presence of Pseudomonas species on the Pseudomonas plates
  - Typical (green) colonies (likely to be Ps.aeruginosa)
  - Atypical (yellow) colonies

- Perform a count on each colony type (e.g. 5 green, 2 yellow) and record on relevant day sheet (sign & date)
- Oxidase each colony type (if enough growth)
    - Oxidase negative – No further work required, record on worksheet only
    - Oxidase Positive – ID each colony type on Vitek/MS and record result on worksheet
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- Client MUST be informed of the presence of any suspect colonies

If insufficient individual colonies or too mixed

- Sub-culture 1 of each colony type for purity onto a Columbia Blood Agar plate
- Incubate 37ºC for 18-24 hrs
- Purity plates of these colonies can then be oxidased &/or sent to the identification section within the laboratory for Vitek/MS ID

Reporting Pseudomonas

If isolate is a presumptive Pseudomonas aeruginosa/sp (i.e. before Vitek ID) :
Follow reporting procedure as for E.coli/coliforms

C. Daily QC of Membrane Filtration Process

Positive Controls

- Reconstitute an E. coli lenticule (after reaching room temperature) in 100ml MRB (Maximum Recovery Broth)
- Leave for a minimum of 10 minutes, mix very well, then filter as per sample
- Place filter onto MLGA plate and incubate at **37˚C 24 Hours**
- Reconstitute a Ps. aeruginosa lenticule (after reaching room temperature) in 100ml MRB (Maximum Recovery Broth)
- Leave for a minimum of 10 minutes, mix very well, then filter as per sample
- Place filter onto Pseudomonas Agar plate and incubate at **37˚C 48 Hours**

Negative Control

- An unfiltered sterile membrane is placed on MLGA plate and incubated at 37˚C 24 Hours
- An unfiltered sterile membrane is placed on Pseudomonas Agar plate and incubated at 37˚C 48 Hours

6.5. Pre & Post Treatment of Effluent Waters

Such samples arrive from Babcock etc; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E.coli counts

- Do not require TVC's

Each requires:

- 1 x MLGA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

Procedure

A. Pre treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on MLGA plate
- Incubate at **37ºC 24 Hours**

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well
- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on MLGA plate
- Incubate at **37ºC 24 Hours**

IQC is performed daily using positive and negative controls. Refer to Section C for full details.

MLGA Plate Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

- Examine Plates as per E.coli/Coliform section above
- Count the first 'countable' plate and multiply that count up by the dilution factor to give a result for coliform cfu/100ml and the same for E.coli.
- Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)
- It is expected that the Post treatment counts will be less than those of the Pre treatment samples. If not, then check that the correct procedure has been followed

Coliform Reporting

- Report both Coliform count and E.coli count
- These results do not need to be phoned to client

6.6. Renal Waters

A. Standard Renal Waters

Such samples arrive usually in 60ml volumes for TVC Only. If a 250ml sample is received then E. Coli/Coliform & Pseudomonas Filtration is usually required. The request form will detail this. If Coliforms and pseudomonas are requested then the procedures for these filtration methods are outlined in sections 8A and 8B of this SOP.

Standard waters TVC enumeration by Pour Plate Method

Label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish

- Mix water sample well before testing
- If the sample bottle is full, pour off a little water to allow adequate mixing
- Pipette 1 ml of sample into each Petri dish
- Aseptically add 15 ml molten **R2Agar** to the Petri dish
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow to set at Room Temperature
- Incubate two plates at 30°C for 5 Days and two at 22°C for 7 days. Label clearly with date and place in a sealed plastic bag to avoid desiccation of the agar

Negative Controls

- 2 x R2A plates with no sample, incubate at 30°C for 5 Days
- 2 x R2A plates with no sample, incubate at 22°C for 7 days

TVC Examination

As detailed for Potable Samples

Reporting TVC's

Reporting procedure as per Potable waters with alert figures as below

Notifiable Counts (TVC): (Defined by Clients)

Alert figure:

>50 cfu/ml (both 30ºC and 22ºC)

B. Ultra pure

Such samples arrive in >= 250ml volumes for TVC by filtration method, using R2Agar.

Ultrapure waters TVC enumeration by Membrane Filtration

Label two ready poured R2Agar plates with each samples' number.

- Invert water sample, mixing well
- Perform membrane filtration as for Coliforms/E Coli
- Place filter onto R2Agar plate
- Perform this twice
- Incubate one plate at 30˚C for 5 Days and one plate at 22˚C for 7 days Label clearly with date and place in a sealed plastic bag to avoid desiccation of the agar

Negative Controls

- An unfiltered sterile membrane is placed on an R2agar plate and incubated at 30ºC for 5 Days in a sealed plastic bag to avoid desiccation
- An unfiltered sterile membrane is placed on an R2agar plate and incubated at 22ºC for 7 days place in a sealed plastic bag to avoid desiccation

TVC Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting
- Count colonies on filter
- Record results on daily worksheet (date & sign)

Reporting TVC's from membrane filtration process

Reporting procedure as per E.coli/Coliforms (Count is cfu/100ml)

Notifiable Counts (TVC): (Defined by Clients)

Alert figure:
>10 cfu/100ml (both 30ºC and 22ºC)

6.7. RHC Theatre & Water Coolers / GJNH (perfussionist) Cardiology Waters

Such samples arrive and will require TVC's, E.coli/Coliforms & Pseudomonas investigation with the addition of Atypical Mycobacterium investigation (AMI)

Process TVC / E. Coli/Coliform & Pseudomonas as per Potable Water Samples

Atypical Mycobacterium Investigation (AMI)

1. Photocopy request form for CL3
2. Once all other work set up, Transfer remaining sample to CL3 for processing for AMI

7. Quality Control Failure

- Any failure of internal QC must be recorded on worksheet
- For the specific QC failure no results can be released (ie Growth noted on negative control for TVC @ 37)
- Notify customer of failed QC and non availability of results & request repeat testing if required
- Record on telephone log & report form
- Pass completed request forms to office to issue final report
- Office staff photocopy, send PDF via generic email. Original form stored
- Worksheet filed in lab for 1 month, after this pass to office for filing

8. References

- Standing Committee of Analysts, The Microbiology of Drinking Water (2002) – Water Quality and Public health, Methods for the Examination of Waters and Associated Materials. London, Drinking Water Inspectorate.
- ED576 The Microbiology of Drinking Water

Microbiology GG&C	LP538v2
	Authorised by: Julie Black
29/08/2019	Author: Julie Black

Non Legionella Water Testing: Potable Water & Endoscopy Analysis	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

The Microbiological examination of water samples from the healthcare environment has a recognised role in the routine monitoring of decontamination procedures and the demonstration of a safe healthcare environment.

Microbiology GG&C	LP538v2
	Authorised by: Julie Black
29/08/2019	Author: Julie Black

This document provides guidance for the processing and reporting of water from Endoscope departments, Pharmacy sterile suites, hydrotherapy pools, renal and potable waters.

Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values. Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, cleaning records and control monitoring charts are part of the quality assurance.

2. Principles & Methods

The microbiological analysis of water and associated materials involves the use of selective procedures and media to ensure that these water samples are of a suitable standard. In addition, the nature of the organisms being isolated and enumerated can present challenges to the analysts. It is therefore important that the media used, and the procedures conducted reflect the quality of the water examined, and that the data generated is reliable
Samples require a combination of TVC's, Coliform and Pseudomonas investigations and on occasion, Atypical Mycobacterium screening.

3. Environmental and Safety Controls

Appropriate PPE must be worn at all times in the laboratory. Gloves must be worn when handling disinfectants, leaking samples and whilst working in the working in the microbiology safety cabinet. Particular care should be taken and heat resistant PPE should be worn when dealing with boiling/water baths and molten agar.
Please refer to Environmental risk assessment R537.

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Handling Cat 2 Organisms	R529	Biological	Good lab Practice
Environmental Laboratory Risk Assessment	R537	General	Good Lab Practice & PPE

4. Types of Sample

- Potable Waters
- Estates Waters
- Pool waters
- Pharmacy water
- Endoscopy Waters
- Raw untreated Waters

5. Required Equipment and Reagents

Common lab consumables, equipment and the following

- Sterile 47mm filter unit
- Manifold
- Vacuum pump
- Water/Boiling bath
- 0.45µ metrical GN-6 membranes
- Sterile petri dishes

6. Calibration Procedures

- Pipettes used in the process are calibrated annually
  - Daily pipette check refer to QF574
- Balance calibrated annually
  - Daily balance check refer to QF574

7. Pre Analytical Procedural Steps

7.1. Media Pre-acceptance

All media received for use within the Environmental Laboratory will be subject to pre-acceptance testing to ensure the media can support the growth of target organism and for sterility. Known ATCC/NCTC reference control strains are used to assess the performance of the media used in water testing.

When new batches of media arrive in the laboratory, reception follows QP520 (Media and Reagent QC) by segregating new stock into a quarantine area until appropriate pre acceptance testing is completed and passed. Pre acceptance instructions are contained within LI567. Environmental laboratory's pre-acceptance testing is recorded using QF533.

If any new batched of media fail QC and do not support growth of target organism's record full details in QF533, retesting should be carried out. If results remain the same inform the Technical Manager. Plates MUST not be put into use.

Manufacturer will then be contacted to discuss this problem batch & a non conformance should be recorded in Q-Pulse for full investigation.

7.2. Sample Transportation and storage

Sampling should be carried out by suitably trained personnel, laboratory staff do not undertake sampling. Samples should be collected in an appropriately sized sterile bottle containing a neutraliser to neutralise any residual disinfectant within the sample. The most commonly used one is Sodium Thiosulphate. The laboratory provides 500ml sterile bottles containing 18mg of Sodium Thiosulphate to customers for sampling. Small volume Renal samples do not require a neutraliser and can be accepted in a small sterile container.

Samples should be transported to the laboratory within 4 hours and stored between 2-8°C if they cannot be processed immediately. Samples ideally should be analysed as soon as is practicable on the day of collection or within a 24hr period.

- If delay in processing is unavoidable, store samples in fridge/cold room at 2 ^{O}C -8^{O}C and test within 4 days
- If unable to be tested within the 4 day limit samples should be rejected and the customer notified of the delay and a repeat requested
- If processed between 24hr and 4 days a comment should be added to the report stating the delay using comment DELAY#

7.3. Specimen Receipt & Booking In

Samples are received from private companies, GG&C hospitals and various health boards. Water samples should be received and booked in as soon as possible.
For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- Following routine acceptance/rejection criteria for samples
- Date stamp the time of receipt of the samples on their request forms using the clock unit
- Ensure the sample details match the request form – mismatched samples should be rejected, telephone customer to advise of this
- Refer to LI659 (Environmental Set Up)for coding and plate requirements
- If the appearance of samples received is anything other than clear, this must be noted on request form to enable inclusion in final report as this may affect recovery of organisms from the sample
    - CLO (cloudy)
    - OI (oily)
    - SS (suspended solids)

- Label samples using designated laboratory numbers stream
- If investigations required are unclear, confirm by checking with Technical Manager, or contacting sender
- The request forms are placed in the laboratory PID tray for the BMS to triage
- Once triaged the BMS will record the details onto the appropriate daily worksheet and pass the request forms to admin staff for PID

Deviating samples
This includes samples which are not correctly preserved, e.g. have exceeded their maximum preservation time, lack the date and time of sampling, are not cooled appropriately etc. As a result, deviating samples may jeopardise the validity of the reported test result.
If sample(s) are received that are considered deviating samples, the customer must be contacted as soon as possible, informed of the status of the sample(s) and further instructions requested.
If the customer requests the samples be processed, a disclaimer must be added to the report stating that the sample was deviating and, as a result, the test result(s) may be invalid.
In practice, if a customer requests that a deviating sample is processed, ensure the following is added to the report:

- At report entry, enter the code SPDEV and on the next line indicate the reason for the deviation, e.g. delay, poor storage etc
- Enter the customers' instructions, e.g. sample processed (or not processed) following discussion with the client

- Record all relevant information of phone call on request form

Rejected samples
This includes samples which are not suitable for testing i.e. mismatch details, >4 days from sampling to analysis and insufficient samples.
Report as Specimen Not Processed (SPNP) and add additional comments as appropriate.

8. Analytical Procedural Steps

8.1. Agar preparation

Review daily workload to establish number and type of pour plates required.
Collect media and place in boiling bath for 30 minutes (ensure molten).
Transfer molten agar to the 50°C holding bath, using heat resistant gloves & safety glasses. Allow media to cool to 50°C before use.

- Yeast Extract Agar (YEA) and R2 Agar are the two types of media used in the pour plate method of TVC enumeration depending on sample type
- Bottles containing these agars are placed in the boiling water as described above. Review sample numbers to only melt required numbers of media
- Each day a set of negative sterility control plates are set up
- Any bottle of agar, opened or unused at the end of the working day should be discarded

8.2. Filter sterilisation

Sterilisation of the filter units is essential. To do this, boiling bath's 1 and 2 should be switched on and the water level replenished. The filter units should be added to the baths and the temperature level turned to maximum. Once both boiling baths have begun to boil, a 30 minute timer should be used to ensure the filter units receive adequate time to sterilize. Great care should be used when working with the boiling baths and PPE requirements always adhered to.

8.3. Daily Process Controls

Daily process controls are carried out on the filtration method to ensure the process is fit for purpose. The controls MUST be set up and processed along side samples to ensure the full process.

Negative Control

- Appropriate agar plates (MLGA, Pseudo and TSA) should be labelled with the sterile Baxter's water Lot number and date
- The negative control should be carried out first using the same filter unit
- Filter 100ml of sample through this membrane. Once all water has passed through filter, place the membrane onto the appropriate agar plate using sterile forceps for each membrane. **See Membrane Filtration Section for Full method**
- Incubate the plates along with the positive control plates accordingly

Positive Controls

- Two lenticules of E.coli (NCTC 9001)and one of Ps.aeruginosa (NCTC 10662) should be removed for the freezer and allow to come to room temperature (10 minutes)

- Appropriate agar plates (MLGA, Pseudo and TSA) should be labelled with the Lenticule batch number, organism type and date
- Reconstitute each lenticule in well labelled 100ml bottle of MRD (Maximum Recovery Diluent)
- Leave for a minimum of 15 minutes until the lenticule has dispersed, mix the solution well and process through the sterile filter unit. The lenticule should be processed within a period of 45 minutes
- Place filter onto the plate and incubate appropriately with the negative control plate

8.4. Membrane Filtration Samples

Membrane filtration is carried out on the following media for the following target organism;

Agar	Target Organism	Incubation/atmosphere
MLGA	Coliforms and E.coli	30°C/4 hours followed by 37°C /14 -16 hours (O_2)
Pseudomonas Agar	Pseudomonas species	37°C/48 hours (O_2)
TSA	Total Viable Count	37°C/48 hours(O_2)
R2A Agar	Total Viable Count	30C°/5 days and 22°C for 7 days (O_2) - bagged to retain moisture
Middlebrooks	Atypical mycobacteria	35°C for 42 days (O_2)

- Mix water sample well before testing
- Sterile filter units should be removed from the boiling baths and placed on the manifold
- The unit should be allowed to cool sufficiently before aseptically placing a 0.45µl filter paper grid side up on the magnetic unit using sterile blunt end forceps
- All manifold valves should remain closed
- If the sample bottle is full, pour off a little water to allow adequate mixing
- Decant 100ml of sample into the filter unit, switch the vacuum pump on and open the manifold valve
- Once all water has passed through the filter, place membrane onto the appropriate agar plate using sterile forceps for each membrane. (**forceps sterilized with 70% IMS between each use**)
- Incubate plates as per section for media type
- **For atypical mycobacteria**, the plates should be sealed with parafilm to retain moisture and incubated in the appropriate daily rack at 35°C for a period of 42 days in the designated incubator within the CL3 suite

8.5. Pour Plate

Total viable counts of waters and renal waters are carried out using a pour plate method using Yeast extract agar and R2A agar. To do this label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish

- If the sample bottle is full, pour off a little water to allow for adequate mixing, mix well
- Pipette 1 ml of sample into each Petri dish
- Aseptically add approximately 15 ml molten Agar to the Petri dish
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow to set at room temperature
- Invert and Incubate two plates at 37°C for 24 hours and two at 22°C for 72 hours

Negative Controls

- 2 x sterile plates with no sample and approximately 15 ml molten YEA Agar incubate at 37°C for 24 hours
- 2 x sterile plates with no sample and approximately 15 ml molten YEA Agar incubate at 22°C for 72 hours

Renal Pour plates

Samples arrive usually in 60ml volumes for TVC Only. If a 250ml sample is received then E. coli/Coliform & Pseudomonas Filtration is usually required. The request form will detail this. If Coliforms and pseudomonas are requested then the procedures for these filtration methods are outlined in section above

- Label 4 sterile Petri dishes for each sample as above
- Process samples as detailed above
- Aseptically add 15 ml molten **R2Agar** to the Petri dish
- Invert and Incubate two plates at 30°C for 5 Days and two at 22°C for 7 days. Label clearly with date and place in a sealed plastic bag to avoid desiccation of the agar

Negative Controls

- 2 x R2A plates with no sample, incubate at 30°C for 5 Days
- 2 x R2A plates with no sample, incubate at 22°C for 7 days

8.6. RHC Theatre & water cooler/ GJNH (perfussionist) Cardiology waters

These water are processed for slow growing atypical mycobacteria and thus cannot be processed on middlebrooks ager. The customer request form should be photocopied and all other tests carried out. A portion of the sample should be decanted into two sterile universals (approx. 50ml), sealed with parafilm and transferred to the CL3 section whereby they will be processed in liquid culture through the MGiT.

8.7. Enumeration of Pre and Post Treatment of Effluent Waters by Membrane Filtration

Such samples arrive from Babcock etc; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E. coli counts
- Do not require TVC's

Each requires:

- 1 x MLGA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

A. Pre treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on MLGA plate
- Incubate at **30°C for 4hrs** then transfer to **37°C for the remaining 14hrs**

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well
- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E. coli
- Place filter on MLGA plate
- Incubate as per membrane filtration section

9. Examination and reporting of samples and process controls

Process control samples must be examined and have passed specific criteria before sample plates can be examined. The examination and reporting of samples must be carried out by a suitable trained member of staff or a member of staff receiving training under supervision. All agar plates should receive the required amount of incubation in the appropriate temperature and atmosphere.

Criteria for acceptability of daily process control results

Plates should be removed from the incubator and read in a timely fashion, If the plates cannot be counted immediately then store between 2-8°C until this can be carried out.

- Count the number of colonies grown on the appropriate area of the filter paper
- Less than 1 cfu/L of both E. Coli NCTC 9001 and P. aeruginosa NCTC 10662 indicates a failed process control. Inform the section Technical Manager and investigate
- Retesting can be carried out if within four days of the sample being taken
- If the batch of samples processed on this particular day contains a positive isolate, then the results may be released although the event should be investigated
- If the process controls fail and all samples test negative for the representative test then the customer must be contacted and made aware of the circumstances and re-testing should be advised
- Reports should be entered into telepath and as TCE – PRFRT which expands to "Process Control Failure. Retesting recommended" and authorised
- A non-conformity (CAPA) should be raised in Q Pulse and root cause analysis carried out

Review of Process Controls

- After the final read of each process control record on appropriate worksheet and populate QF577 held in the Share Drive (North Glasgow Microbiology/Environmental Laboratory/Process Control Data), before releasing final results
- The mean and standard deviation values objectively assess our daily controls
- Any value out with three standard deviations (3SDs)either side of the mean will be investigated by the technical manager and samples that have been run alongside this process control will be assessed for validity
- Any value recorded out with 3SDs will be annotated on the graph and the corrective action completed on QF577. If warranted a full NC will be raised in the CAPA module of Q-Pulse

Examination and enumeration of Coliform/E.coli

- Examine plates at no less than 18 hours and ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Coliforms and E.coli on the MLGA plates
- Coliforms are lactose positive and will appear yellow
- E.coli is both lactose positive and possesses glucuronidase, this make it appear green. In some cases an E.coli can be a weak glucuronidase producer and will appear yellow
- Perform a count on each colony type (e.g. 5 green, 2 yellow) and record on the appropriate day sheet, sign & date
- If sufficient individual colonies and purity allows, send any green or yellow isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day

- Report as number of Coliforms/E.coli per 100ml taking into account any dilution factors
- Pre/post treatment samples - Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)
- It is expected that the Post treatment counts will be less than those of the Pre treatment samples. If not, then check that the correct procedure has been followed
- See reporting criteria for positive results

Examination and enumeration of Pseudomonas species

- Examine plates at 48 hours and ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Pseudomonas species on the Pseudomonas plates;
    - Typical (green) colonies (likely to be Ps.aeruginosa)
    - Atypical (yellow) colonies or any growth
- Perform an oxidase test on all growth
- Perform a count on each colony type (e.g. 5 green, 2 yellow) and record on relevant day sheet (sign & date)
- If sufficient individual colonies and purity allows, send any suspect isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- See reporting criteria for positive results

Examination of membrane filtration TVC plates – Endoscopy waters only

Endoscopy rinse waters have a Total viable count performed on them using membrane filtration and two TSA plates

- Ensure all QC Plates have passed IQC before reading sample plates
- Count number of colonies on the filters on both TSA plates
- Record on endoscopy water day sheet
- See reporting criteria for positive results

10. Retention of Isolates and samples

Isolates should be stored in the -80°C freezer within the main laboratory in case further investigations are required. Isolates include;

- Any isolate involved in an infection control issue
- Any pseudomonas aeruginsosa isolated from an endoscopy water
- All culture plates showing notifiable growth should be retained for a period of 7 days
- All samples once processed should be retained in the cold room (2-8°C) for a period of 8 days until all investigations have been concluded

11. Reporting Procedures

Reporting Criteria
Any results out with the specified limits in the following tables should be telephoned/emailed to the customer as per their requirements.

Water	TVC @37/30	TVC @22	TVC @30	Coliform/ E.coli	Pseudo aeruginosa	Pseudo Species	Atypical Mycobacteria
POTW/POOL	>10	>100		>0	>0	>0	>0
RFS		>50	>50	>0	>0	>0	-
DFU		>10	>10	>0	>0	>0	-
ERW	*	-	-	-	>0	-	>0
Raw treatment water	-	-	-	Any	-	-	-

*Endoscopy samples have varying limits for TVC reporting please see the following table;

GGC Guidance for Final Rinse Water:	Action/Alert
RO Unit Alert = 34-68 cfu/100ml	>68 cfu/100ml
EWD Bath Alert = 41-82 cfu/100ml	>82 cfu/100ml
Washer supply water filter bank	41-82 cfu/100ml

Interim Result

Results should be transferred from the daily worksheet to the customer request form. Telephone the customer with interim notifiable count and record details of the call on the customer request form. Enter the results into LIMS entering telephone details in TCE. Email the result to customer if required via the admin staff.

Final Result

Results should be transferred from day worksheet to customer request form and entered into LIMS and authorised.

- Forms are labelled for admin staff to issue email result to customer and passed through to the office
- If a notifiable count is recorded on the final day of testing, then the report can be reported with no requirement for a telephone call
- Forms are labelled for admin staff to issue email result to customer and passed through to the office
- Admin staff issue PDF report via email and the form is filed appropriately
- Worksheet filed in lab for 2 months, after this pass to office for filing

12. Examination and enumeration of Atypical mycobacteria

- Plates should be examined weekly for bacterial growth
- Colony count, colonial morphology and any further work to be carried out (e.g. purity plates) should be recorded on LF573, dated and signed
- Any plate showing bacterial growth should be transferred to the Class 1 safety cabinet designated for Category A work
- A film for Ziehl-Neelson (ZN) staining should be prepared, stating the full laboratory number and heat fixed for a minimum of 20 minutes
- A ZN stain should be carried out following LI546 and examined under oil immersion X100 light microscopy
- **Positive mycobacteria** will appear as red bacilli
- Any positive film should be checked by another trained member of staff and countersigned
- The slide should have a positive label added with the full laboratory number, sample identifier and date and stored in the positive slide box
- A negative film will show green stained organisms of different structure e.g. bacilli, cocci

Atypical Mycobacterium Reporting

- Results should be recorded on the LI546 and the customer request form stating the colony forming units
- Any positive result should be reported in the LIMS as an interim result and the appropriate customer/infection control medic notified
- The customer request form should then be emailed to the appropriate customer distribution list by the admin staff and returned to CL3 until the culture has finalised at 42 days
- In many cases a new presumptive mycobacteria isolate should be referred to the Mycobacteria Reference Laboratory in Edinburgh for identification, this should be discussed with a consultant as to their requirements and a middlebrooks slope used
- All positive isolates should be retained for a period of 1 year in the -20 Freezer within the CL3 suite
- All culture plates should be disposed of via the category 3 waste route for autoclaving
- Results for Endoscopy waters should be entered into LIMS into the appropriate singleton test filed (AMS) field
- For other water types, results should be entered into LIMS and should state;
    - Atypical mycobacteria Detected _____CFU/100ml
    - Atypical mycobacteria Not Detected
- Customer request forms should be updated with results/counts and entered and authorised in the LIMS. The customer request form should then be emailed to the appropriate distribution list and filed by the admin staff

13. Quality Control Procedures

- The laboratory participates in external quality assurance scheme PHE
- Process control as detailed below
    - Each new Lot number of E. coli and P. aeruginosa process controls (lenticules) are run through our entire process twenty times
    - The Colony Forming Unit counts achieved for these are recorded on the Batch Control Record Form QF593. This data is then transferred to the spreadsheet QF577, which is held in the North Glasgow Share Drive
    - From this, the mean value as well as one, two and three standard deviation values are calculated via the spreadsheet and a chart generated
    - The mean and standard deviation values generated from this chart will be used as reference values to objectively assess our daily water process controls
    - Expected CFU ranges of control lenticules can be obtained from the Sigma Aldrich website using the lenticule batch number
    - A copy of the lenticule certificate of analysis can also be found in the Environmental section of the share drive
    - For each new batch a new process control chart should be generated with the last complete one saved to Q-Pulse

14. References

- Standing Committee of Analysts, The Microbiology of Drinking Water (2002) – Water Quality and Public health, Methods for the Examination of Waters and Associated Materials. London, Drinking Water Inspectorate.
- ED576 The Microbiology of Drinking Water
- Central Decontamination Unit, Pan Glasgow Document no EDUG22 – Held electronically - [redacted] Share Drive\Endoscopy EDU\Logbooks\Water Sampling
- Health Technical Memorandum 01-06: Decontamination of flexible endoscopes: Part E: Testing methods

Microbiology GG&C	LP538v3
	Authorised by: Julie Black
20/04/20	Author: Julie Black

Non Legionella Water Testing: Potable Water & Endoscopy Analysis	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

The Microbiological examination of water samples from the healthcare environment has a recognised role in the routine monitoring of decontamination procedures and the demonstration of a safe healthcare environment.

This document provides guidance for the processing and reporting of water from Endoscopy departments, Pharmacy sterile suites, hydrotherapy pools, renal and potable waters.

Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values. Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, cleaning records and control monitoring charts are part of the quality assurance.

2. Principles & Methods

The microbiological analysis of water and associated materials involves the use of selective procedures and media to ensure that water samples are of a suitable standard. In addition, the nature of the organisms being isolated and enumerated can present challenges to the analysts. It is therefore important that the media used, and the procedures conducted reflect the quality of the water examined, and that the data generated is reliable
Samples require a combination of TVC's, Coliform and Pseudomonas investigations and on occasion, Atypical Mycobacterium screening.

3. Environmental and Safety Controls

Appropriate PPE must be worn at all times within the laboratory. Gloves must be worn when handling disinfectants, leaking samples and whilst working in the microbiology safety cabinet. Particular care should be taken and heat resistant PPE should be worn when dealing with boiling/water baths and molten agar. Please refer to Environmental risk assessment R537.

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Handling Cat 2 Organisms	R529	Biological	Good lab Practice
Environmental Laboratory Risk Assessment	R537	General	Good Lab Practice & PPE

4. Sample Type

- Potable Waters
- Estates Waters
- Pool waters
- Pharmacy water
- Endoscopy Waters
- Renal fluids
- Raw untreated Waters

5. Equipment and Common lab consumables

- Sterile 47mm filter unit
- Manifold
- Vacuum pump
- Water/Boiling bath
- 0.45µ metrical GN-6 membranes
- Sterile petri dishes

6. Calibration Procedures

- Daily pipette check refer to QF574
- Daily balance check refer to QF574

Equipment	Calibration
Pipettes	Annually
Thermometers	Annually
Balance	Annually
Filter Units	Annually
Centrifuges	Annually
Timers	Annually
Check Weights	Annually – Immediately after Balance Calibration
Thermocouples (TMS)	Annually
Temperature mapping	Every 3 Years
Agar Tempering	Every 5 Years

A re-verification of all testing methods should be carried out every 5 years to ensure the method remains fit for purpose.

7. Pre Analytical Procedural Steps

7.1. Media Pre-acceptance

All media received for use within the Environmental Laboratory will be subject to pre-acceptance testing to ensure the media can support the growth of target organism and for sterility. Known ATCC/NCTC reference control strains are used to assess the performance of the media used in water testing. All details should be recorded on QF533.

When new batches of media arrive within the laboratory, reception staff follow QP520 (Media and Reagent QC) by segregating new stock into a quarantine area until appropriate pre-acceptance testing is completed and passed. Pre-acceptance instructions are stated within LI567.

Retesting should be carried out immediately on any new batch of media that fails quality control and all details should be recorded on QF533 and the section Technical manager should be informed. Plates must NOT be put into use. The manufacturer will then be contacted to discuss the problem and a non conformance should be initiated in Q-Pulse for full investigation. Additional media should be ordered if required.

7.2. Sample Transportation and storage

Sampling should be carried out by suitably trained personnel, laboratory staff do not undertake sampling. Samples should be collected in an appropriately sized sterile bottle containing a neutraliser to neutralise any residual disinfectant within the sample. The most commonly used one is Sodium Thiosulphate. The laboratory provides 500ml sterile bottles containing 18mg of Sodium Thiosulphate to customers for sampling. Small volume renal samples do not require a neutraliser and can be accepted in a small sterile container.

Samples should be transported to the laboratory within 4 hours and stored between 2-8°C if they cannot be processed immediately. Samples ideally should be analysed as soon as is practicable on the day of collection or within a 24hr period.

If samples cannot be tested within the 24 hour limit, samples should be rejected and the customer notified and a repeat requested

7.3. Specimen Receipt & Booking In

Samples are received from private companies, GG&C hospitals and various health boards. Water samples should be received and booked in as soon as possible. For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- All details of the days processing should be recorded on the Daily governance worksheet (QF612). This covers process controls, media, reagents, processing staff and laboratory numbers and ensures all work is traceable.
- Follow routine acceptance/rejection criteria for samples
- Date stamp the time of receipt of the samples on their request forms
- Ensure the sample details match the customer request form – mismatched samples should be rejected, telephone customer to advise of this
- Refer to LI659 (Environmental Set Up)for coding and plate requirements
- If the appearance of samples received is anything other than clear, this must be noted on request form to enable inclusion in final report as this may affect recovery of organisms from the sample
    - CLO (cloudy)
    - OI (oily)
    - SS (suspended solids)

- Label samples using designated laboratory numbers stream
- If investigations required are unclear, confirm by checking with the section Technical Manager or contact sender
- The request forms are placed in the laboratory PID tray for the BMS triage
- Once triaged the BMS will record sender details and laboratory numbers onto the appropriate daily worksheet and pass the request forms to admin staff for PID

7.4. Deviating samples

This includes samples which are not correctly preserved, e.g. have exceeded their maximum preservation time, lack the date and time of sampling, are not cooled

appropriately etc. As a result, deviating samples may jeopardise the validity of the reported test result.
If sample(s) are received that are considered deviating samples, the customer must be contacted as soon as possible, informed of the status of the sample(s) and further instructions requested.
If the customer requests the samples be processed, a disclaimer must be added to the report stating that the sample was deviating and, as a result, the test result(s) may be invalid.
In practice, if a customer requests that a deviating sample is processed, ensure the following is added to the report:

- At report entry, enter the code SPDEV and on the next line indicate the reason for the deviation, e.g. delay, poor storage etc
- Enter the customers' instructions, e.g. sample processed (or not processed) following discussion with the client
- Record all relevant information of phone call on request form

7.5. Rejected samples

This includes samples which are not suitable for testing i.e. mismatch details, >24 hours from sampling to analysis and insufficient samples.
Report as Specimen Not Processed (SPNP) and add additional comments as appropriate.

7.6. Infection control Investigations

On occasion the laboratory will be required to assist in the investigation of infection control issues that may affect a particular ward or hospital location. All ad-hoc investigation work must be arranged via the laboratory Service manager, Quality manager and the Environmental Technical manager prior to work being carried out. An Off SOP request form (MF530) should be completed and the methodology determined with regard to the particular organism sought.

7.7. QEUH 6A – Children's Haematology ward

Twelve weekly samples will be received from QEUH 6A for routine potable water testing and investigations for **gram negative bacilli**. An additional TSA plate (37°C/48H) should be set up on these waters and any bacteria that grow should be sub-cultured. Any gram positives bacteria can be disregarded. All gram negative bacteria should be identified, reported and stored long term. **Any positive isolates should be telephoned to Dr Alistair Leonard or the appropriate infection control lead.**

7.8. RHC Theatre & water cooler/ GJNH (perfussionist) Cardiology waters

These water are processed for slow growing atypical mycobacteria and thus cannot be processed on middlebrooks ager. The customer request form should be photocopied and all other tests carried out. A portion of the sample should be decanted into two sterile universals (approx. 50ml), sealed with parafilm and transferred to the CL3 section with the photocopied form where they will be processed in liquid culture through the MGiT.

7.9. Enumeration of Pre and Post Treatment of Effluent Waters by Membrane Filtration

Such samples arrive from Babcock etc; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E. coli counts
- Do not require TVC's

Each requires:

- 1 x MLGA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

A. Pre treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on MLGA plate
- Incubate at **30°C for 4hrs** then transfer to **37°C for the remaining 14hrs**

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well
- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E. coli
- Place filter on MLGA plate
- Incubate as per membrane filtration section

7.10. Agar preparation

Review daily workload to establish the number and type of pour plates required. Collect media and place in boiling bath for 30 minutes (ensure molten).

Transfer molten agar to the 45°C holding bath, using appropriate PPE. Allow media to cool to 45°C before use. No more than 15 bottles of agar should be prepared at any one time and media should be allowed to cool appropriately.

Number of bottles	Cooling time required
5	30 Minutes
7	40 Minutes
10	40 Minutes
15	70 Minutes

- Yeast Extract Agar (YEA) and R2 Agar are the two types of media used in the pour plate method of TVC enumeration depending on sample type
- Daily process controls are carried out **see 8.3**
- Any bottle of agar, opened or unused at the end of the working day should be discarded

7.11. Filter sterilisation

Sterilisation of the filter units is essential. To do this, boiling bath's 1 and 2 should be switched on and the water level replenished. The filter units should be added to the baths and the temperature level turned to maximum. Once both boiling baths have begun to boil, a 30 minute timer should be used to ensure the filter units receive adequate time to sterilize. Great care should be used when working with the boiling baths and PPE requirements always adhered to.

7.12. Daily Process Controls

Daily process controls are carried out on each singleton test method to ensure the process is fit for purpose. The controls MUST be set up and processed along side samples to ensure the full process is robust.

Media	Plates required	Control Lenticule	Negative Control/Sterility Check	Sample volume
MLGA	4	E.coli NCTC 9001 K.oxytoca NCTC 8167 Ps.aeruginosa NCTC 10662 (non target)	Baxters Water	100ml
PYO	3	Ps.aeruginosa NCTC 10662 E.coli NCTC 9001 (non target)	Baxters Water	100ml
TSA	2	E.coli NCTC 9001	Baxters Water	100ml
YEA	8	E.faecalis NCTC 775	Baxters Water	1ml
R2A (Pour)	8	E.faecalis NCTC 775	Baxters Water	1ml
R2A (pre-pour)	4	E.faecalis NCTC 775	Baxters Water	100ml
Middlebrooks	1	-	Baxters Water	100ml

Negative Control

- Appropriate agar plates should be labelled with the sterile Baxter's water Lot number and date
- The negative control should be carried out first using the same filter unit

- Filter 100ml of the vortexed sample through the membrane. Once all water has passed through, place the membrane onto the appropriate agar plate using sterile forceps for each membrane. **See Membrane Filtration Section for Full method**
- Incubate the plates along with the positive control plates accordingly

Positive Controls

- The required amount of lenticules should be removed for the freezer and allowed to come to room temperature (10 minutes)
- Appropriate agar plates should be labelled with the Lenticule batch number, organism type and date
- Reconstitute each lenticule in well labelled 100ml bottle of MRD (Maximum Recovery Diluent)
- Leave for a minimum of 15 minutes until the lenticule has dispersed, mix the solution well and process through the sterile filter unit. The lenticule should be processed within a period of 45 minutes
- Place filter onto the plate and incubate appropriately with the negative control plate and any sample plates
- Process controls should be carried out in parallel with a batch of samples every day

7.13. Generating New Process Control Data – New Lenticule Batch

- When a new batch number of lenticules are received, the following process is carried out;
    - Each new Lot number of process controls lenticules are run through our entire process twenty times ideally by 4 different individuals
    - The Colony Forming Unit counts achieved for these are recorded on the Batch Control Record Form QF593. This data is then transferred to the spreadsheet QF577, which is held in the North Glasgow Share Drive
    - From this, the mean value as well as one, two and three standard deviation values are calculated via the spreadsheet and a chart generated
    - The mean and standard deviation values generated from this chart will be used as reference values to objectively assess our daily water process controls
    - Expected CFU ranges of control lenticules can be obtained from the Sigma Aldrich website using the lenticule batch number
    - A copy of the lenticule certificate of analysis can also be found in the Environmental section of the share drive
    - For each new batch a new process control chart should be generated with the last complete one saved to Q-Pulse

7.14. Membrane Filtration Samples

Membrane filtration is carried out on the following media for the following target organism;

Agar	Target Organism	Incubation/atmosphere
MLGA	Coliforms and E.coli	30°C/4 hours followed by 37°C /14 -16 hours (O_2)

Pseudomonas Agar	Pseudomonas species	37°C/48 hours (O_2)
TSA	Total Viable Count	37°C/48 hours(O_2)
R2A Agar	Total Viable Count	30C°/5 days and 22°C for 7 days (O_2) – bagged to retain moisture
Middlebrooks	Atypical mycobacteria	35°C for 42 days (O_2)

- Mix water sample well before testing
- Sterile filter units should be removed from the boiling baths and placed on the manifold
- The unit should be allowed to cool sufficiently before aseptically placing a 0.45µl filter paper grid side up on the magnetic unit using sterile blunt end forceps
- All manifold valves should remain closed
- If the sample bottle is full, pour off a little water to allow adequate mixing
- Decant 100ml of sample into the filter unit, switch the vacuum pump on and open the manifold valve
- Once all water has passed through the filter, place membrane onto the appropriate agar plate using sterile forceps for each membrane. (**forceps sterilized with 70% IMS between each use**)
- Incubate plates as per section for media type. The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours
- **For atypical mycobacteria**, the plates should be sealed with parafilm to retain moisture and incubated in the appropriate daily rack at 35°C for a period of 42 days in the designated incubator within the CL3 suite

7.15. Pour Plate

Total viable counts of waters and renal waters are carried out using a pour plate method using Yeast extract agar and R2A agar. To do this label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish. Ensure process controls have been carried out.

- If the sample bottle is full, pour off a little water to allow for adequate mixing, mix well
- Pipette 1 ml of sample into each Petri dish
- Aseptically add approximately 15 – 20ml of molten Agar to the Petri dish within 20 minutes of the 1ml being dispensed
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow agar to set at room temperature
- Invert and Incubate plates at the required temperature/atmosphere

8. Result Interpretation

8.1. TVC Plate Examination

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

A colony count should be carried out as soon as the plates are removed from the incubator. If the plates cannot be counted immediately, store at 4ºC.
Spreading colonies can interfere with counts, and the following gives guidance on what should be counted as a single colony.

- A chain of colonies caused by the disintegration of a clump of organisms
- A spreading growth that develops as a film at the bottom of a Petri dish
- A colony that forms in a film of water at the edge or over the surface of the agar

8.2. Colony Counting Method using Stuart Counter

Before beginning any count examine the plate to ensure that the colonies are spread evenly over the whole plate.
If the appearance of colonies shows no uniform spread, perhaps colonies only present on one side, this would indicate that the initial mixing of the sample was not performed correctly. Unless uniform spread then the count cannot be reliably reported and the test should be repeated.

- Switch on light and ensure that the tally counter is zeroed and free to move when the counting area is depressed
- Place the plate to be counted, without lid, on the grid
- With a fine felt tip pen mark each colony, applying sufficient pressure to cause the tally counter to move
- Record the number of colonies counted from each plate on the relevant Daily Worksheet
- Calculate the mean count and record on the daily worksheet
- Rounding up an average count is acceptable e.g. two counts of 15 and 16 = a average of 15.5 Thus the average should be recorded as 16 CFU

If the number of colonies to be counted is too great e.g. >300 the plate may be divided into a half, quartered or a reduced area counted and multiplied by the reciprocal of that area i.e.

- Count 5 large squares using counter grid which are ≅ 1/10 plate and multiply by 10 for final count/ml
- Count 1 small square using counter grid which is ≅ 1/450 plate and multiply by 9 to give one large square, then multiply by 50 for final count/ml
- If there are more than 20 colonies in 1 small square record the count as greater than 10^4 organisms per ml

8.3. Criteria for acceptability of daily process control results

Process control samples must be examined and have passed specific criteria before sample plates can be examined. The examination and reporting of samples must be carried out by a suitable trained member of staff or a member of staff receiving training under supervision. All agar plates should receive the required amount of incubation in the appropriate temperature and atmosphere.
Plates should be removed from the incubator and read in a timely fashion, If the plates cannot be counted immediately then store between 2-8°C until this can be carried out.

- Count the number of colonies that have grown on the positive control plate
- Less than 1 cfu of any positive control organism indicates a failed process control. Inform the section Technical Manager and investigate
- If the batch of samples processed on this particular day contains a positive isolate, then the results may be released although the event should be investigated
- If the process controls fail and all samples test negative for the representative test then the customer must be contacted and made aware of the circumstances and re-testing should be advised
- Reports should be entered into telepath and as TCE – PRFRT which expands to "Process Control Failure. Retesting recommended" and authorised
- A non-conformity (CAPA) should be raised in Q Pulse and root cause analysis carried out

8.4. Review of Process Controls

- Record the count of all process controls on the daily governance worksheet (QF612) worksheet and populate QF577 held in the Share Drive (North Glasgow Microbiology/Environmental Laboratory/Process Control Data), before releasing final results
- The mean and standard deviation values objectively assess our daily controls
- Any value out with three standard deviations (3SDs)either side of the mean will be investigated by the technical manager and samples that have been run alongside this process control will be assessed for validity
- Any value recorded out with 3SDs will be annotated on the graph and the corrective action completed on QF577. If warranted a full NC will be raised in the CAPA module of Q-Pulse

8.5. Reporting TVC Results

- The TVC reported is the mean of the colony count from the two duplicate plates from an undiluted sample
- Results are recorded on daily worksheet and transcribed to the customer request form as cfu/1ml
- Report as per section 8.9 – reporting criteria

8.6. Examination and enumeration of Coliform/E.coli

Testing for coliform bacteria and *Escherichia coli* (*E. coli*) is one of the most important microbiological examinations carried out on drinking water. The test provides a sensitive means for detecting faecal contamination and for assessing water quality, the effectiveness of water treatment and disinfection.

- Examine plates at no less than 18 hours and within 15 minutes of being removed from the incubator, as the yellow colouration may change on cooling and standing
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Coliforms and E.coli on the MLGA plates. Coliforms are lactose positive and will appear yellow and E.coli is both lactose positive and possesses glucuronidase, this makes it appear green. In some cases an E.coli can be a weak glucuronidase producer and will appear yellow.

- Count all yellow and green colonies (however faint) irrespective of size (e.g. 5 green, 2 yellow), taking note of different colonial morphology if necessary and record on the appropriate day worksheet, sign & date. The maximum number of colonies that should be counted from a single membrane is 100.
- If the growth of pink colonies is considered to be such that they obscure lactose-fermenting colonies, a further portion of the retained sample may be re-examined using an appropriate dilution factor to enable isolated colonies to develop
- If sufficient individual colonies and purity allows, send any green or yellow isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Coliforms/E.coli per 100ml taking into account any dilution factors
- Pre/post treatment samples - Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)
- It is expected that the Post treatment counts will be less than those of the Pre treatment samples. If not, then check that the correct procedure has been followed
- See reporting criteria for positive results

8.7. Examination and enumeration of Pseudomonas species

- Examine plates at 24 hours and finally at 48 hours The number of colonies at 24 hours should be noted on the daily worksheet as growth between 24 and 48 hours may be such that colonial growth results in the merging of colonies, and the number of colonies at 48 hours may seem less than the number of colonies at 24 hours.
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Pseudomonas species on the Pseudomonas plates;
    - Typical (green) colonies (likely to be Ps.aeruginosa)
    - Atypical (yellow) colonies or any growth
- Perform an oxidase test on all growth
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- If sufficient individual colonies and purity allows, send any suspect isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- See reporting criteria for positive results

8.8. Examination of membrane filtration TVC plates – Endoscopy waters only

Endoscopy rinse waters have a Total viable count performed on them using membrane filtration and two TSA plates

- Ensure all QC Plates have passed IQC before reading sample plates
- Count number of colonies present on the filters on both TSA plates
- Record on endoscopy water day sheet and average the two results
- See reporting criteria for positive results

8.9. Reporting Procedures

Reporting Criteria

Any results out with the specified limits in the following tables should be telephoned /emailed to the customer as per their requirements.

Water	TVC @37	TVC @22	TVC @30	Coliform/ E.coli	Pseudo aeruginosa	Pseudo Species	Atypical Mycobacteria
POTW/POOL	>10	>100	-	>0	>0	>0	>0
RFS		>50	>50	>0	>0	>0	-
DFU		>10	>10	>0	>0	>0	-
ERW	*	-	-	-	>0	-	>0
Raw treatment water	-	-	-	Any	-	-	-

*Endoscopy samples have varying limits for TVC reporting please see the following table;

GGC Guidance for Final Rinse Water:	Action/Alert
RO Unit Alert = 34-68 cfu/100ml	>68 cfu/100ml
EWD Bath Alert = 41-82 cfu/100ml	>82 cfu/100ml
Washer supply water filter bank	41-82 cfu/100ml

Interim Result

Results should be transferred from the daily worksheet to the customer request form. Telephone the customer with interim notifiable count and record details of the call on the customer request form. Enter the results into LIMS entering telephone details in TCE. Email the result to customer if required via the admin staff.

Final Result

Results should be transferred from day worksheet to customer request form and entered into LIMS and authorised.

- Forms are labelled and transferred to admin staff who issue a final email report to customer
- If a notifiable count is recorded on the final day of testing, then the report can be reported with no requirement for a telephone call
- Admin staff will Dart scan the customer request form before the form is filed appropriately
- Daily Worksheets filed in lab for 2 months, after this pass to office for dart scanning and filing

8.10. Examination and enumeration of Atypical mycobacteria

- Plates should be examined weekly for bacterial growth

- Colony count, colonial morphology and any further work to be carried out (e.g. purity plates) should be recorded on LF573, dated and signed
- Any plate showing bacterial growth should be transferred to the Class 1 safety cabinet designated for Category A work
- A film for Ziehl-Neelson (ZN) staining should be prepared, stating the full laboratory number and heat fixed for a minimum of 20 minutes
- A ZN stain should be carried out following LI546 and examined under oil immersion X100 light microscopy
- **Positive mycobacteria** will appear as red bacilli
- Any positive film should be checked by another trained member of staff and countersigned
- The slide should have a positive label added with the full laboratory number, sample identifier and date and stored in the positive slide box
- A negative film will show green stained organisms of different structure e.g. bacilli, cocci

8.11. Atypical Mycobacterium Reporting

- Results should be recorded on the LI546 and the customer request form stating the colony forming units
- Any positive result should be reported in the LIMS as an interim result and the appropriate customer/infection control medic notified
- The customer request form should then be emailed to the appropriate customer distribution list by the admin staff and returned to CL3 until the culture has finalised at 42 days
- In many cases a new presumptive mycobacteria isolate should be referred to the Mycobacteria Reference Laboratory in Edinburgh for identification, this should be discussed with a consultant as to their requirements and a middlebrooks slope used
- All positive isolates should be retained for a period of 1 year in the -20 Freezer within the CL3 suite
- All culture plates should be disposed of via the category 3 waste route for autoclaving
- Results for Endoscopy waters should be entered into LIMS into the appropriate singleton test filed (AMS) field
- For other water types, results should be entered into LIMS and should state;
    - Atypical mycobacteria Detected _____CFU/100ml
    - Atypical mycobacteria Not Detected
- Customer request forms should be updated with results/counts and entered and authorised in the LIMS. The customer request form should then be emailed to the appropriate distribution list and filed by the admin staff

9. Retention of Isolates and samples

All culture plates showing notifiable growth should be retained for a period of 7 days within the environmental storage cabinet within the main laboratory.
Any isolate involved in an infection control incident or any Ps.aeruginsosa isolated from endoscopy water should be stored in the -80°C freezer (recorded on LF558 – stored Isolate form) within the main laboratory in case further investigations are required.

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All samples once processed should be retained in the cold room (2-8°C) for a period of 8 days until all investigations have been concluded

10. References

- Central Decontamination Unit, Pan Glasgow Document no EDUG22 – Held electronically - [redacted] Share Drive\Endoscopy EDU\Logbooks\Water Sampling
- Health Technical Memorandum 01-06: Decontamination of flexible endoscopes: Part E: Testing methods
- Standing Committee of Analysts The Microbiology of Drinking Water Part 1 – Water quality, epidemiology and public health
- Standing Committee of Analysts The Microbiology of Drinking Water Part 3 - Practices and procedures for laboratories
- Standing Committee of Analysts The Microbiology of Drinking Water Part 4 - Methods for the Isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157 H7)
- Standing Committee of Analysts The Microbiology of Drinking Water Part 7 - Methods for the enumeration of heterotrophic bacteria
- Standing Committee of Analysts The Microbiology of Drinking Water Part 8 – Methods for the isolation and enumeration of Aeromonas and Pseudomonas aeruginosa
- EDTNA/ERCA Guidelines on Control and Monitoring of Microbiological Contamination in Water for Dialysis
- Guideline on water treatment systems, dialysis water and dialysis fluid quality for haemodialysis and related therapies Clinical Practice Guideline Prepared on behalf of The Renal Association1 and The Association of Renal Technologists2 January 2016
- Eurachem guide EA – 4/10 : Accreditation for Microbiology Laboratories, Second edition (2013)

Microbiology GG&C	LP538v4
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Non Legionella Water Testing: Potable Water & Endoscopy Analysis	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Routine Testing

The Microbiological examination of water samples from the healthcare environment has a recognised role in the routine monitoring of decontamination procedures and the demonstration of a safe healthcare environment.
This document provides guidance for the processing and reporting of water from Endoscopy departments, Pharmacy sterile suites, and hydrotherapy pools, renal and potable waters.

Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values. Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, cleaning records and control monitoring charts are part of the quality assurance.

Infection Control Investigation Testing

On occasion the laboratory will be required to assist in the investigation of infection control issues that may affect a particular ward or hospital location. All 'ad-hoc' investigation work must be requested by the Infection Control Consultant through arrangement with Laboratory Senior Management prior to work being carried out. Refer to MI521 Ad Hoc Request Procedure for the full details.
For each agreed episode of Ad-hoc/Additional Environmental testing a completed request form (LF592) should be in place. This details the expected volume of work and the clear instructions of what the investigation or target organisms are and the reporting criteria.

2. Principles & methods of the procedure

The microbiological analysis of water and associated materials involves the use of selective procedures and media to ensure that water samples are of a suitable standard. In addition, the nature of the organisms being isolated and enumerated can present challenges to the analysts. It is therefore important that the media used, and the procedures conducted reflect the quality of the water examined, and that the data generated is reliable
Samples require a combination of TVC's, Coliform and Pseudomonas investigations and on occasion, Atypical Mycobacterium screening.

3. Environmental & Safety Controls

- Laboratory coats must be worn at all times in the laboratory. Gloves must be worn when handling leaking samples
- Heat resistant gloves and visor must always be worn when working with the boiling baths
- A spill kit is located under the Legionella processing bench – all staff should be aware of the protocol to be followed in the event of a minor / major spillage

- Good laboratory practice and aseptic technique should always be practiced when dealing with ACDP category 2 pathogens and chemicals

Risk Assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	Good Lab Practice & PPE
GGC Waste Policy & RA	H&SP506 R542	Biological Chemical	Good Lab Practice & PPE
Hazard Group 2 & 3 Pathogens: Microbiological Manipulations & Exposure	R531	Biological	Good Lab Practice & PPE
Working in the Environmental Laboratory	R537	General	Good Lab Practice & PPE
Distel	COSHH508	Chemical	Good Lab Practice & PPE

Laboratory environmental monitoring

Environmental monitoring of the laboratory is carried out on a weekly basis by the section CSW. This is to reduce the likelihood of environmental contamination of any given sample which can render results invalid. Results are interpreted by the section Biomedical Scientist.
Refer to LP539for full details
Any breaches in alert levels should be relayed to a Technical Manager as soon as possible and a deep clean of the laboratory and/or equipment along with retesting should be carried out.
Benches are cleaned daily and all equipment cleaned once a week with Distel (recorded on H&SF516).

Laboratory settle plates monitoring

Settle plates should be set out and exposed to the environment for a 1 hour period. Plates should then be sealed using parafilm and incubated accordingly. All results should be reported on LF521

Settle plate	Temperature(˚C)	Atmosphere	Duration	Alert limit
SAB	22˚C	O_2	7 Days	>10 Fungi
SAB	30˚C	O_2	7 Days	>10 Fungi
TSA	30˚C	O_2	7 Days	>20 Bacteria
GVPC	37˚C	O_2	10 Days	>0 Legionella

Water bath Monitoring

TVC testing is performed weekly on each of the laboratories holding baths e.g. Water bath 3 (Potable 45˚C) and 4 (Legionella 50˚C). Record all results on LF518.

	Temperature	Atmosphere	Duration	Alert limit
TVC per 1ml	22˚C	O_2	72 Hours	>10 CFU
	37˚C	O_2	48 Hours	>10 CFU

Water baths are routinely emptied on a Friday afternoon, cleaned and reinstated on a Monday morning.

4. Sample Types

- Potable Waters
- Estates Waters
- Pool waters
- Pharmacy water
- Endoscopy Waters
- Renal fluids
- Raw untreated Waters

5. Equipment and Common lab consumables

- Sterile 47mm filter unit
- Manifold
- Vacuum pump
- Water/Boiling bath
- 0.45µ metrical GN-6 membranes
- Sterile petri dishes

6. Calibration Procedures

- Daily pipette & balance check refer to QF574

Equipment	Calibration
Pipettes	Annually
Thermometers	Annually
Balance	Annually
Filter Units	Annually
Stuart Counter	Annually
Centrifuges	Annually
Timers	Annually
Check Weights	Annually -Immediately after Balance Calibration
Thermocouples (TMS)	Annually
Temperature mapping	Every 3 Years
Agar Tempering	Every 5 Years

A re-verification of all testing methods should be carried out every 5 years to ensure the method remains fit for purpose.

7. Pre Analytical Procedural Steps

7.1. Media Pre-acceptance

All media received for use within the Environmental Laboratory will be subject to pre-acceptance testing to ensure the media can support the growth of target organism and for sterility. Known ATCC/NCTC reference control strains are used to assess the performance of the media used in water testing. All details should be recorded on QF533.

When new batches of media arrive within the laboratory, reception staff follow QP520 (Media and Reagent QC) by segregating new stock into a quarantine area until appropriate pre-acceptance testing is completed and passed. Pre-acceptance instructions are stated within LI567.

Retesting should be carried out immediately on any new batch of media that fails quality control and all details should be recorded on QF533 and the section Technical manager should be informed. Plates must NOT be put into use. The manufacturer will then be contacted to discuss the problem and a non-conformance should be initiated in Q-Pulse for full investigation. Additional media should be ordered if required.

7.2. Sample transportation & storage

Sampling should be carried out by suitably trained personnel, laboratory staff do not undertake sampling. Samples should be collected in an appropriately sized sterile bottle containing a neutraliser to neutralise any residual disinfectant within the sample. The most commonly used one is Sodium Thiosulphate. The laboratory provides 500ml sterile bottles containing 18mg of Sodium Thiosulphate to customers for sampling. Small volume renal samples do not require a neutraliser and can be accepted in a small sterile container.

Samples should be transported to the laboratory within 4 hours and stored between 2-8°C if they cannot be processed immediately. Samples ideally should be analysed as soon as is practicable on the day of collection or within a 24hr period.

If samples cannot be tested within the 24 hour limit, samples should be rejected and the customer notified and a repeat requested

7.3. Specimen receipt & booking in

Samples are received from private companies, GG&C hospitals and various health boards. Water samples should be received and booked in as soon as possible. For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- All details of the days processing should be recorded on the Daily governance worksheet (QF612). This covers process controls, media, reagents, processing staff and laboratory numbers and ensures all work is traceable
- Date stamp the time of receipt of the samples on their request forms
- Ensure the sample details match the customer request form – mismatched samples should be rejected, telephone customer to advise of this
- Refer to LI659 (Environmental Set Up)for coding and plate requirements for standard testing, for any ad hoc requests refer to LF592 for the full details as directed by Infection Control Consultant
- If the appearance of samples received is anything other than clear, this must be noted on request form to enable inclusion in final report as this may affect recovery of organisms from the sample
    - CLO (cloudy)
    - OI (oily)
    - SS (suspended solids)

- Label samples using designated laboratory numbers stream
- If investigations required are unclear, confirm by checking with the section Technical Manager or contact sender
- The request forms are placed in the laboratory PID tray for the BMS triage

- Once triaged the BMS will record sender details and laboratory numbers onto the appropriate daily worksheet and pass the request forms to admin staff for PID

7.4. Deviating & rejection criteria and process

Deviating Samples - This is samples that are not correctly preserved, e.g. have exceeded their maximum preservation time, >24 hours from sampling, lack the date and time of sampling, are not cooled appropriately etc. As a result, deviating samples may jeopardise the validity of the reported test result.
If sample(s) are received that are considered deviating samples, the customer must be contacted as soon as possible, informed of the status of the sample(s) and further instructions requested.
If the customer requests the samples be rejected see section below.
If the customer request the samples to be processed, a disclaimer must be added to the report stating that the sample was deviating and, as a result, the test result(s) may be invalid.
In practice, if a customer requests that a deviating sample is processed, ensure the following is added to the report:

- At report entry, enter the code **SPDEV** and additional codes to indicate the reason for the deviation, e.g. delay, poor storage etc
- Enter the customers' instructions, e.g. sample processed (or not processed) following discussion with the client
- Record all relevant information of phone call on request form

Rejected Samples
This includes samples which are not suitable for testing i.e. mismatch details, insufficient samples or any that the customer has agreed based on deviating nature above.

Report as Specimen Not Processed (SPNP) and add additional comments as appropriate.

- At report entry, enter the code **SPNP** and additional codes to indicate the reason for the rejection e.g. insufficient sample, incorrect sample etc.
- Record all relevant information of phone call on request form

8. Procedural Steps

8.1. Agar Preparation

Review daily workload to establish the number and type of pour plates required.
Collect media and place in boiling bath for 30 minutes (ensure molten).
Transfer molten agar to the 45ºC holding bath, using appropriate PPE. Allow media to cool to 45ºC before use. No more than 15 bottles of agar should be prepared at any one time and media should be allowed to cool appropriately.

Number of bottles	Cooling time required

5	30 Minutes
7	40 Minutes
10	40 Minutes
15	70 Minutes

- Yeast Extract Agar (YEA) and R2 Agar are the two types of media used in the pour plate method of TVC enumeration depending on sample type
- Daily process controls are carried out **see section 8.3**
- Any bottle of agar, opened or unused at the end of the working day should be discarded

8.2. Filter sterilisation

Sterilisation of the filter units is essential. To do this, boiling bath's 1 and 2 should be switched on and the water level replenished. The filter units should be added to the baths and the temperature level turned to maximum. Once both boiling baths have begun to boil, a 30 minute timer should be used to ensure the filter units receive adequate time to sterilize. Great care should be used when working with the boiling baths and PPE requirements always adhered to.

8.3. Daily Process Controls

Daily process controls are carried out on each singleton test method to ensure the process is fit for purpose. The controls MUST be set up and processed along side samples to ensure the full process is robust.

Media	Plates required	Sample volume	Positive Control Lenticule	Negative Control	Incubation
CCA	4	100ml	E.coli NCTC 9001 K.oxytoca NCTC 8167 Ps.aeruginosa NCTC 10662 (non target)	Baxters Water	37°C/18H
PYO	3	100ml	Ps.aeruginosa NCTC 10662 E.coli NCTC 9001 (non target)	As Above	37°C/48H
TSA	2	100ml	E.coli NCTC 9001	As Above	37°C/48H
YEA	8	1ml	E.faecalis NCTC 775	As Above	37°C/48H 22°C/72H
R2A (Pour)	8	1ml	E.faecalis NCTC 775	As Above	33°C/5D 22°C/7D
R2A (pre-pour)	4	100ml	E.faecalis NCTC 775	As Above	As Above
Middlebrooks	2	100ml	M.fortuitum NCTC 10394	As Above	35°C/42D
SAB	4	100ml	C.albicans NCPF 3255	As Above	30/22°C/7D

Negative Control

- Appropriate agar plates should be labelled with the sterile Baxter's water Lot number and date
- The negative control should be carried out first using the same filter unit
- Filter 100ml of the vortexed sample through the membrane. Once all water has passed through, place the membrane onto the appropriate agar plate

using sterile forceps for each membrane. **See Membrane Filtration Section for Full method**

- Incubate the plates along with the positive control plates accordingly

Positive Controls

- The required amount of lenticules should be removed for the freezer and allowed to come to room temperature (10 minutes)
- Appropriate agar plates should be labelled with the Lenticule batch number, organism type and date
- Reconstitute each lenticule in well labelled 100ml bottle of MRD (Maximum Recovery Diluent)
- Leave for a minimum of 15 minutes until the lenticule has dispersed, mix the solution well and process through the sterile filter unit. The lenticule should be processed within a period of 45 minutes
- Place filter onto the plate and incubate appropriately with the negative control plate and any sample plates
- Process controls should be carried out in parallel with a batch of samples every day

8.4. Generating New Process Control Data – New Lenticule Batch

- When a new batch number of lenticules are received, the following process is carried out;
    - Each new Lot number of process controls lenticules are run through our entire process twenty times ideally by 4 different individuals
    - The Colony Forming Unit counts achieved for these are recorded on the Batch Control Record Form QF593. This data is then transferred to the spreadsheet QF577, which is held in the North Glasgow Share Drive
    - From this, the mean value as well as one, two and three standard deviation values are calculated via the spreadsheet and a chart generated
    - The mean and standard deviation values generated from this chart will be used as reference values to objectively assess our daily water process controls
    - Expected CFU ranges of control lenticules can be obtained from the Sigma Aldrich website using the lenticule batch number
    - A copy of the lenticule certificate of analysis can also be found in the Environmental section of the share drive
    - For each new batch a new process control chart should be generated with the last complete one saved to Q-Pulse

9. Methods

9.1 Membrane Filtration Procedure

Membrane filtration is carried out on the following media for the following target organism:
Refer to LI659 for full details

Agar	Target Organism	Incubation/atmosphere
CCA	Coliforms and E.coli	37°C/18 hours (O_2)
Pseudomonas Agar	Pseudomonas species	37°C/48 hours (O_2)
TSA	Total Viable Count Or Target GNB	37°C/48 hours(O_2)
R2A Agar	Total Viable Count	30° C /5 days and 22°C for 7 days (O_2) – bagged to retain moisture
Middlebrooks	Atypical mycobacteria	35°C for 42 days (O_2) – sealed with parafilm
SAB	Yeast & Fungi	30°C 7 days (O_2) 22°C 7 days (O_2)

- Mix water sample well before testing
- Sterile filter units should be removed from the boiling baths and placed on the manifold
- The unit should be allowed to cool sufficiently before aseptically placing a 0.45µm filter paper grid side up on the magnetic unit using sterile blunt end forceps
- All manifold valves should remain closed
- If the sample bottle is full, pour off a little water to allow adequate mixing
- Decant 100ml of sample into the filter unit, switch the vacuum pump on and open the manifold valve
- Once all water has passed through the filter, place membrane onto the appropriate agar plate using sterile forceps for each membrane. (**forceps sterilized with 70% IMS between each use**)
- Incubate plates as per section for media type. The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours
- **For Fungal cultures**, plates should be sealed with parafilm to avoid fungal contamination of incubators which can be problematic
- **For atypical mycobacteria**, the plates should be sealed with parafilm to retain moisture and incubated in the appropriate daily rack at 35°C for a period of 42 days in the designated incubator within the CL3 suite

9.2 Pour Plate Procedure

Total viable counts of waters and renal waters are carried out using a pour plate method using Yeast extract agar or R2A agar. To do this label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish. Ensure process controls have been carried out.

- If the sample bottle is full, pour off a little water to allow for adequate mixing, mix well
- Pipette 1 ml of sample into each Petri dish
- Aseptically add approximately 15 – 20ml of molten Agar to the Petri dish within 20 minutes of the 1ml being dispensed
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow agar to set at room temperature
- Invert and Incubate plates at the required temperature/atmosphere (see LI659)

9.3 RHC Theatre & water cooler/ GJNH (perfussionist) Cardiology waters Procedure

These water are processed for slow growing atypical mycobacteria and thus cannot be processed on Middlebrooks ager. The customer request form should be photocopied and all other tests carried out. A portion of the sample should be decanted into two sterile universals (approx. 50ml), sealed with parafilm and transferred to the CL3 section with the photocopied form where they will be processed in liquid culture through the MGiT (See LI693 for PID instruction and LP505 for processing guidelines)

9.4 Enumeration of Pre and Post Treatment of Effluent Waters by Membrane Filtration Procedure

Such samples arrive from Babcock etc.; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E. coli counts
- Do not require TVC's

Each requires:

- 1 x CCA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

A. Pre-treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on CCA plate
- Incubate at 37°C/O_2 for a minimum of 18 hours

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well

- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E. coli
- Place filter on CCA plate
- Incubate at 37°C/O_2 for a minimum of 18 hours

10 Result Interpretation & Reporting

10.1 Criteria for acceptability of daily process control results

Process control samples must be examined and have passed specific criteria before sample plates can be examined. The examination and reporting of samples must be carried out by a suitable trained member of staff or a member of staff receiving training under supervision. All agar plates should receive the required amount of incubation in the appropriate temperature and atmosphere.
Plates should be removed from the incubator and read in a timely fashion, If the plates cannot be counted immediately then store between 2-8°C until this can be carried out.

- Count the number of colonies that have grown on the positive control plate
- Less than 1 cfu of any positive control organism indicates a failed process control. Inform the section Technical Manager and investigate
- If the batch of samples processed on this particular day contains a positive isolate, then the results may be released although the event should be investigated first by the section TM and Quality department
- If the process controls fail and all samples test negative for the representative test then the customer must be contacted and made aware of the circumstances and re-testing should be advised
- Reports should be entered into telepath and as TCE – PRFRT which expands to “Process Control Failure. Retesting recommended” and authorised
- A non-conformity (CAPA) should be raised in Q Pulse and root cause analysis carried out

Review of Process Controls

- Record the count of all process controls on the daily governance worksheet (QF612) worksheet and populate QF577 held in the Share Drive (North Glasgow Microbiology/Environmental Laboratory/Process Control Data), before releasing final results
- The mean and standard deviation values objectively assess our daily controls
- Any one value recorded out with 3SDs represents a large sudden shift from the average and will be annotated on the graph and highlighted in yellow. This can be considered a sporadic occurrence. Any corrective action should be noted on QF577
- If two out of three successive counts exceed 3SDs then an investigation should be carried out by the section TM in conjunction with the Quality

department. In most cases a non-conformance should be raised unless deemed unnecessary. All values should be highlighted in yellow

- Nine consecutive counts falling on the same side of the mean represents a shift and an investigation should be carried out by the section TM to find the potential cause for this. This may reflect the original values being unreliable and result in a new process control data and chart being compiled to reflect the shift
- Six consecutive counts continually rise/fall denotes a trend and should be investigated by the section TM. The quality of reference material may need to questioned

10.2 Examination & Enumeration of Total Viable Counts (TVC)

TVC – Pour Plate Interpretation

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

A colony count should be carried out as soon as the plates are removed from the incubator. If the plates cannot be counted immediately, store at 4ºC.
Spreading colonies can interfere with counts, and the following gives guidance on what should be counted as a single colony.

- A chain of colonies caused by the disintegration of a clump of organisms
- A spreading growth that develops as a film at the bottom of a Petri dish
- A colony that forms in a film of water at the edge or over the surface of the agar
- Due to the variation in organism distribution within water, split samples e.g. TVC's done in duplicate, can display significant variation in enumerations. For example if one TVC plate is enumerated at 5 CFU/1ml then the 95% confidence interval for the 2nd plate would be an enumeration between 0-15 CFU/1ml. Please see Annex A for acceptable 95% confidence intervals. Thus in 5% of cases (1 in every 20 samples) the difference in enumeration between TVC plate 1 and 2 can be larger than the expected count, however if counts exceed 5% of difference then an investigation into this reason must be initiated and a non-conformance raised in Q-pulse.

Colony Counting Method using Stuart Counter

Before beginning any count examine the plate to ensure that the colonies are spread evenly over the whole plate.
If the appearance of colonies shows no uniform spread, perhaps colonies only present on one side, this would indicate that the initial mixing of the sample was not performed correctly. Unless uniform spread then the count cannot be reliably reported and the test should be repeated.

- Switch on light and ensure that the tally counter is at zero and free to move when the counting area is depressed
- Place the plate to be counted, without lid, on the grid
- With a fine felt tip pen mark each colony, applying sufficient pressure to cause the tally counter to move

- Record the number of colonies counted from each plate on the relevant Daily Worksheet
- Calculate the mean count and record on the daily worksheet
- Rounding up an average count is acceptable e.g. two counts of 15 and 16 = a average of 15.5 Thus the average should be recorded as 16 CFU

If the number of colonies to be counted is too great e.g. >300 the plate may be divided into a half, quartered or a reduced area counted and multiplied by the reciprocal of that area i.e.

- Count 5 large squares using counter grid which are ≅ 1/10 plate and multiply by 10 for final count/ml
- Count 1 small square using counter grid which is ≅ 1/450 plate and multiply by 9 to give one large square, then multiply by 50 for final count/ml
- If there are more than 20 colonies in 1 small square record the count as greater than 104 organisms per ml

Reporting of Pour Plate TVC Results

- The TVC reported is the mean of the colony count from the two duplicate plates from an undiluted sample
- Results are recorded on daily worksheet and transcribed to the customer request form as cfu/1ml
- Refer to Reporting criteria section

TVC – Membrane Filtration Interpretation – Endoscopy Waters Only

Endoscopy rinse waters have a Total viable count performed on them using membrane filtration and two TSA plates

- Ensure all QC Plates have passed IQC before reading sample plates
- Count number of colonies present on the filters on both TSA plates
- Record on endoscopy water day sheet and average the two results
- Refer to Reporting criteria section

10.3 Examination & Enumeration of Coliform / E. coli

Testing for coliform bacteria and *Escherichia coli* (*E. coli*) is one of the most important microbiological examinations carried out on drinking water. The test provides a sensitive means for detecting faecal contamination and for assessing water quality, the effectiveness of water treatment and disinfection**. It should be noted that CCA agar does not inhibit the growth of gram positive bacteria and can in some cases hinder the identification of enterobacteriaceae, any samples showing high counts of gram positive bacteria should be diluted and retested (result should be multiplied by dilution factor) to ensure target organisms are isolated.**

- Examine CCA plates at no less than 18 hours and within 15 minutes of being removed from the incubator
- Ensure all QC Plates have passed IQC before reading sample plates

- Check for presence of Coliforms and E.coli on the CCA plates. Coliforms are will appear as red/pink colonies and E.coli will appear as violet/blue colonies.
- Count all red/pink and violet/blue colonies (however faint) irrespective of size (e.g. 5 red, 2 blue), taking note of different colonial morphology if necessary and record on the appropriate day worksheet, sign & date. The maximum number of colonies that should be counted from a single membrane is 100.
- If the growth of clear colonies (non – target organism) is considered to be such that they obscure potential Coliform growth then a repeat sample from the customer may be required – discuss with section TM
- If sufficient individual colonies and purity allows, send any red/pink or violet/blue isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Coliforms/E.coli per 100ml taking into account any dilution factors
- Pre/post treatment samples - Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)
- It is expected that the Post treatment counts will be less than those of the Pre-treatment samples. If not, then check that the correct procedure has been followed
- Refer to Reporting criteria section

10.4 Examination & Enumeration of Pseudomonas species

- Examine plates at 24 hours and finally at 48 hours The number of colonies at 24 hours should be noted on the daily worksheet as growth between 24 and 48 hours may be such that colonial growth results in the merging of colonies, and the number of colonies at 48 hours may seem less than the number of colonies at 24 hours.
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Pseudomonas species on the Pseudomonas plates;
    - Typical (green) colonies (likely to be Ps.aeruginosa)
    - Atypical (yellow) colonies or any growth
- Perform an oxidase test on all growth
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- If sufficient individual colonies and purity allows, send any suspect isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- Refer to Reporting criteria section

10.5 Examination & Enumeration of Ad Hoc Target Gram Negative Bacteria

- Examine TSA plates at 48 hours
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of any GNB on the TSA plate;
- Perform a Gram film on all colony types
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- All gram positive bacteria can be disregarded and plates stored for 7 days
- If sufficient individual colonies and purity allows, GNB isolates should be sent to the main laboratory for identification using Vitek/MS. and result recorded on the daily worksheet
- Any isolate that fails initial MSID should be retested on the MS before a VITEK2 gram negative identification is carried out
- Any isolate that fails identification by the above method should be reported as Environmental GNB
- **Mixed colonies** – purity plate onto Blood agar and incubate at 37ºC for 18-24 hrs. Gram film/Send to the main laboratory for identification the following day
- Report as number of Target Organism per 100ml
- Refer to Reporting criteria section
- Store all target organism in the -80 freezer in the Environmental section and complete LF558

Reporting of Ad-hoc/Additional Environmental GNB results

- Gram Negative Testing – If the request has been for All GNB. All GNB should be reported on customer request forms and in LIMS. Where no GNB but other organisms may be present such as Gram Positive bacteria, samples should be reported as “No environmental Gram-negative bacilli Isolated” (comment/barcode NEGNB) See LI670
- Target Organism Testing e.g. when a specific organism is requested such as Cupriavidus then this is all that should be reported as either detected or not detected (NOTDET). No laboratory work or other named organisms identified during the testing should be submitted to the requestor (Including ICD). All this information should be classed as lab use only and be available only on the ‘back of form’, it should not be transcribed to LIMs or emailed as a result
- All bacterial speciation can be reported in LIMS via NSI – AISO, whereby organisms can be reported with specific counts
- All completed LF592’s (Ad Hoc/Additional Environmental requests) should remain in the laboratory with the appropriate customer request form until all results have finalised and a copy sent to the Quality Department on completion
- All results should be emailed by the admin staff to the named personal detailed with the LF592 and Dart scanned

10.6 Examination & Enumeration of Fungi

- Examine SAB plates at day 2 for yeast species and day 7 for fungi
- Ensure all QC Plates have passed IQC before reading sample plates
- Examine plates for the presence of yeasts, performing either a wet prep or gram film for confirmation – further identification if required can be done via the Malditof (MS) from fresh purity plates
- Re-incubate all plates until day 7
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- All filamentous fungi should be referred to the mycology section using LF539 (Mycology Referral Isolate form) for further identification using LP560 – Non superficial Mycology (ensure the plate is sealed with parafilm to avoid the release of spores)
- Report as number yeast/fungi per 100ml as an interim result on the customer request form and within LIMS
- Refer to Reporting criteria section

Reporting of Fungal results

- All identifications returned from mycology should be transcribed onto the daily worksheet (LF533), customer request form and input and authorised within LIMS as a final report using LI670
- All results are entered into the LIMS by HCPC registered BMS
- All yeast species should be reported as Yeast species unless a full identification has been specifically requested (LF592)
- Exophiala species (Black yeast) should be confirmed using a gram film/wet prep and reported as Exophiala species – a referral to mycology is not required and full identification should only be carried out if requested
- All results should be emailed by the admin staff to the appropriate distribution list and Dart scanned

10.7 Examination & Enumeration of Atypical mycobacterium

- Ensure all process control plates show the expected results before reading sample plates
- Plates should be examined weekly for bacterial growth
- Colony count, colonial morphology and any further work to be carried out (e.g. purity plates) should be recorded on LF573, dated and signed
- Any plate showing bacterial growth should be transferred to the Class 1 safety cabinet designated for Category A work
- A film for Ziehl-Neelson (ZN) staining should be prepared, stating the full laboratory number and heat fixed for a minimum of 20 minutes
- A ZN stain should be carried out following LI546 and examined under oil immersion X100 light microscopy
- **Positive mycobacteria** will appear as red bacilli
- Any positive film should be checked by another trained member of staff and countersigned

- The slide should have a positive label added with the full laboratory number, sample identifier and date and stored in the positive slide box
- A negative film will show green stained organisms of different structure e.g. bacilli, cocci

Reporting of Atypical Mycobacterium Results

- Ensure all process control plates have passed IQC before reading sample plates – ensure QF612 (Daily governance worksheet) and QF577 (Enviro process control charts) are updated to reflect this information
- Results should be recorded on the LF573 and the customer request form stating the colony forming units
- Fungal contamination may occur as plates are incubated for long periods of time – Consideration should be given to the validity of results. For major contamination, discard plates and record details on the AMS worksheet and customer request form. The auto comment "CON1" and "CON2" should be entered into TCE in telepath, this expands to "Due to contamination issue, unable to report – If available, Please send another sample". Contact the customer to notify them of the circumstance
- Any positive result should be reported in the LIMS as an interim result and the appropriate customer/infection control medic notified
- The customer request form should then be emailed to the appropriate customer distribution list by the admin staff and returned to CL3 until the culture has finalised at 42 days
- In many cases a new presumptive mycobacteria isolate should be referred to the Mycobacteria Reference Laboratory in Edinburgh for identification, this should be discussed with a consultant as to their requirements and a Middlebrooks slope used
- All positive isolates should be retained for a period of 1 year in the -20 Freezer within the CL3 suite
- All sealed culture plates should be disposed of via the category 3 waste route for autoclaving
- Customer request forms should be updated with results/counts and entered and authorised in LIMS at 42 days
- Results should be entered into LIMS into the appropriate singleton test field, stating "N" for not detected or "D" for detected. If the result is positive then an enumeration per 100ml should be entered into the appropriate telepath field
- AMSPR (AMS in Progress) should be removed from the comments section
- The customer request form should then be emailed to the appropriate distribution list, dart scanned and filed by the admin staff
- All complete AMS worksheets should be sent to the office to be dart scanned and returned. These forms shore be retained in the records room in the appropriate box file

11 Reporting Criteria

Any results out with the specified limits in the following tables should be telephoned /emailed to the customer as per their requirements.

Microbiology GG&C	LP538v4
	Authorised by: Julie Black
24/08/2021	Author: Julie Black

								Requests	
Water	TVC @37	TVC @22	TVC @30	Coliform/ E.coli	Pseudo aeruginosa	Pseudo Species	Atypical Mycobacteria	Target Org / GNB	Fungi @22 or 30
POTW/POOL	>10	>100	-	>0	>0	>0	>0	>0	>0
RFS		>50	>50	>0	>0	>0	-	-	-
DFU		>10	>10	>0	>0	>0	-	-	-
ERW	*	-	-	-	>0	-	>0	-	-
Raw treatment water	-	-	-	Any	-	-	-	-	-

*Endoscopy samples have varying limits for TVC reporting please see the following table:

GGC Guidance for Final Rinse Water:	**Action/Alert**
RO Unit Alert = 34-68 cfu/100ml	>68 cfu/100ml
EWD Bath Alert = 41-82 cfu/100ml	>82 cfu/100ml
Washer supply water filter bank	41-82 cfu/100ml

Interim Reporting

Results should be transferred from the daily worksheet to the customer request form. Telephone the customer with interim notifiable count and record details of the call on the customer request form. Enter the results into LIMS entering telephone details in TCE/TELWAT. Email the result to customer if required or if this is the customers preferred method for communication via the admin staff.

Final Reporting

Results should be transferred from day worksheet to customer request form and entered/authorised into LIMS by HCPC registered BMS staff.

- All Negatives are entered using barcode scripts (LI670)
- All report codes/comments can be found on LI670
- All positives should be entered manually

- Specific organism identifications with counts should be reported via the organism table (NSI, AISO)
- Once all reporting complete, release as final 'PRF' and 'NRQ'
- Check the OUTW/WINP queues daily for any outstanding results – complete LF524
- Customer request forms are labelled with an instruction card stating what work should be carried out by the admin staff e.g. email, dart scan, file etc
- If a notifiable count is recorded on the final day of testing, then the report can be reported with no requirement for a telephone call
- All Environmental forms should be Dart scanned and filed appropriately
- Daily Worksheets are held in the environmental laboratory for 2 months, after this pass to office for dart scanning and filing in the records room

Reports are authorised by;

- **PRI -** Interim report (There can be multiple interims if required)

- **PRF -** Final report (There must only be 1 final report issued, anything after this MUST be additional)
- **PRA -** Additional report
- **NRQ** - to release results

ALL RESULTS ARE AUTHORISED BY THE BMS FROM THE SUPERVISOR AUTHORISATION QUEUE

This queue should be checked every time a result is released to ensure that all reports are authorised.

Referral of Isolates

- Isolates should only be referred to a reference laboratory at the request of medical staff
- All isolates being referred to Reference labs must be parcelled & sent as per Postal SOP LP542 and details recorded on LF515
- Each send away sample must have a postal declaration form LF516 completed
- LIMS should be updated in comments "REFER" with the organism identification and released as an Interim report

Reports returned from Reference labs

- All reference reports are received by specimen reception / office and will be scanned to DART &/or portal before being handed to the laboratory
- Once the reference lab reports are received by the bench the BMS should enter RLRENV and enter the date the report was received
- All details reported on the reference report should be entered manually into the comments section of telepath to enable the user to see results - **results are not viewable via portable**
- The BMS must complete LF515 and then authorise the report to the appropriate status required

12 Retention of Isolates & Samples

All culture plates showing notifiable growth should be retained for a period of 7 days within the environmental storage cabinet within the main laboratory.
Any isolate reported in an infection control incident or any Ps.aeruginsosa isolated from endoscopy water should be stored in the -80°C freezer (recorded on LF558 – stored Isolate form) within the main laboratory in case further investigations are required.

All samples once processed should be retained in the cold room (2-8°C) for a period of 8 days until all investigations have been concluded

13 References

- Central Decontamination Unit, Pan Glasgow Document no EDUG22 – Held electronically - [redacted] Share Drive\Endoscopy EDU\Logbooks\Water Sampling
- Health Technical Memorandum 01-06: Decontamination of flexible endoscopes: Part E: Testing methods

- Standing Committee of Analysts The Microbiology of Drinking Water Part 1 – Water quality, epidemiology and public health : 2002
- The Microbiology of Water and Associated Materials (2017)
  Practices and Procedures for Laboratories
  Methods for the Examination of Waters and Associated Materials
- Standing Committee of Analysts The Microbiology of Drinking Water Part 4 - Methods for the Isolation and enumeration of coliform bacteria and Escherichia coli (including E. coli O157 H7) : 2016
- Standing Committee of Analysts The Microbiology of Drinking Water Part 7 - Methods for the enumeration of heterotrophic bacteria : 2020
- Standing Committee of Analysts The Microbiology of Drinking Water Part 8 – Methods for the isolation and enumeration of Aeromonas and Pseudomonas aeruginosa : 2015
- BS EN ISO 13959 : 2015 Water for haemodialysis and related therapies
- EDTNA/ERCA Guidelines on Control and Monitoring of Microbiological Contamination in Water for Dialysis
- Guideline on water treatment systems, dialysis water and dialysis fluid quality for haemodialysis and related therapies Clinical Practice Guideline Prepared on behalf of The Renal Association1 and The Association of Renal Technologists2 January 2016
- Eurachem guide EA – 4/10 : Accreditation for Microbiology Laboratories, Second edition (2013)

Annex A : 95 % Confidence intervals for the expected count from the second TVC sample.

First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count
0	0-5	26	13-43	52	33-75	78	55-105
1	0-7	27	14-44	53	34-76	79	56-106
2	0-9	28	15-45	54	35-77	80	57-107
3	0-11	29	16-47	55	36-78	81	58-108
4	0-12	30	16-48	56	37-79	82	58-110
5	0-14	31	17-49	57	38-80	83	59-111
6	1-16	32	18-50	58	38-82	84	60-112
7	1-17	33	19-52	59	39-83	85	61-113
8	2-19	34	19-53	60	40-84	86	62-114
9	2-20	35	20-54	61	41-85	87	63-115
10	3-22	36	21-55	62	42-86	88	63-117
11	3-23	37	22-56	63	42-88	89	64-118
12	4-24	38	22-58	64	43-89	90	65-119
13	5-26	39	23-59	65	44-90	91	66-120
14	5-27	40	24-60	66	45-91	92	67-121
15	6-28	41	25-61	67	46-92	93	68-122
16	6-30	42	26-63	68	47-93	94	69-123
17	7-31	43	26-64	69	47-95	95	69-125

18	8-32	44	27-65	70	48-96	96	70-126
19	8-34	45	28-66	71	49-97	97	71-127
20	9-35	46	29-67	72	50-98	98	71-128
21	10-36	47	29-69	73	51-99	99	73-129
22	10-38	48	30-70	74	52-100	100	74-130
23	11-39	49	31-71	75	52-102		
24	12-40	50	32-72	76	53-103		
25	13-41	51	33-73	77	54—104		

Microbiology GG&C	LP538v5
	Authorised by: Mary Ann Ritchie
08/12/2022	Author: Mary Ann Ritchie

Non Legionella Water Testing: Potable Water & Endoscopy Analysis	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Routine Testing

The Microbiological examination of water samples from the healthcare environment has a recognised role in the routine monitoring of decontamination procedures and the demonstration of a safe healthcare environment.
This document provides guidance for the processing and reporting of water from Endoscopy departments, Pharmacy sterile suites, and hydrotherapy pools, renal and potable waters.

Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values. Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, including cleaning records and control monitoring charts.

Infection Control Investigation Testing

On occasion the laboratory will be required to assist in the investigation of infection control issues that may affect a particular ward or hospital location. All 'ad-hoc' investigation work must be requested by the Infection Control Consultant through arrangement with Laboratory Senior Management prior to work being carried out. Refer to MI521 Ad Hoc Request Procedure for the full details.
For each agreed episode of Ad-hoc/Additional Environmental testing a completed request form (LF592) should be in place. This details the expected volume of work and the clear instructions of what the investigation or target organisms are and the reporting criteria.

2. Principles & methods of the procedure

The microbiological analysis of water and associated materials involves the use of selected procedures and microbiological culture media to ensure that the nature and enumeration of target organisms reflects the quality of the water being examined and that the data generated is reliable. Analysis includes a combination of Total Viable Counts (TVC), coliform and E. coli isolation, Pseudomonas investigations and Atypical Mycobacterium screening (AMS).

This Standard Operating Procedure describes the UKAS accredited tests offered and performed within Greater Glasgow and Clyde's Environmental Testing Service based at Glasgow Royal Infirmary. In addition there are other tests and methods within this procedure that are not part of our accredited scope. As such, the table below highlights those tests for which we are UKAS accredited. Other methodology and tests must be considered as off-scope testing. Tests not covered under accreditation will have a comment to this effect on the final laboratory report.

Tests have been verified with reports and data held in the Q-pulse electronic Quality Management System. A re-verification of all testing methods will be carried out every 5 years to ensure the methods and procedures used for testing remain fit for purpose and valid.

Table of Tests within UKAS 17025 2017 Accredited Scope (Laboratory 4211)

Water Type	Accreditation Tests Offered
Endoscopy Rinse Water	TVC, Pseudomonas aeruginosa & AMS
Potable/Pool Water	TVC, coliform, E. coli, Pseudomonas sp. & Pseudomonas aeruginosa

Renal Dialysis Water	TVC, coliform, E. coli, Pseudomonas sp. & Pseudomonas aeruginosa

3. Environmental & Safety Controls

- Laboratory coats must be worn at all times in the laboratory. Gloves must be worn when handling leaking samples
- Heat resistant gloves and visor must always be worn when working with the boiling baths. Tongs must also be used to removing items from boiling baths.
- A spill kit is located under the Legionella processing bench – all staff should be aware of the protocol to be followed in the event of a minor/major spillage
- Good laboratory practice and aseptic technique should always be practiced when dealing with ACDP category 2 pathogens and chemicals

Risk Assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	Good Lab Practice & PPE
GGC Waste Policy & RA	H&SP506 R542	Biological Chemical	Good Lab Practice & PPE
Hazard Group 2 & 3 Pathogens: Microbiological Manipulations & Exposure	R531	Biological	Good Lab Practice & PPE
Working in the Environmental Laboratory	R537	General	Good Lab Practice & PPE
Chemgene	R560	Chemical	Good Lab Practice & PPE
ZN Staining	R536	Biological & Chemical	Good Lab Practice & PPE
North Sector - Code of Practice for CL3 Working	LP518	General	Good Lab Practice & PPE

Laboratory environmental monitoring

Environmental monitoring of the laboratory is carried out on a weekly basis by the section CSW. This is to reduce the likelihood of environmental contamination of any given sample which can render results invalid. Results are interpreted by the section Biomedical Scientist. Refer to LP539 for full details.

Any breaches in alert levels should be relayed to a Technical Manager as soon as possible and a deep clean of the laboratory and/or equipment along with retesting should be carried out.

Benches are cleaned daily and all equipment cleaned once a week with Chemgene (recorded on H&SF516).

Laboratory settle plates monitoring

Settle plates should be set out and exposed to the environment for a 1 hour period. Plates should then be sealed using parafilm and incubated accordingly. All results should be reported on LF521

Settle plate	Temperature(˚C)	Atmosphere	Duration	Alert limit
SAB	22˚C	O_2	7 Days	>10 Fungi
SAB	30˚C	O_2	7 Days	>10 Fungi
TSA	30˚C	O_2	7 Days	>20 Bacteria
GVPC	37˚C	O_2	10 Days	>0 Legionella

Water bath Monitoring

TVC testing is performed weekly on all water baths. Record all results on LF518.

	Temperature	Atmosphere	Duration	Alert limit
TVC per 1ml	22˚C	O_2	72 Hours	>10 CFU
	37˚C	O_2	48 Hours	>10 CFU

Water baths are routinely emptied on a Friday afternoon, cleaned with Chemgene and reinstated on a Monday morning.

4. Sample Types

- Potable Waters
- Estates Waters
- Pool waters
- Pharmacy water
- Endoscopy Waters
- Renal fluids
- Raw untreated Waters

5. Equipment and Common lab consumables

- Sterile 47mm filter unit
- Manifold
- Vacuum pump
- Water/Boiling bath
- 0.45µm metrical GN-6 membranes
- Sterile petri dishes
- 1ml pipette

6. Calibration Procedures

- Daily pipette & balance check refer to QF574

Equipment	Calibration
Pipettes	Annually
Thermometers	Annually
Balance	Annually

Filter Units	Annually
Stuart Counter	Annually
Timers	Annually
Check Weights	Annually -Immediately after Balance Calibration
Thermocouples (TMS)	Annually
Temperature mapping	Every 3 Years
Agar Tempering	Every 5 Years

A re-verification of all testing methods should be carried out every 5 years to ensure the method remains fit for purpose.

7. Pre Analytical Procedural Steps

7.1. Media/Diluent Pre-acceptance

All media and diluents received for use within the Environmental Laboratory will be subject to pre-acceptance testing to ensure the media can support the growth of target organism and for sterility. Known ATCC/NCTC reference control strains are used to assess the performance of the media and diluents used in water testing, see QF530 and QF534. All details should be recorded on QF618.

When new batches of media or diluents arrive within the laboratory, reception staff follow QP520 (Media and Reagent QC) by segregating new stock into a quarantine area until appropriate pre-acceptance testing is completed and passed. Pre-acceptance instructions are stated within LI567.

Retesting should be carried out immediately on any new batch of media or diluent that fails quality control and all details should be recorded on QF618 and the section Technical manager should be informed. Items must NOT be put into use. The manufacturer will then be contacted to discuss the problem and a non-conformance should be initiated in Q-Pulse for full investigation. Additional stock should be ordered if required.

7.2. Sample transportation & storage

Sampling should be carried out by suitably trained personnel, laboratory staff do not undertake sampling. Samples should be collected in an appropriately sized sterile bottle containing a neutraliser to neutralise any residual disinfectant within the sample. The most commonly used is Sodium Thiosulphate. The laboratory provides 500ml sterile bottles containing 18mg/L of Sodium Thiosulphate to customers for sampling. Small volume renal samples do not require a neutraliser and can be accepted in a small sterile container.

Samples should be transported to the laboratory within 4 hours and stored between 2-5°C if they cannot be processed immediately. Samples ideally should be analysed as soon as is practicable on the day of collection or within a 24hr period.

7.3. Specimen receipt & booking in

Samples are received from private companies, GG&C hospitals and various health boards. Water samples should be received and booked in as soon as possible.

For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- All details of the days processing should be recorded on the Daily governance worksheet (QF612). This covers process controls, media, reagents, processing staff and laboratory numbers and ensures all work is traceable
- Date stamp the time of receipt of the samples on their request forms
- Ensure the sample details match the customer request form – mismatched samples should be rejected, telephone customer to advise of this
- Refer to LI659 (Environmental Set Up) for coding and plate requirements for standard testing, for any ad hoc requests refer to LF592 for the full details as directed by Infection Control Consultant
- If the appearance of samples received is anything other than clear, this must be noted on request form to enable inclusion in final report as this may affect recovery of organisms from the sample
    - CLO (cloudy)
    - OI (oily)
    - SS (suspended solids)

- Label samples using designated laboratory number stream
- If investigations required are unclear, confirm by checking with the section Technical Manager or contact sender
- The request forms are placed in the laboratory PID tray for the BMS triage
- Once triaged the BMS will record sender details and laboratory numbers onto the appropriate daily worksheet and pass the request forms to admin staff for PID

7.4. Deviating & rejection criteria and process

Deviating Samples - this is samples that are not correctly preserved, e.g. have exceeded their maximum preservation time, >24 hours from sampling, lack the date and time of sampling, are not cooled appropriately etc. As a result, deviating samples may jeopardise the validity of the reported test result.
If sample(s) are received that are considered deviating samples, the customer must be contacted as soon as possible, informed of the status of the sample(s) and further instructions requested.
If the customer requests the samples be rejected see section below.
If the customer request the samples to be processed, a disclaimer must be added to the report stating that the sample was deviating and, as a result, the test result(s) may be invalid.
In practice, if a customer requests that a deviating sample is processed, ensure the following is added to the report:

- At report entry, enter the code **SPDEV** and additional codes to indicate the reason for the deviation, e.g. delay, poor storage etc
- Enter the customers' instructions, e.g. sample processed (or not processed) following discussion with the client
- Record all relevant information of phone call on request form

Rejected Samples

This includes samples which are not suitable for testing i.e. mismatch details, insufficient samples or any that the customer has agreed based on deviating nature above.

Report as Specimen Not Processed (SPNP) and add additional comments as appropriate.

- At report entry, enter the code **SPNP** and additional codes to indicate the reason for the rejection e.g. insufficient sample, incorrect sample etc.
- Record all relevant information of phone call on request form

8. Procedural Steps

8.1. Agar Preparation

Review daily workload to establish the number and type of pour plates required. Collect media and place in boiling bath for 30 minutes (ensure molten).
Transfer molten agar to the 45ºC holding bath, using appropriate PPE. Allow media to cool to 45ºC before use. No more than 15 bottles of agar should be prepared at any one time and media should be allowed to cool appropriately.

Number of bottles	Cooling time required
5	30 Minutes
7	40 Minutes
10	40 Minutes
15	70 Minutes

- Yeast Extract Agar (YEA) and R2 Agar are the two types of media used in the pour plate method of TVC enumeration depending on sample type
- Daily process controls are carried out **see section 8.3**
- Any bottle of agar opened at the end of the working day should be discarded

8.2. Filter sterilisation

Sterilisation of the filter units before each sample is processed is essential. To do this, water bath's 1 and 2 should be switched on, the water level replenished and allowed to come to 99°C. The filter unit cup should then be fully submerged in the water bath. Once the bath has again reached 99°C, a 5 minute timer should be used to ensure the filter cups receive adequate time to sterilise. Great care should be used when working with the boiling baths and PPE requirements always adhered to. Filter cup base should be wiped with an alcohol wipe and allowed to air dry before next use.
In addition all filter units (cup and base) will be autoclaved once a week to maintain sterility

8.3. Daily Process Controls

Daily process controls are carried out on each singleton test method to ensure the process is fit for purpose. The controls MUST be set up and processed along side samples to ensure the full process is robust.

Media	Plates required	Sample volume	Positive Control Lenticule	Negative Control	Incubation
CCA	4	100ml	E.coli NCTC 9001 K.oxytoca NCTC 8167 Ps.aeruginosa NCTC 10662 (non target)	Baxters Water	37°C/18H
PYO	3	100ml	Ps.aeruginosa NCTC 10662 E.coli NCTC 9001 (non target)	As Above	37°C/48H
TSA	2	100ml	E.coli NCTC 9001	As Above	37°C/48H
TSA	2	100ml	E.faecalis NCTC 775	As Above	30°C/5D
YEA	8	1ml	E.faecalis NCTC 775	As Above	37°C/48H 22°C/72H
R2A (Pour)	8	1ml	E.faecalis NCTC 775	As Above	30°C/5D 22°C/7D
R2A (pre-pour)	4	100ml	E.faecalis NCTC 775	As Above	As Above
Middlebrooks	2	100ml	M.fortuitum NCTC 10394	As Above	35°C/42D
SAB	4	100ml	C.albicans NCPF 3255	As Above	30/22°C/7D

Negative Control

- Appropriate agar plates should be labelled with the sterile Baxter's water Lot number and date
- The negative control should be carried out as follows:
- Filter 100ml of the vortexed sample through the membrane. Once all water has passed through, place the membrane onto the appropriate agar plate using sterile forceps for each membrane. **See Membrane Filtration Section for Full method**
- Incubate the plates along with the positive control plates accordingly

Positive Controls

- The required amount of lenticules should be removed for the freezer and allowed to come to room temperature (10 minutes)
- Appropriate agar plates should be labelled with the Lenticule batch number, organism type and date
- Reconstitute each lenticule in well labelled 100ml bottle of MRD (Maximum Recovery Diluent)
- Leave for a minimum of 15 minutes until the lenticule has dispersed, mix the solution well and process through the sterile filter unit. The lenticule should be processed within a period of 45 minutes
- Place filter onto the plate and incubate appropriately with the negative control plate and any sample plates
- Process controls should be carried out in parallel with a batch of samples every day

8.4. Generating New Process Control Data – New Lenticule Batch

- When a new batch number of lenticules are received, the following process is carried out;
    - Each new Lot number of process controls lenticules are run through our entire process twenty times ideally by 4 different individuals

- The Colony Forming Unit counts achieved for these are recorded on the Batch Control Record Form QF593. This data is then transferred to the spreadsheet QF577, which is held in the North Glasgow Share Drive
- From this, the mean value as well as one, two and three standard deviation values are calculated via the spreadsheet and a chart generated
- The mean and standard deviation values generated from this chart will be used as reference values to objectively assess our daily water process controls
- Expected CFU ranges of control lenticules can be obtained from the Sigma Aldrich website using the lenticule batch number
- A copy of the lenticule certificate of analysis can also be found in the Environmental section of the share drive

9. Methods

9.1 Membrane Filtration Procedure

Membrane filtration is carried out on the following media for the following target organism:
Refer to LI659 for full details

Agar	Target Organism	Incubation/atmosphere
CCA	Coliforms and E.coli	37°C/18 hours (O_2)
Pseudomonas Agar	Pseudomonas species	37°C/48 hours (O_2)
TSA TSA	Target GNB TVC (Endoscopy waters)	37°C/48 hours(O_2) target GNB and 37°C for 48 hours(O_2) Endoscopy waters
R2A Agar	Total Viable Count	30° C /5 days and 22°C for 7 days (O_2) – bagged to retain moisture
Middlebrooks	Atypical mycobacteria	35°C for 42 days (O_2) – sealed with parafilm
SAB	Yeast & Fungi	30°C 7 days (O_2) – sealed with parafilm 22°C 7 days (O_2) – sealed with parafilm

- Mix water sample vigorously before testing. If the sample bottle is full, pour off a little water to allow adequate mixing
- Sterile filter units should be removed from the boiling baths and placed on the manifold
- The unit should be allowed to cool sufficiently before aseptically placing a 0.45µm filter paper grid side up on the magnetic unit using sterile blunt end forceps
- All manifold valves should remain closed
- Decant 100ml of sample into the filter unit, switch the vacuum pump on and open the manifold valve
- Once all water has passed through the filter, close the manifold valve, and place membrane onto the appropriate agar plate using sterile forceps for each membrane. (**forceps sterilized with 70% IMS between each use**)
- Incubate plates as per section for media type. The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours

- **For Fungal cultures**, plates should be sealed with parafilm to avoid fungal contamination of incubators which can be problematic
- **For atypical mycobacteria**, the plates should be sealed with parafilm to retain moisture and incubated in the appropriate daily rack at 35°C for a period of 42 days in the designated incubator within the CL3 suite

9.2 Pour Plate Procedure

Total viable counts of waters and renal waters are carried out using a pour plate method using Yeast extract agar or R2A agar. To do this label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish. Ensure process controls have been carried out.

- If the sample bottle is full, pour off a little water to allow for adequate mixing, mix well
- Pipette 1 ml of sample into each Petri dish
- Aseptically add approximately 15 – 20ml of molten Agar to the Petri dish within 20 minutes of the 1ml being dispensed
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow agar to set at room temperature
- Invert and Incubate plates at the required temperature/atmosphere (see LI659)

9.3 RHC Theatre & water cooler/ GJNH (perfussionist) Cardiology waters Procedure

These water are processed for slow growing atypical mycobacteria and thus cannot be processed on Middlebrooks ager. The customer request form should be photocopied and all other tests carried out. A portion of the sample should be decanted into two sterile universals, sealed with parafilm and transferred to the CL3 section with the photocopied form where they will be processed in liquid culture through the MGiT (See LI693 for PID instruction and LP505 for processing guidelines)

9.4 Enumeration of Pre and Post Treatment of Effluent Waters by Membrane Filtration Procedure

Such samples arrive from Babcock etc.; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E. coli counts
- Do not require TVC's

Each requires:

- 1 x CCA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

A. Pre-treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well

- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on CCA plate
- Incubate at 37ºC/O_2 for a minimum of 18 hours

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well
- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E. coli
- Place filter on CCA plate
- Incubate at 37ºC/O_2 for a minimum of 18 hours

10 Result Interpretation & Reporting

At the weekend any cultures, both controls and samples, which are not able to be read on the day they are due to be reported should be refrigerated until they can be read. LF598 to be signed by the staff member moving plates from the incubator to the cold room. **It is the responsibility of the BMS reading the plates to ensure that correct incubation times for tests have been carried out before results are recorded and reported to customer. If refrigeration of plates has been necessary, the BMS must check that LF598 has been completed thus ensuring that there is an auditable trail of refrigeration having occurred.**

10.1 Criteria for acceptability of daily process control results

Process control samples must be examined and have passed specific criteria before sample plates can be examined. The examination and reporting of samples must be carried out by a suitable trained member of staff or a member of staff receiving training under supervision. All agar plates should receive the required amount of incubation in the appropriate temperature and atmosphere.

Plates should be removed from the incubator and read in a timely fashion, if the plates cannot be counted immediately then store between 2-8°C until this can be carried out.

- Count the number of colonies that have grown on the positive control plate
- Less than 1 cfu of any positive control organism indicates a failed process control. Inform the section Technical Manager and investigate
- If the batch of samples processed on this particular day contains a positive isolate, then the results may be released although the event should be investigated first by the section TM and Quality department
- If the process controls fail and all samples test negative for the representative test then the customer must be contacted and made aware of the circumstances and re-testing should be advised
- Reports should be entered into telepath and as TCE – PRFRT which expands to "Process Control Failure. Retesting recommended" and authorised
- A non-conformity (CAPA) should be raised in Q Pulse and root cause analysis carried out

Review of Process Controls

- Record the count of all process controls on the daily governance worksheet (QF612) worksheet and populate QF577 held in the Share Drive (North Glasgow Microbiology/Environmental Laboratory/Process Control Data), before releasing final results
- The mean and standard deviation values objectively assess our daily controls
- Any one value recorded out with 3SDs represents a large sudden shift from the average and will be annotated on the graph and highlighted in yellow. This can be considered a sporadic occurrence. Any corrective action should be noted on QF577
- If two out of three successive counts exceed 3SDs then an investigation should be carried out by the section TM in conjunction with the Quality department. In most cases a non-conformance should be raised unless deemed unnecessary. All values should be highlighted in yellow
- Nine consecutive counts falling on the same side of the mean represents a shift and an investigation should be carried out by the section TM to find the potential cause for this. This may reflect the original values being unreliable and result in a new process control data and chart being compiled to reflect the shift
- Six consecutive counts continually rise/fall denotes a trend and should be investigated by the section TM. The quality of reference material may need to questioned

10.2 Examination & Enumeration of Total Viable Counts (TVC)

TVC – Pour Plate Interpretation

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

A colony count should be carried out as soon as the plates are removed from the incubator. If the plates cannot be counted immediately, store at 2-8ºC.
Spreading colonies can interfere with counts, and the following gives guidance on what should be counted as a single colony.

- A chain of colonies caused by the disintegration of a clump of organisms
- A spreading growth that develops as a film at the bottom of a Petri dish
- A colony that forms in a film of water at the edge or over the surface of the agar
- Due to the variation in organism distribution within water, split samples e.g. TVC's done in duplicate, can display significant variation in enumerations. For example if one TVC plate is enumerated at 5 CFU/1ml then the 95% confidence interval for the 2nd plate would be an enumeration between 0-15 CFU/1ml. Please see Appendix A for acceptable 95% confidence intervals. If counts exceed 95% CI, then an investigation into must be initiated: inform the section TM, or QM.

Colony Counting Method using Stuart Counter

Before beginning any count examine the plate to ensure that the colonies are spread evenly over the whole plate.
If the appearance of colonies shows no uniform spread, perhaps colonies only present on one side, this would indicate that the initial mixing of the sample was not performed correctly. Unless uniform spread then the count cannot be reliably reported and the test should be repeated.

- Switch on light and ensure that the tally counter is at zero and free to move when the counting area is depressed
- Place the plate to be counted, without lid, on the grid
- With a fine felt tip pen mark each colony, applying sufficient pressure to cause the tally counter to move
- Record the number of colonies counted from each plate on the relevant Daily Worksheet
- Calculate the mean count and record on the daily worksheet
- Rounding up an average count is acceptable e.g. two counts of 15 and 16 = a average of 15.5 Thus the average should be recorded as 16 CFU

If the number of colonies to be counted is too great e.g. >300 the plate may be divided into a half, quartered or a reduced area counted and multiplied by the reciprocal of that area i.e.

- Count 5 large squares using counter grid which are ≅ 1/10 plate and multiply by 10 for final count/ml
- Count 1 small square using counter grid which is ≅ 1/450 plate and multiply by 9 to give one large square, then multiply by 50 for final count/ml
- If there are more than 20 colonies in 1 small square record the count as greater than 104 organisms per ml

Reporting of Pour Plate TVC Results

- The TVC reported is the mean of the colony count from the two duplicate plates from an undiluted sample

- Results are recorded on daily worksheet and transcribed to the customer request form as cfu/1ml
- Refer to Reporting criteria section

TVC – Membrane Filtration Interpretation – Endoscopy Waters Only

Endoscopy rinse waters have a Total viable count performed on them using membrane filtration and two TSA plates

- Ensure all QC Plates have passed IQC before reading sample plates
- Count number of colonies present on the filters on both TSA plates. The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- Record on endoscopy water day sheet and average the two results
- Refer to Reporting criteria section

10.3 Examination & Enumeration of Coliform / E. coli

Testing for coliform bacteria and *Escherichia coli* (*E. coli*) is one of the most important microbiological examinations carried out on drinking water. The test provides a sensitive means for detecting faecal contamination and for assessing water quality, the effectiveness of water treatment and disinfection**. It should be noted that CCA agar does not inhibit the growth of gram positive bacteria and can in some cases hinder the identification of enterobacteriaceae, any samples showing high counts of gram positive bacteria should be diluted and retested (result should be multiplied by dilution factor) to ensure target organisms are isolated.**

- Examine CCA plates at no less than 18 hours and within 15 minutes of being removed from the incubator
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Coliforms and E.coli on the CCA plates. Coliforms are will appear as red/pink colonies and E.coli will appear as violet/blue colonies.
- Count all red/pink and violet/blue colonies (however faint) irrespective of size (e.g. 5 red, 2 blue), taking note of different colonial morphology if necessary and record on the appropriate day worksheet, sign & date. The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- If the growth of clear colonies (non – target organism) is considered to be such that they obscure potential Coliform growth then a repeat sample from the customer may be required – discuss with section TM
- If sufficient individual colonies and purity allows, send any red/pink or violet/blue isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Coliforms/E.coli per 100ml taking into account any dilution factors
- Pre/post treatment samples - Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)

- It is expected that the Post treatment counts will be less than those of the Pre-treatment samples. If not, then check that the correct procedure has been followed
- Refer to Reporting criteria section

10.4 Examination & Enumeration of Pseudomonas species

- Examine plates at 24 hours and finally at 48 hours The number of colonies at 24 hours should be noted on the daily worksheet as growth between 24 and 48 hours may be such that colonial growth results in the merging of colonies, and the number of colonies at 48 hours may seem less than the number of colonies at 24 hours.
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Pseudomonas species on the Pseudomonas plates;
    - Typical (green) colonies (likely to be Ps.aeruginosa)
    - Atypical (yellow) colonies or any growth
- Perform an oxidase test on all growth
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- If sufficient individual colonies and purity allows, send any suspect isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- Refer to Reporting criteria section

10.5 Examination & Enumeration of Ad Hoc Target Gram Negative Bacteria

- Examine TSA plates at 48 hours
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of any GNB on the TSA plate;
- Perform a Gram film on all colony types
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- All gram positive bacteria can be disregarded and plates stored for 7 days
- If sufficient individual colonies and purity allows, GNB isolates should be sent to the main laboratory for identification using Vitek/MS. and result recorded on the daily worksheet
- Any isolate that fails initial MSID should be retested on the MS before a VITEK2 gram negative identification is carried out
- Any isolate that fails identification by the above method should be reported as Environmental GNB
- **Mixed colonies** – purity plate onto Blood agar and incubate at 37ºC for 18-24 hrs. Gram film/Send to the main laboratory for identification the following day
- Report as number of Target Organism per 100ml

- Refer to Reporting criteria section
- Store all target organisms in the -80 freezer in the Environmental section and complete LF558

Reporting of Ad-hoc/Additional Environmental GNB results

- Gram Negative Testing – If the request has been for All GNB. All GNB should be reported on customer request forms and in LIMS. Where no GNB but other organisms may be present such as Gram Positive bacteria, samples should be reported as "No environmental Gram-negative bacilli Isolated" (comment/barcode NEGNB) See LI670
- Target Organism Testing e.g. when a specific organism is requested such as Cupriavidus then this is all that should be reported as either detected or not detected (NOTDET). No laboratory work or other named organisms identified during the testing should be submitted to the requestor (Including ICD). All this information should be classed as lab use only and be available only on the 'back of form', it should not be transcribed to LIMs or emailed as a result
- All bacterial speciation can be reported in LIMS via NSI – AISO, whereby organisms can be reported with specific counts
- All completed LF592's (Ad Hoc/Additional Environmental requests) should remain in the laboratory with the appropriate customer request form until all results have finalised and a copy sent to the Quality Department on completion
- All results should be emailed by the admin staff to the named personal detailed with the LF592 and Dart scanned

10.6 Examination & Enumeration of Fungi

- Examine SAB plates at day 7 for yeast species and fungi
- Ensure all QC Plates have passed IQC before reading sample plates
- Examine plates for the presence of yeasts, including suspected performing either a wet prep or gram film for confirmation – further identification if required can be achieved via Maldi-ToF (MS) from fresh purity plates
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- All filamentous fungi should be referred to the mycology section using LF539 (Mycology Referral Isolate form) for further identification using LP560 – Non superficial Mycology (ensure the plate is sealed with parafilm to avoid the release of spores)
- Black colonies should be confirmed with gram stain/wet prep and reported as Exophiala species
- Pink colonies should be confirmed with gram stain/wet prep and reported as Rhodotorula species

- Report as number yeast/fungi per 100ml as a final report or an interim result if further ID is required on the customer request form and within LIMS
- Refer to Reporting criteria section

Reporting of Fungal results

- All identifications returned from mycology should be transcribed onto the daily worksheet (LF533), customer request form and input and authorised within LIMS as a final report using LI670
- All results are entered into the LIMS by HCPC registered BMS
- All white yeast species should be reported as Yeast species unless a full identification has been specifically requested (LF592)
- Exophiala species (black yeast) and Rhodotorula species (pink yeast) – a referral to mycology is not required and full identification should only be carried out if requested
- All results should be emailed by the admin staff to the appropriate distribution list and Dart scanned

10.7 Examination & Enumeration of Atypical mycobacterium

- Ensure all process control plates show the expected results before reading sample plates
- Plates should be examined weekly for bacterial growth
- Colony count, colonial morphology and any further work to be carried out (e.g. purity plates) should be recorded on LF573, dated and signed
- Any plate showing bacterial growth should be transferred to the Class 1 safety cabinet designated for Category A work
- A film for Ziehl-Neelson (ZN) staining should be prepared, stating the full laboratory number and heat fixed for a minimum of 20 minutes
- A ZN stain should be carried out following LI546 and examined under oil immersion X100 light microscopy
- **Positive mycobacteria** will appear as red bacilli
- Any positive film should be checked by another trained member of staff and countersigned
- The slide should have a positive label added with the full laboratory number, sample identifier and date and retain in the positive slide box.
- A negative film will show green stained organisms of different structure e.g. bacilli, cocci

Reporting of Atypical Mycobacterium Results

- Ensure all process control plates have passed IQC before reading sample plates – ensure QF612 (Daily governance worksheet) and QF577 (Enviro process control charts) are updated to reflect this information
- Results should be recorded on the LF573 and the customer request form stating the colony forming units
- Fungal contamination may occur as plates are incubated for long periods of time – Consideration should be given to the validity of results. For major contamination, discard plates and record details on the AMS worksheet and customer request form. The auto comment “CON1” and “CON2” should be

entered into TCE in telepath, this expands to “Due to contamination issue, unable to report – If available, please send another sample”. Notify the customer of the contamination.

- Any positive result should be reported in the LIMS as an interim result and the appropriate customer/infection control medic notified
- The customer request form should then be emailed to the appropriate customer distribution list by the admin staff and returned to CL3 until the culture has finalised at 42 days
- In many cases a new presumptive mycobacteria isolate should be referred to the Mycobacteria Reference Laboratory in Edinburgh for identification, this should be discussed with a consultant as to their requirements and a Middlebrooks slope used
- All positive isolates should be retained for a period of 1 year in the -20 Freezer within the CL3 suite
- All sealed culture plates should be disposed of via the category 3 waste route for autoclaving
- Customer request forms should be updated with results/counts and entered and authorised in LIMS at 42 days
- Results should be entered into LIMS into the appropriate singleton test field, stating “N” for not detected or “D” for detected. If the result is positive then an enumeration per 100ml should be entered into the appropriate telepath field
- AMSPR (AMS in Progress) should be removed from the comments section
- The customer request form should then be emailed to the appropriate distribution list, dart scanned and filed by the admin staff
- All complete AMS worksheets should be sent to the office to be dart scanned and returned. These forms shore be retained in the records room in the appropriate box file

11 Reporting Criteria

Any results out with the specified limits in the following tables should be telephoned /emailed to the customer as per their requirements.

	Standard Testing							For Ad Hoc Requests	
Water	TVC @37	TVC @22	TVC @30	Coliform/ E.coli	Pseudo aeruginosa	Pseudo Species	Atypical Mycobacteria	Target Org / GNB	Fungi @22 or 30
POTW/POOL	>10	>100	-	>0	>0	>0	>0	>0	>0
RFS		>50	>50	>0	>0	>0	-	-	-
DFU		>10	>10	>0	>0	>0	-	-	-
ERW	*	-	-	-	>0	-	>0	-	-
Raw treatment water	-	-	-	Any	-	-	-	-	-

*Endoscopy samples have varying limits for TVC reporting please see the following table:

GGC Guidance for Final Rinse Water:	Action/Alert

RO Unit Alert = 34-68 cfu/100ml	>68 cfu/100ml
EWD Bath Alert = 41-82 cfu/100ml	>82 cfu/100ml
Washer supply water filter bank = 41 – 82 cfu/100ml	>82 cfu/100ml

Interim Reporting

Results should be transferred from the daily worksheet to the customer request form. Telephone the customer with interim notifiable count and record details of the call on the customer request form. Enter the results into LIMS entering telephone details in TCE/TELWAT. Email the result to customer if required or if this is the customers preferred method for communication via the admin staff.

Final Reporting

Results should be transferred from day worksheet to customer request form and entered/authorised into LIMS by HCPC registered BMS staff.

- All Negatives are entered using barcode scripts (LI670)
- All report codes/comments can be found on LI670
- All positives should be entered manually
- Specific organism identifications with counts should be reported via the organism table (NSI, AISO)
- Once all reporting complete, release as final 'PRF' and 'NRQ'
- Check the OUTW/WINP queues daily for any outstanding results – complete LF524
- Customer request forms are labelled with an instruction card stating what work should be carried out by the admin staff e.g. email, dart scan, file etc
- If a notifiable count is recorded on the final day of testing, then the report can be reported with no requirement for a telephone call
- All Environmental forms should be Dart scanned and filed appropriately
- Daily Worksheets are held in the environmental laboratory for 2 months, after this pass to office for dart scanning and filing in the records room

Reports are authorised by;

- **PRI -** Interim report (There can be multiple interims if required)
- **PRF -** Final report (There must only be 1 final report issued, anything after this MUST be additional)
- **PRA -** Additional report
- **NRQ** - to release results

ALL RESULTS ARE AUTHORISED BY THE BMS FROM THE SUPERVISOR AUTHORISATION QUEUE

This queue should be checked every time a result is released to ensure that all reports are authorised.

Referral of Isolates

- Isolates should only be referred to a reference laboratory at the request of medical staff

- All isolates being referred to Reference labs must be parcelled & sent as per Postal SOP LP542 and details recorded on LF515
- Each send away sample must have a postal declaration form LF516 completed
- LIMS should be updated in comments "REFER" with the organism identification and released as an Interim report

Reports returned from Reference labs

- All reference reports are received by specimen reception / office and will be scanned to DART &/or portal before being handed to the laboratory
- Once the reference lab reports are received by the bench the BMS should enter RLRENV and enter the date the report was received
- All details reported on the reference report should be entered manually into the comments section of telepath to enable the user to see results - **results are not viewable via portal**
- The BMS must complete LF515 and then authorise the report to the appropriate status required

12 Retention of Isolates & Samples

All culture plates showing notifiable growth should be retained for a period of 7 days within the environmental storage cabinet within the main laboratory.
Any isolate reported in an infection control incident or any P. aeruginosa isolated from endoscopy water should be stored in the -80°C freezer (recorded on LF558 – stored Isolate form) within the main laboratory in case further investigations are required.

All samples once processed should be retained in the cold room (2-5°C) for a period of 8 days until all investigations have been concluded

13 References

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- Standing Committee of Analysts The Microbiology of Drinking Water Part 8 – Methods for the isolation and enumeration of Aeromonas and Pseudomonas aeruginosa : 2015
- EDTNA/ERCA Guidelines on Control and Monitoring of Microbiological Contamination in Water for Dialysis
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- Preparation and quality management of fluids for haemodialysis and related therapies – Part 3: Water for haemodialysis and related therapies (ISO 23500-3:2019)
- Protocol for Environmental Sampling, Processing and Culturing of Water and Air Samples for the Isolation of Slow-Growing Mycobacteria Standard operating procedure. Public Health England. 2016
- ISO 9308-1:2014(E) Water quality — Enumeration of Escherichia coli and coliform bacteria —Part 1: Membrane filtration method for waters with low bacterial background flora
- Marek *et al*. 2014. Endoscopy Supply Water and Final Rinse Water Testing: Five Years of Experience. Science Direct. Volume 88 Issue 4, pp 207-212

Appendix A: 95 % Confidence intervals for the expected count from the second TVC sample.

First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count
0	0-5	26	13-43	52	33-75	78	55-105
1	0-7	27	14-44	53	34-76	79	56-106
2	0-9	28	15-45	54	35-77	80	57-107
3	0-11	29	16-47	55	36-78	81	58-108
4	0-12	30	16-48	56	37-79	82	58-110
5	0-14	31	17-49	57	38-80	83	59-111
6	1-16	32	18-50	58	38-82	84	60-112
7	1-17	33	19-52	59	39-83	85	61-113
8	2-19	34	19-53	60	40-84	86	62-114
9	2-20	35	20-54	61	41-85	87	63-115
10	3-22	36	21-55	62	42-86	88	63-117
11	3-23	37	22-56	63	42-88	89	64-118
12	4-24	38	22-58	64	43-89	90	65-119
13	5-26	39	23-59	65	44-90	91	66-120
14	5-27	40	24-60	66	45-91	92	67-121
15	6-28	41	25-61	67	46-92	93	68-122
16	6-30	42	26-63	68	47-93	94	69-123
17	7-31	43	26-64	69	47-95	95	69-125
18	8-32	44	27-65	70	48-96	96	70-126
19	8-34	45	28-66	71	49-97	97	71-127
20	9-35	46	29-67	72	50-98	98	71-128
21	10-36	47	29-69	73	51-99	99	73-129
22	10-38	48	30-70	74	52-100	100	74-130
23	11-39	49	31-71	75	52-102		
24	12-40	50	32-72	76	53-103		
25	13-41	51	33-73	77	54—104		

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	Authorised by: Mary Ann Ritchie
16/10/2023	Author: Mary Ann Ritchie

Non Legionella Water Testing: Potable Water & Endoscopy Analysis	
LOCATION OF COPIES	Environmental Laboratory

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Routine Testing

The Microbiological examination of water samples from the healthcare environment has a recognised role in the routine monitoring of decontamination procedures and the demonstration of a safe healthcare environment.
This document provides guidance for the processing and reporting of water samples received from Endoscopy departments, Pharmacy sterile suites, hydrotherapy pools,

Methods should be capable of establishing, within limits of deviation and detection, whether samples contain numbers of selected micro-organisms which contravene prescribed values. Effective quality assurance should cover the whole process from sample collecting to reporting and interpretation of results, including cleaning records and control monitoring charts.

Infection Control Investigation Testing

On occasion the laboratory will be required to assist in the investigation of infection control issues that may affect a particular ward or hospital location. All 'ad-hoc' investigation work must be requested by the Infection Control Consultant through arrangement with Laboratory Senior Management prior to work being carried out. Refer to MI521 Ad Hoc Request Procedure for the full details.
For each agreed episode of Ad-hoc/Additional Environmental testing a completed request form (LF592) should be in place. This details the expected volume of work and provides clear instructions for investigation(s) or target organism(s) are and the reporting criteria.

2. Principles & methods of the procedure

The microbiological analysis of water and associated materials involves the use of selected procedures and microbiological culture media to ensure that the nature and enumeration of target organisms reflects the quality of the water being examined and that the data generated is reliable. Analysis includes a combination of Total Viable Counts (TVC), coliform and E. coli isolation, Pseudomonas investigations and Atypical Mycobacterium screening (AMS).

This Standard Operating Procedure describes the UKAS accredited tests offered and performed within Greater Glasgow and Clyde's Environmental Testing Service based at Glasgow Royal Infirmary. In addition there are other tests and methods within this procedure that are not part of our accredited scope. As such, the table below highlights those tests for which we are UKAS accredited. Other methodology and tests must be considered as off-scope testing. Tests not covered under accreditation will have a comment to this effect on the final laboratory report.

Tests have been verified with reports and data held in the Q-pulse electronic Quality Management System. A re-verification of all testing methods will be carried out every 5 years to ensure the methods and procedures used for testing remain fit for purpose and valid.

Table of Tests within UKAS 17025 2017 Accredited Scope (Laboratory 4211)

Water Type	Accreditation Tests Offered
Endoscopy Rinse Water	TVC, Pseudomonas aeruginosa & AMS
Potable/Pool Water	TVC, coliform, E. coli, Pseudomonas sp. & Pseudomonas aeruginosa

Renal Dialysis Water	TVC, coliform, E. coli, Pseudomonas sp. & Pseudomonas aeruginosa

3. Environmental & Safety Controls

- Laboratory coats must be worn at all times in the laboratory. Gloves must be worn when handling leaking samples, and whilst cleaning with laboratory disinfectants.
- Heat resistant gloves and visor must always be worn when working with the boiling baths. Tongs must also be used to remove items from boiling baths.
- A spill kit is located under the Legionella processing bench – all staff should be aware of the protocol to be followed in the event of a minor/major spillage
- Good laboratory practice and aseptic technique should always be practiced when dealing with ACDP category 2 pathogens and chemicals

Risk Assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	Good Lab Practice & PPE
GGC Waste Policy & RA	H&SP506 R542	Biological Chemical	Good Lab Practice & PPE
Hazard Group 2 & 3 Pathogens: Microbiological Manipulations & Exposure	R531	Biological	Good Lab Practice & PPE
Working in the Environmental Laboratory	R537	General	Good Lab Practice & PPE
Chemgene	R560	Chemical	Good Lab Practice & PPE
ZN Staining	R536	Biological & Chemical	Good Lab Practice & PPE
North Sector - Code of Practice for CL3 Working	LP518	General	Good Lab Practice & PPE
Use of Bleach (Teepol)	COSHH532	Chemical	Good Lab Practice & PPE

Laboratory environmental monitoring

Environmental monitoring of the laboratory is carried out on a weekly basis by the section CSW. This is to reduce the likelihood of environmental contamination of any given sample which can render results invalid. Results are interpreted by the section Biomedical Scientist. Refer to LP539 for full details.

Any breaches in alert levels should be relayed to a Technical Manager as soon as possible and a deep clean of the laboratory and/or equipment along with retesting should be carried out.

Benches are cleaned daily and all equipment cleaned once a week with 10% Chemgene (recorded on H&SF516).

Laboratory settle plates monitoring

Settle plates should be set out and exposed to the environment for a 1 hour period. Plates should then be sealed using parafilm and incubated accordingly. All results should be reported on LF521

Settle plate	Temperature(˚C)	Atmosphere	Duration	Alert limit
SAB	22˚C	O_2	7 Days	>10 Fungi
SAB	30˚C	O_2	7 Days	>10 Fungi
TSA	30˚C	O_2	7 Days	>20 Bacteria
GVPC	37˚C	O_2	10 Days	>0 Legionella

Water bath Monitoring

TVC testing is performed weekly on all water baths. Record all results on LF518.

TVC per 1ml	Temperature	Atmosphere	Duration	Alert limit
	22˚C	O_2	72 Hours	>10 CFU
	37˚C	O_2	48 Hours	>10 CFU

Water baths are routinely emptied on a Friday afternoon, cleaned with Chemgene and reinstated on a Monday morning.

4. Sample Types

- Potable Waters
- Estates Waters
- Pool waters
- Pharmacy water
- Endoscopy Waters Renal fluids
- Raw untreated Waters

5. Equipment and Common lab consumables

- Sterile 47mm filter unit
- Manifold
- Vacuum pump
- Water/Boiling bath
- 0.45µm metrical GN-6 membranes
- Sterile petri dishes
- 1ml pipette/pipette tips

6. Calibration Procedures

- Daily pipette & balance check refer to QF574

Equipment	Calibration
Pipettes	Annually
Thermometers	Annually

Balance	Annually
Filter Units	Annually
Stuart Counter	Annually
Timers	Annually
Check Weights	Annually -Immediately after Balance Calibration
Thermocouples (TMS)	Annually
Temperature mapping	Every 3 Years
Agar Tempering	Every 5 Years

A re-verification of all testing methods should be carried out every 5 years to ensure the method remains fit for purpose.

7. Pre Analytical Procedural Steps

7.1. Media/Diluent Pre-acceptance

All media and diluents received for use within the Environmental Laboratory will be subject to pre-acceptance testing to ensure the media can support the growth of target organism and for sterility. Known ATCC/NCTC reference control strains are used to assess the performance of the media and diluents used in water testing, see QF530 and QF534. All details should be recorded on QF618.

When new batches of media or diluents arrive within the laboratory, reception staff follow QP520 (Media and Reagent QC) by segregating new stock into a quarantine area until appropriate pre-acceptance testing is completed and passed. Pre-acceptance instructions are stated within LI567.

Retesting should be carried out immediately on any new batch of media or diluent that fails quality control and all details should be recorded on QF618 and the section Technical manager should be informed. Items must NOT be put into use. The manufacturer will then be contacted to discuss the problem and a non-conformance should be initiated in Q-Pulse for full investigation. Additional stock should be ordered if required.

7.2. Sample transportation & storage

Sampling should be carried out by suitably trained personnel, laboratory staff do not undertake sampling. Samples should be collected in an appropriately sized sterile bottle containing a neutraliser to neutralise any residual disinfectant within the sample. The most commonly used is Sodium Thiosulphate. The laboratory provides 500ml sterile bottles containing 18mg/L of Sodium Thiosulphate to customers for sampling. Small volume renal samples do not require a neutraliser and can be accepted in a small sterile container.

Samples should be transported to the laboratory within 4 hours and stored between 2-5°C if they cannot be processed immediately. Samples ideally should be analysed as soon as is practicable on the day of collection or within a 24hr period.

7.3. Specimen receipt & booking in

Samples are received from private companies, GG&C hospitals and various health boards. Water samples should be received and booked in as soon as possible.
For all samples ensure sufficient information is recorded on the request form to issue a report (i.e. comments required)

- All details of the days processing should be recorded on the Daily governance worksheet (QF612). This covers process controls, media, reagents, processing staff and laboratory numbers and ensures all work is traceable
- Date stamp the time of receipt of the samples on their request forms
- Ensure the sample details match the customer request form – mismatched samples should be rejected, telephone customer to advise of this
- Refer to LI659 (Environmental Set Up) for coding and plate requirements for standard testing, for any ad hoc requests refer to LF592 for the full details as directed by Infection Control Consultant
- If the appearance of samples received is anything other than clear, this must be noted on request form to enable inclusion in final report as this may affect recovery of organisms from the sample
    - CLO (cloudy)
    - OI (oily)
    - SS (suspended solids)

- Label samples using designated laboratory number stream
- If investigations required are unclear, confirm by checking with the section Technical Manager or contact sender
- The request forms are placed in the laboratory PID tray for the BMS triage
- Once triaged the BMS will record sender details and laboratory numbers onto the appropriate daily worksheet and pass the request forms to admin staff for PID

7.4. Deviating & rejection criteria and process

Deviating Samples - this is samples that are not correctly preserved, e.g. have exceeded their maximum preservation time, >24 hours from sampling, lack the date and time of sampling, are not cooled appropriately etc. As a result, deviating samples may jeopardise the validity of the reported test result.
If sample(s) are received that are considered deviating samples, the customer must be contacted as soon as possible, informed of the status of the sample(s) and further instructions requested.
If the customer requests the samples be rejected see section below.
If the customer request the samples to be processed, a disclaimer must be added to the report stating that the sample was deviating and, as a result, the test result(s) may be invalid.
In practice, if a customer requests that a deviating sample is processed, ensure the following is added to the report:

- At report entry, enter the code **SPDEV** and additional codes/freetext comments to indicate the reason for the deviation, e.g. delay, poor storage etc
- Enter the customers' instructions, e.g. sample processed (or not processed) following discussion with the client
- Record all relevant information of phone call on request form

Rejected Samples

This includes samples which are not suitable for testing i.e. mismatch details, insufficient samples or any that the customer has agreed based on deviating nature above.

Report as Specimen Not Processed (SPNP) and add additional comments as appropriate.

- At report entry, enter the code **SPNP** and additional codes/freetext comments to indicate the reason for the rejection e.g. insufficient sample, incorrect sample etc.
- Record all relevant information of phone call on request form

8. Procedural Steps

8.1. Agar Preparation

Review daily workload to establish the number and type of pour plates required. Collect media and place in boiling bath for 30 minutes (ensure molten). Transfer molten agar to the 45ºC holding bath, using appropriate PPE. Allow media to cool to 45ºC before use. No more than 15 bottles of agar should be prepared at any one time and media should be allowed to cool appropriately.

Number of bottles	Cooling time required
5	30 Minutes
7	40 Minutes
10	40 Minutes
15	70 Minutes

- Yeast Extract Agar (YEA) and R2 Agar are the two types of media used in the pour plate method of TVC enumeration depending on sample type
- Daily process controls are carried out **see section 8.3**
- Any bottle of agar opened at the end of the working day should be discarded

8.2. Filter and manifold sterilisation

Sterilisation of the filter units before each sample is processed is essential. To do this, water bath's 1 and 2 should be switched on, the water level replenished and allowed to come to 99°C. The filter cup should then be fully submerged in the water bath. Once the bath has again reached 99°C, a 5 minute timer should be used to ensure the filter cups receive adequate time to sterilise. Great care should be used when working with the boiling baths and PPE requirements always adhered to. Filter cup base should be wiped with an alcohol wipe and allowed to air dry before next use.

In addition all filter units (cup and base) will be autoclaved, using the free steam autoclave cycle setting, on alternate days to maintain sterility. Filtration units are also cleaned by immersion in <5% sodium hypochlorite (Teepol) on a weekly basis in

addition to a free steam cycle. The manifold itself is autoclaved on the first Monday of each month, using a free steam cycle.

8.3. Daily Process Controls

Daily process controls are carried out on each singleton test method to ensure the process is fit for purpose. The controls MUST be set up and processed along side samples to ensure the full process is robust.

Media	Plates required	Sample volume	Positive Control Lenticule	Negative Control	Incubation
CCA	4	100ml	E.coli NCTC 9001 K.oxytoca NCTC 8167 Ps.aeruginosa NCTC 10662 (non target)	Baxters Water	37°C/18H
PYO	3	100ml	Ps.aeruginosa NCTC 10662 E.coli NCTC 9001 (non target)	As Above	37°C/48H
TSA	2	100ml	E.coli NCTC 9001	As Above	37°C/48H
TSA	2	100ml	E.coli NCTC 9001	As Above	30°C/5D
YEA	8	1ml	E.faecalis NCTC 775	As Above	37°C/48H 22°C/72H
R2A (Pour)	8	1ml	E.faecalis NCTC 775	As Above	30°C/5D 22°C/7D
R2A (pre-pour)	4	100ml	E.faecalis NCTC 775	As Above	As Above
Middlebrooks	2	100ml	M.fortuitum NCTC 10394	As Above	35°C/42D
SAB	4	100ml	C.albicans NCPF 3255	As Above	30/22°C/7D

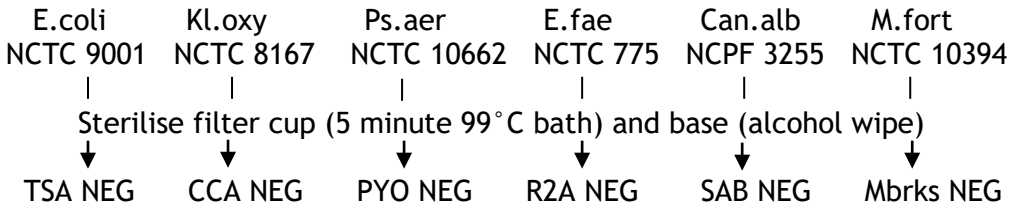
Positive Controls

- The required amount of lenticules should be removed for the freezer and allowed to come to room temperature (10 minutes)
- Appropriate agar plates should be labelled with the Lenticule batch number, organism type and date
- Reconstitute each lenticule in well labelled 100ml bottle of MRD (Maximum Recovery Diluent)
- Leave for a minimum of 15 minutes until the lenticule has dispersed, mix the solution well and process through the sterile filter unit. The lenticule should be processed within a period of 45 minutes

- Place filter onto the plate and incubate appropriately with the negative control plate and any sample plates
- Process controls should be carried out in **parallel with a batch of samples every day**

Negative Control

- Appropriate agar plates should be labelled as negative control and dated
- To demonstrate the efficacy of the filter unit cleaning practices a negative control should always follow a positive control with the standard sterilisation procedures carried out in between, see below:



- Filter 100ml of the vortexed sample through the membrane. Once all water has passed through, place the membrane onto the appropriate agar plate using sterile forceps for each membrane. **See Membrane Filtration Section for Full method**
- Incubate the plates along with the positive control plates accordingly

8.4. Generating New Process Control Data and Assessing Productivity – New Lenticule Batch

8.4.1 Generating Process Control Data

When a new lot number of lenticules is received/new batch of in house AMS controls are produced (see LI738), the following process is carried out

- Each new Lot number of process controls lenticules are run through our entire process twenty times ideally, 10 on receipt of new batch and a subsequent 10 from daily process control data by 4 different individuals.
- The Colony Forming Unit counts achieved for these are recorded on the Batch Control Record Form QF593. This data is then transferred to the spreadsheet QF577, which is held in the North Glasgow Share Drive
- From this, the mean value as well as one, two and three standard deviation values are calculated via the spreadsheet and a chart generated
- The mean and standard deviation values generated from this chart will be used as reference values to objectively assess our daily water process controls
- Expected CFU ranges of control lenticules can be obtained from the Sigma Aldrich website using the lenticule batch number
- A copy of the lenticule certificate of analysis can also be found in the Environmental section of the share drive

8.4.2 Assessing Productivity of Selective Agar

When a new lot number of specific control strain lenticules is received, ten lenticules of the new lot undergo the membrane filtration process onto specific selective media currently in-use (see table below for relevant control strains and the respective selective media to be tested). The process is repeated for a reference media – the reference media for both CCA and Pseudomonas agar is TSA. Following incubation of plates, the average of the ten test media counts is divided by the average of the ten reference media counts, to arrive at a productivity value. A value of ≥0.7 is deemed acceptable. A value of <0.7, or >1.4 must be investigated by the Technical Manager/Quality team, and remedial action taken. The lot number of the selective media and CFU counts are recorded electronically. This process is used to periodically ensure the viability of ready-made selective media following transport and storage, by ensuring satisfactory numbers of organisms can be recovered. Details of selective plates to be productivity-tested, and the relevant control lenticules to be used, are listed in the table below.

Test Media	Reference Media	Control strain
CCA	TSA	E.coli NCTC 9001;Klebsella oxytoca NCTC 8167
Pseudomonas agar	TSA	Pseudomonas aeruginosa NCTC 10562

9. Methods

9.1 Membrane Filtration Procedure

Membrane filtration is carried out on the following media for the following target organism:

Refer to LI659 for full details

Agar	Target Organism	Incubation/atmosphere
CCA	Coliforms and E.coli	37°C/18 hours (O_2)
Pseudomonas Agar	Pseudomonas species	37°C/48 hours (O_2)
TSA TSA	Target GNB TVC (Endoscopy waters)	37°C/48 hours(O_2) target GNB and 30°C for 5 days (O_2) Endoscopy waters
R2A Agar	Total Viable Count	30°C/5 days and 22°C for 7 days (O_2) – bagged to retain moisture
Middlebrooks	Atypical mycobacteria	35°C for 42 days (O_2) – sealed with parafilm
SAB	Yeast & Fungi	30°C 7 days (O_2) – sealed with parafilm 22°C 7 days (O_2) – sealed with parafilm

- Mix water sample vigorously before testing. If the sample bottle is full, pour off a little water to allow adequate mixing
- Clean the base of filter unit thoroughly with alcohol wipe, the filter cup can then be removed from the boiling baths and placed on the manifold

- The unit should be allowed to cool sufficiently before aseptically placing a 0.45µm filter paper grid side up on the magnetic unit using sterile blunt end forceps (Forceps must be decontaminated with 70% IMS and allowed to dry before use)
- All manifold valves should remain closed
- Decant 100ml of sample into the filter unit, switch the vacuum pump on and open the manifold valve
- Once all water has passed through the filter, close the manifold valve, and place membrane onto the appropriate agar plate using sterile forceps for each membrane. Forceps must be sterilized with 70% IMS between each use- (**Forceps decontaminated with 70% IMS must be allowed to fully air dry prior to manipulating membrane filters.**)
- Incubate plates as per section for media type. The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours
- **For Fungal cultures**, plates should be sealed with parafilm to avoid fungal contamination of incubators which can be problematic
- **For atypical mycobacteria**, the plates should be sealed with parafilm to retain moisture and incubated in the appropriate daily rack at 35°C for a period of 42 days in the designated incubator within the CL3 suite

9.2 Pour Plate Procedure

Total viable counts of waters and renal waters are carried out using a pour plate method using Yeast extract agar or R2A agar. To do this label 4 sterile Petri dishes for each sample by placing a barcode label on side of each petri dish. Ensure process controls have been carried out.

- If the sample bottle is full, pour off a little water to allow for adequate mixing, mix well
- Pipette 1 ml of sample into each Petri dish
- Aseptically add approximately 15 – 20ml of molten Agar to the Petri dish within 20 minutes of the 1ml being dispensed.
- Mix well (to and fro, clockwise, side to side and anti-clockwise)
- Allow agar to set at room temperature
- Invert and Incubate plates at the required temperature/atmosphere (see LI659)

9.3 RHC Theatre & water cooler/ GJNH (perfussionist) Cardiology waters Procedure

These water are processed for slow growing atypical mycobacteria and thus cannot be processed on Middlebrooks agar. The customer request form should be photocopied and all other tests carried out. A portion of the sample should be decanted into two sterile universals, sealed with parafilm and transferred to the CL3 section with the photocopied form where they will be processed in liquid culture through the MGiT (See LI693 for PID instruction and LP505 for processing guidelines)

9.4 Enumeration of Pre and Post Treatment of Effluent Waters by Membrane Filtration Procedure

Such samples arrive from Babcock etc.; usually samples of pre and post treatment of effluent water e.g. Pre UV and Final Effluent are received

- Only looking for Total Coliform/E. coli counts
- Do not require TVCs

Each requires:

- 1 x CCA per dilution
- 1 x 99ml Ringers ¼ Strength
- 6 x 90ml Ringers ¼ Strength (3 for pre & 3 for post)

A. Pre-treatment sample

- Mix water sample well before testing
- Add 1ml of sample to **99**ml Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well
- Take 10ml from 10^{-3} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-4} sample)
- Mix well
- Take 10ml from 10^{-4} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-5} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions
- Perform membrane filtration as for Coliforms/E Coli
- Place filter on CCA plate
- Incubate at 37ºC/O_2 for a minimum of 18 hours

B. Final Effluent/Post Treatment sample

- Mix water sample well before testing
- Undiluted sample is the NEAT sample
- Add 10ml of sample to **90**ml Ringers (This will become a 10^{-1} sample)
- Mix well
- Take 10ml from 10^{-1} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-2} sample)
- Mix well
- Take 10ml from 10^{-2} sample after mixing and add to a **90**ml of Ringers (This will become a 10^{-3} sample)
- Mix well before filtration of each dilution (4 in total)
- The 4 samples to be filtered are the Neat, 10^{-1}, 10^{-2}, 10^{-3} dilutions
- Perform membrane filtration as for Coliforms/E. coli
- Place filter on CCA plate
- Incubate at 37ºC/O_2 for a minimum of 18 hours

10 Result Interpretation & Reporting

At the weekend any cultures, both controls and samples, which are not able to be read on the day they are due to be reported should be refrigerated until they can be read. LF598 to be signed by the staff member moving plates from the incubator to the cold room. **It is the responsibility of the BMS reading the plates to ensure that correct incubation times for tests have been carried out before results are recorded and reported to customer. If refrigeration of plates has been necessary, the BMS must check that LF598 has been completed thus ensuring that there is an auditable trail of refrigeration having occurred.**

10.1 Criteria for acceptability of daily process control results

Process control samples must be examined and have passed specific criteria before sample plates can be examined. The examination and reporting of samples must be carried out by a suitable trained member of staff or a member of staff receiving training under supervision. All agar plates should receive the required amount of incubation in the appropriate temperature and atmosphere.
Plates should be removed from the incubator and read in a timely fashion, if the plates cannot be counted immediately then store between 2-8°C until this can be carried out.

- Count the number of colonies that have grown on the positive control plate
- Less than 1 cfu of any positive control organism indicates a failed process control. Inform the section Technical Manager and investigate
- If the batch of samples processed on this particular day contains a positive isolate, then the results may be released although the event should be investigated first by the section TM and Quality department
- If the process controls fail and all samples test negative for the representative test then the customer must be contacted and made aware of the circumstances and re-testing should be advised
- Reports should be entered into telepath and as TCE – PRFRT which expands to “Process Control Failure. Retesting recommended” and authorised
- A non-conformity (CAPA) should be raised in Q Pulse and root cause analysis carried out

Review of Process Controls

- Record the count of all process controls on the daily governance worksheet (QF612) worksheet and populate QF577 held in the Share Drive (North Glasgow Microbiology/Environmental Laboratory/Process Control Data), before releasing final results
- The mean and standard deviation values objectively assess our daily controls
- Any one value recorded out with 3SDs represents a large sudden shift from the average and will be annotated on the graph and highlighted in yellow. This can be considered a sporadic occurrence. Any corrective action should be noted on QF577
- If two out of three successive counts exceed 3SDs then an investigation should be carried out by the section TM in conjunction with the Quality department. In most cases a non-conformance should be raised unless deemed unnecessary. All values should be highlighted in yellow
- Nine consecutive counts falling on the same side of the mean represents a shift and an investigation should be carried out by the section TM to find the potential cause for this. This may reflect the original values being unreliable

and result in a new process control data and chart being compiled to reflect the shift

- Six consecutive counts continually rise/fall denotes a trend and should be investigated by the section TM. The quality of reference material may need to questioned

10.2 Examination & Enumeration of Total Viable Counts (TVC)

TVC – Pour Plate Interpretation

- Ensure all QC Plates have passed IQC before reading sample plates
- Where any QC failures noted, refer to Failed QC Section for follow up and reporting

A colony count should be carried out as soon as the plates are removed from the incubator. If the plates cannot be counted immediately, store at 2-8ºC.
Spreading colonies can interfere with counts, and the following gives guidance on what should be counted as a single colony.

- A chain of colonies caused by the disintegration of a clump of organisms
- A spreading growth that develops as a film at the bottom of a Petri dish
- A colony that forms in a film of water at the edge or over the surface of the agar
- Due to the variation in organism distribution within water, split samples e.g. TVCs done in duplicate, can display significant variation in enumerations. For example if one TVC plate is enumerated at 5 CFU/1ml then the 95% confidence interval for the 2nd plate would be an enumeration between 0-15 CFU/1ml. Please see Appendix A for acceptable 95% confidence intervals. If counts exceed 95% CI, then an investigation into must be initiated: inform the section TM or QM.

Colony Counting Method using Stuart Counter

Before beginning any count examine the plate to ensure that the colonies are spread evenly over the whole plate.
If the appearance of colonies shows no uniform spread, perhaps colonies only present on one side, this would indicate that the initial mixing of the sample was not performed correctly. Unless uniform spread then the count cannot be reliably reported and the test should be repeated.

- Switch on light and ensure that the tally counter is at zero and free to move when the counting area is depressed
- Place the plate to be counted, without lid, on the grid
- With a fine felt tip pen mark each colony, applying sufficient pressure to cause the tally counter to move
- Record the number of colonies counted from each plate on the relevant Daily Worksheet
- Calculate the mean count and record on the daily worksheet
- Rounding up an average count is acceptable e.g. two counts of 15 and 16 = a average of 15.5 Thus the average should be recorded as 16 CFU

If the number of colonies to be counted is too great e.g. >300 the plate may be divided into a half, quartered or a reduced area counted and multiplied by the reciprocal of that area i.e.

- Count 5 large squares using counter grid which are ≅ 1/10 plate and multiply by 10 for final count/ml
- Count 1 small square using counter grid which is ≅ 1/450 plate and multiply by 9 to give one large square, then multiply by 50 for final count/ml
- If there are more than 20 colonies in 1 small square record the count as $>10^4$ organisms per millilitre.

Reporting of Pour Plate TVC Results

- The TVC reported is the mean of the colony count from the two duplicate plates from an undiluted sample
- Results are recorded on daily worksheet and transcribed to the customer request form as cfu/1ml
- Refer to Reporting criteria section

TVC – Membrane Filtration Interpretation – Endoscopy Waters Only

Endoscopy rinse waters have a Total viable count performed on them using membrane filtration and two TSA plates

- Ensure all QC Plates have passed IQC before reading sample plates
- Count number of colonies present on the filters on both TSA plates. The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- Record on endoscopy water day sheet and average the two results
- Refer to Reporting criteria section

10.3 Examination & Enumeration of Coliform / E. coli

Testing for coliform bacteria and *Escherichia coli* (*E. coli*) is one of the most important microbiological examinations carried out on drinking water. The test provides a sensitive means for detecting faecal contamination and for assessing water quality, the effectiveness of water treatment and disinfection**. It should be noted that CCA agar does not inhibit the growth of gram positive bacteria and can in some cases hinder the identification of Enterobacterales. Any samples showing high counts of gram positive bacteria should be diluted and retested (result should be multiplied by dilution factor) to ensure target organisms are isolated.**

- Examine CCA plates at no less than 18 hours and within 15 minutes of being removed from the incubator
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of coliforms and E.coli on the CCA plates. Coliforms are will appear as red/pink colonies and E.coli will appear as violet/blue colonies.
- Count all red/pink and violet/blue colonies (however faint) irrespective of size (e.g. 5 red, 2 blue), taking note of different colonial morphology if necessary and record on the appropriate day worksheet, sign & date. The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100

- If the growth of clear colonies (non – target organism) is considered to be such that they obscure potential coliform growth then a repeat sample from the customer may be required – discuss with section TM
- If sufficient individual colonies and purity allows, send any red/pink or violet/blue isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of coliforms/E.coli per 100ml taking into account any dilution factors
- Pre/post treatment samples - Be aware that these counts may come from two different plates/dilutions (e.g. 39 colonies on a 10-3 plate equates to 39,000cfu/100ml)
- It is expected that the post treatment counts will be less than those of the pre-treatment samples. If not, then check that the correct procedure has been followed
- Refer to Reporting criteria section

10.4 Examination & Enumeration of Pseudomonas species

- Examine plates at 24 hours and finally at 48 hours The number of colonies at 24 hours should be noted on the daily worksheet as growth between 24 and 48 hours may be such that colonial growth results in the merging of colonies, and the number of colonies at 48 hours may seem less than the number of colonies at 24 hours.
- Ensure all QC Plates have passed IQC before reading sample plates
- Check for presence of Pseudomonas species on the Pseudomonas plates;
    - Typical (green) colonies (likely to be Ps.aeruginosa)
    - Atypical (yellow) colonies or any growth
- Perform an oxidase test on all growth
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- If sufficient individual colonies and purity allows, send any suspect isolates to the main laboratory for identification using Vitek/MS and record result on worksheet
- Purity plate any mixed isolates onto blood agar and incubate at 37ºC for 18-24 hrs. Send to the main laboratory for identification the following day
- Report as number of Ps. aeruginosa or Ps. species per 100ml
- Refer to Reporting criteria section

10.5 Examination & Enumeration of Ad Hoc Target Gram Negative Bacteria

- Examine TSA plates at 48 hours
- Ensure all QC plates have passed IQC before reading sample plates
- Check for presence of any GNB on the TSA plate;
- Perform a Gram film on all colony types
- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a

single membrane is 100 thus counts exceeding 100 should be reported as >100
- All gram positive bacteria can be disregarded and plates stored for 7 days
- If sufficient individual colonies and purity allows, GNB isolates should be sent to the main laboratory for identification using Vitek/MS. and result recorded on the daily worksheet
- Any isolate that fails initial MSID should be retested on the MS before a VITEK2 gram negative identification is carried out
- Any isolate that fails identification by the above method should be reported as Environmental GNB
- **Mixed colonies** – purity plate onto Blood agar and incubate at 37ºC for 18-24 hrs. Gram film/Send to the main laboratory for identification the following day
- Report as number of Target Organism per 100ml
- Refer to reporting criteria section
- Store all target organisms in the Environmental section of the -80ºC freezer and complete LF558

Reporting of Ad-hoc/Additional Environmental GNB results

- Gram Negative Testing – If the request has been for All GNB. All GNB should be reported on customer request forms and in LIMS. Where no GNB but other organisms may be present such as Gram Positive bacteria, samples should be reported as "No environmental Gram-negative bacilli Isolated" (comment/barcode NEGNB) See LI670
- Target Organism Testing e.g. when a specific organism is requested such as Cupriavidus then this is all that should be reported as either detected or not detected (NOTDET). No laboratory work or other named organisms identified during the testing should be submitted to the requestor (Including ICD). All this information should be classed as lab use only and be available only on the 'back of form', it should not be transcribed to the LIMS or emailed as a result
- All bacterial speciation can be reported in LIMS via NSI – AISO, whereby organisms can be reported with specific counts. **The comment code 'UKAS' must be added to these reports.**
- All completed LF592 forms (Ad Hoc/Additional Environmental requests) should remain in the laboratory with the appropriate customer request form until all results have finalised and a copy sent to the Quality Department on completion
- All results should be emailed by the admin staff to the named personal detailed with the LF592 and Dart scanned

10.6 Examination & Enumeration of Fungi

- Examine SAB plates at day 7 for yeast species and fungi
- Ensure all QC Plates have passed IQC before reading sample plates
- Examine plates for the presence of yeasts, including suspected performing either a wet prep or gram film for confirmation – further identification if required can be achieved via MALDI-ToF (MS) from fresh purity plates

- Perform a count on each colony type and record on relevant day sheet (sign & date). The maximum number of colonies that should be counted from a single membrane is 100 thus counts exceeding 100 should be reported as >100
- All filamentous fungi should be referred to the mycology section using LF539 (Mycology Referral Isolate form) for further identification using LP560 – Non superficial Mycology (ensure the plate is sealed with parafilm to avoid the release of spores)
- Black colonies should be confirmed with gram stain/wet prep and reported as Exophiala species
- Pink colonies should be confirmed with gram stain/wet prep and reported as Rhodotorula species
- Report as number yeast/fungi per 100ml as a final report or an interim result if further ID is required on the customer request form and within LIMS
- Refer to Reporting criteria section

Reporting of Fungal results

- All identifications returned from mycology should be transcribed onto the daily worksheet (LF533), customer request form and input and authorised within LIMS as a final report using LI670
- All results are entered into the LIMS by a HCPC registered BMS
- All white yeast species should be reported as Yeast species unless a full identification has been specifically requested (LF592)
- Exophiala species (black yeast) and Rhodotorula species (pink yeast) – a referral to mycology is not required and full identification should only be carried out if requested. Any fungi identified to a genus/species level must be reported along with **comment code 'UKAS'.**
- All results should be emailed by the admin staff to the appropriate distribution list and Dart scanned

10.7 Examination & Enumeration of Atypical mycobacterium

- Ensure all process control plates show the expected results before reading sample plates
- Plates should be examined weekly for bacterial growth
- Colony count, colonial morphology and any further work to be carried out (e.g. purity plates) should be recorded on LF573, dated and signed
- Any plate showing bacterial growth should be transferred to the Class 1 safety cabinet designated for Category A work
- A film for Ziehl-Neelson (ZN) staining should be prepared, stating the full laboratory number.
- A ZN stain should be carried out following LI546 and examined under oil immersion X100 light microscopy
- **Positive mycobacteria** will appear as red bacilli
- Any positive film should be checked by another trained member of staff and countersigned
- The slide should have a positive label added with the full laboratory number, sample identifier and date and retain in the positive slide box.

- A negative film will show green stained organisms of different structure e.g. bacilli, cocci

Reporting of Atypical Mycobacterium Results

- Ensure all process control plates have passed IQC before reading sample plates – ensure QF612 (Daily governance worksheet) and QF577 (Enviro process control charts) are updated to reflect this information
- Results should be recorded on the LF573 and the customer request form stating the colony forming units
- Fungal contamination may occur as plates are incubated for long periods of time – Consideration should be given to the validity of results. For major contamination, discard plates and record details on the AMS worksheet and customer request form. The auto comment "CON1" and "CON2" should be entered into TCE in telepath, this expands to "Due to contamination issue, unable to report – If available, please send another sample". Notify the customer of the contamination.
- Any positive result should be reported in the LIMS as an interim result and the appropriate customer. Endoscopy and Air positives must be notified to infection control consultant.
- The customer request form should then be emailed to the appropriate customer distribution list by the admin staff and returned to CL3 until the culture has finalised at 42 days
- In some cases a new presumptive mycobacteria isolate should be referred to the Mycobacteria Reference Laboratory in Edinburgh for identification. This should be discussed with a consultant as to their requirements and a Middlebrooks slope used. **All air isolates must be referred**
- All positive isolates should be retained in the -80 Freezer within the CL3 suite
- All sealed culture plates should be disposed of via the category 3 waste route for autoclaving
- Customer request forms should be updated with results/counts and entered and authorised in LIMS at 42 days
- Results should be entered into LIMS into the appropriate singleton test field, stating "N" for not detected or "D" for detected. If the result is positive then an enumeration per 100ml should be entered into the appropriate telepath field
- AMSPR (AMS in Progress) should be removed from the comments section
- The customer request form should then be emailed to the appropriate distribution list, dart scanned and filed by the admin staff
- All complete AMS worksheets should be sent to the office to be Dart scanned and returned. These forms shore be retained in the records room in the appropriate box file

11 Reporting Criteria

Any results out with the specified limits in the following tables should be telephoned /emailed to the customer as per their requirements.

	Standard Testing							For Ad Hoc Requests	
Water	TVC	TVC	TVC	Coliform/	Pseudo	Pseudo	Atypical	Target	Fungi

	@37	@22	@30	E.coli	aeruginosa	Species	Mycobacteria	Org / GNB	@22 or 30
POTW/POOL	>10	>100	-	>0	>0	>0	>0	>0	>0
RFS		>50	>50	>0	>0	>0	-	-	-
DFU		>10	>10	>0	>0	>0	-	-	-
ERW	-	-	*	-	>0	-	>0	-	-
Raw treatment water	-	-	-	Any	-	-	-	-	-

*Endoscopy samples have varying limits for TVC reporting please see the following table:

GGC Guidance for Final Rinse Water:	**Action/Alert**
Unsatisfactory TVC result	≥10-100 cfu/100ml*
Unacceptable TVC result	>100 cfu/100ml*
Free of *Pseudomonas aeruginosa*	>0 cfu/100ml
Free of Atypical Mycobacteria	>0 cfu/100ml
*Alert user of any TVC ≥10 cfu/100ml	

Interim Reporting

Results should be transferred from the daily worksheet to the customer request form. Telephone the customer with interim notifiable count and record details of the call on the customer request form. Enter the results into LIMS entering telephone details in TCE/TELWAT. Email the result to customer if required or if this is the customers preferred method for communication via the admin staff.

Final Reporting

Results should be transferred from day worksheet to customer request form and entered/authorised into LIMS by HCPC registered BMS staff.

- All Negatives are entered using barcode scripts (LI670)
- All report codes/comments can be found on LI670
- All positives should be entered manually
- Specific organism identifications with counts should be reported via the organism table (NSI, AISO)
- Once all reporting complete, release as final ‘PRF’ and ‘NRQ’
- Check the OUTW/WINP lists periodically for any outstanding reports- record on LF52 when complete.
- Customer request forms are labelled with an instruction card stating what work should be carried out by the admin staff e.g. email, dart scan, file etc
- If a notifiable count is recorded on the final day of testing, then the report can be reported with no requirement for a telephone call
- All Environmental forms should be Dart scanned and filed appropriately

- Daily Worksheets are held in the environmental laboratory for 2 months, after this pass to office for dart scanning and filing in the records room

Reports are authorised by;

- **PRI -** Interim report (There can be multiple interims if required)
- **PRF -** Final report (There must only be 1 final report issued, anything after this MUST be additional)
- **PRA -** Additional report
- **NRQ** - to release results

ALL RESULTS ARE AUTHORISED BY THE BMS FROM THE SUPERVISOR AUTHORISATION QUEUE

This queue should be checked every time a result is released to ensure that all reports are authorised.

Referral of Isolates

- Isolates should only be referred to a reference laboratory at the request of medical staff. **All Air AMS isolates must be referred.**
- All isolates being referred to Reference labs must be parcelled & sent as per Postal SOP LP542 and details recorded on LF515
- Each send away sample must have a postal declaration form LF516 completed
- LIMS should be updated in comments "REFER" with the organism identification and released as an Interim report

Reports returned from Reference labs

- All reference reports are received by specimen reception/office and will be scanned to DART &/or portal before being handed to the laboratory
- Once the reference lab reports are received by the bench the BMS should enter RLENV and enter the date the report was received
- All details reported on the reference report should be entered manually into the comments section of telepath to enable the user to see results - **results are not viewable via portal**
- The BMS must complete LF515 and then authorise the report to the appropriate status required

12 Retention of Isolates & Samples

All culture plates showing notifiable growth should be retained for a period of 7 days within the environmental storage cabinet within the main laboratory.
All target isolates are stored -according to the investigation requested-(recorded on LF558 – stored Isolate form) within the main laboratory in case further investigations are required. All Atypical Mycobacteria isolates should be retained in the -80°C freezer within the CL3 suite

All samples once processed should be retained in the cold room (2-5°C) for a period of 7 days until all investigations have been concluded

13 References

- Scottish Health Technical Memorandum 2030 part 3 Version 2 October 2001
- Health Technical Memorandum 01-06: Decontamination of flexible endoscopes: Part E: Testing methods
- Standing Committee of Analysts: The Microbiology of Drinking Water Part 1 – Water quality, epidemiology and public health : 2002
- Standing Committee of Analysts: The Microbiology of Water and Associated Materials. Practices and Procedures for Laboratories. Methods for the Examination of Waters and Associated Materials. 2017
- Standing Committee of Analysts The Microbiology of Drinking Water Part 7 - Methods for the enumeration of heterotrophic bacteria : 2020
- Standing Committee of Analysts The Microbiology of Drinking Water Part 8 – Methods for the isolation and enumeration of Aeromonas and Pseudomonas aeruginosa : 2015
- EDTNA/ERCA Guidelines on Control and Monitoring of Microbiological Contamination in Water for Dialysis
- Guideline on water treatment systems, dialysis water and dialysis fluid quality for haemodialysis and related therapies Clinical Practice Guideline Prepared on behalf of The Renal Association1 and The Association of Renal Technologists2 January 2016
- Preparation and quality management of fluids for haemodialysis and related therapies – Part 3: Water for haemodialysis and related therapies (ISO 23500-3:2019)
- Protocol for Environmental Sampling, Processing and Culturing of Water and Air Samples for the Isolation of Slow-Growing Mycobacteria Standard operating procedure. Public Health England. 2016
- ISO 9308-1:2014(E) Water quality — Enumeration of Escherichia coli and coliform bacteria —Part 1: Membrane filtration method for waters with low bacterial background flora
- ISO 11133:2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media.

Appendix A: 95 % Confidence intervals for the expected count from the second TVC sample.

First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count	First TVC Count	95% CL for 2nd TVC count
0	0-5	26	13-43	52	33-75	78	55-105
1	0-7	27	14-44	53	34-76	79	56-106
2	0-9	28	15-45	54	35-77	80	57-107
3	0-11	29	16-47	55	36-78	81	58-108
4	0-12	30	16-48	56	37-79	82	58-110
5	0-14	31	17-49	57	38-80	83	59-111
6	1-16	32	18-50	58	38-82	84	60-112
7	1-17	33	19-52	59	39-83	85	61-113
8	2-19	34	19-53	60	40-84	86	62-114
9	2-20	35	20-54	61	41-85	87	63-115
10	3-22	36	21-55	62	42-86	88	63-117
11	3-23	37	22-56	63	42-88	89	64-118
12	4-24	38	22-58	64	43-89	90	65-119
13	5-26	39	23-59	65	44-90	91	66-120
14	5-27	40	24-60	66	45-91	92	67-121
15	6-28	41	25-61	67	46-92	93	68-122
16	6-30	42	26-63	68	47-93	94	69-123
17	7-31	43	26-64	69	47-95	95	69-125
18	8-32	44	27-65	70	48-96	96	70-126
19	8-34	45	28-66	71	49-97	97	71-127
20	9-35	46	29-67	72	50-98	98	71-128
21	10-36	47	29-69	73	51-99	99	73-129
22	10-38	48	30-70	74	52-100	100	74-130
23	11-39	49	31-71	75	52-102		
24	12-40	50	32-72	76	53-103		
25	13-41	51	33-73	77	54—104		

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INVESTIGATION OF BLOOD CULTURES USING BacT/ALERT 3D

LABORATORY PROCEDURE

NUMBER / VERSION	1
DATE OF ISSUE	30/1/15
REVIEW INTERVAL	2 years
AUTHORISED BY	S Kerr
AUTHOR	J McCulloch / M Noble
COPY 3	Master file in Q-Pulse
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DOCUMENT REVIEW HISTORY
All review / revision details are available in Q-Pulse

Date	Amendment	Initials

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1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood is essential for microbiological diagnosis of bacteraemia, fungaemia, infective endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (eg joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a lighter colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO2 is generated, more light is reflected. This information is compared to the initial CO2 level in the bottle. If there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® 3D has been performed see **[T040]** Validation of BacT/ALERT” in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

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7. Required Equipment and Reagents

- BacT/ALERT 3D Incubator Modules
- BacT/ALERT 3D Control Module
- BacT/ALERT Reflectance Standards
- Digital Thermometers
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes
- Glass Microscope Slides
- Solid Culture Media as required

8. Environmental and Safety Controls

Risk assessment	Reference	Hazard	Precautions
Safety Manual	**Refer to local Safety Manual**	General	Good lab practice
Blood culture analysis by automated system	**R013**	Biological	Good lab practice
Handling Cat 2+ organisms	**R054**	Biological	Harvest *Neisseria sp* in Microbiological Safety Cabinet
Handling Cat 2 organisms	**R127**	Biological	Good lab practice. Hand washing etc
Handling ACDP Cat 3 organisms	**R058**	Biological	Good lab practice

9.Calibration Procedures

A cell which has failed the automatic internal diagnostic check no longer records bottle readings and will not be indicated as available when loading bottles. The cell must be calibrated or disabled.
See “Calibrating an Instrument Cell” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.
Temperature calibration is required: “Anytime the optimal (setpoint) temperature is changed; or anytime the periodic check of the Reference Thermometer reveals that the temperature is more than 0.5°C above or below the module's setpoint”.
See “Calibrating an Incubation Module Temperature” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

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10.Procedural Steps

Overview

The BacT/ALERT® 3D Microbial Detection System is a totally automated test syste capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once placed in the unit, handling of a specimen bottle is not required until a result is obtained. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep. If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle:- No growth 2 days
Anaerobic Bottle:- No growth 2 days
OR
Paediatric Bottle:- No growth 2 days
Culture will be continued, no further report will be issued unless this becomes positive.
Authorised by Automatic release by system.
Final Report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative. The system will also indicate the negative samples that are ready for removal from the system when prompted.
Because the system handles bottles individually, testing of new specimens may begin at any time. The system also utilises barcode technology to assist in specimen and data tracking.

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10.1 Receipt, Booking In and Incubation of Bottles

- Use appropriate PPE as per Reception SOP
- Follow routine reception rejection criteria
- Examine bottle(s) visually for positive indicator on arrival. If positive, process according to positive protocol then load bottle into BacT/ALERT 3D.
- Examine for any external contamination of bottles and disinfect if appropriate.
- Label bottle(s) and request form with laboratory barcode number labels.
- Remove bottle bar code labels and attach to request form, if unavailable, manually transcribe the alpha numeric details onto the request form.
- Enter PID/sample details into LIMS.
- Load bottles as soon as possible into the BacT/ALERT 3D.
- Electronic report to user confirming receipt of sample issued automatically by LIMS.
- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 POSITIVE BOTTLES (either on receipt or flagged by BacT/ALERT)

Note : Results must be accessible at all stages for laboratory clinical staff as soon as they become available.
See "Blood Culture Communication Protocol" LI504

- Confirm bottle and request form details match.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment
- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- Disinfect rubber septum with alcohol wipe.
- Using a sterile sub-vent unit, prepare a film for Gram stain and inoculate media. **(See Table 1)**
- Record Gram film result on request form.
- Enter Gram film results into Telepath indicating which bottle.
- Release interim report.
- BMS Authorises.
- Inform Clinical Staff.

Table 1

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

<u>If no bacteria seen check growth curve for bottle on Bact/Alert.</u>
If curve not suggestive of bacterial activity i.e. no steep climb. Return bottle to Bact/Alert. Examine growth curve and record appearance.
Enter in LIMS Comment Code BCFP "No Organisms Seen Bottle Reloaded"

Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation.
If no growth return bottle status in Telepath to Negative.

If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve .
Re-film bottle, if no organism seen, seek second opinion of microscopy if possible.
Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
Inform clinical staff of findings.
Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation.

(See Table 2)

If bacteria seen.

- Set up direct sensitivities and any appropriate additional tests.
- For coliform like organisms dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.
  (See Table 2)

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Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity.	Await results of solid media subculture.	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C. Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, Abiotrophia species.
No bacteria seen and growth curve not suggestive of bacterial activity.	Await results of solid media subculture	Return bottle to system.
GPC	BC1 Set on MH Agar O2 BC2 Set on BMH CO2	If GPC in clusters inoculate SAID agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	BC2 Set on BMH CO2	
GPB (large, square-ended)	BC1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	BCGNB1 and BCGNB2 on MH Dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.	CLED agar
GNB ? *Haemophilus*	Haemophilus/Moraxella Set BCGNB1 and BCGNB2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

When organisms seen and no growth on solid media excluding Propionibacteria:

- Refilm bottle
- Check growth curve
- Depending on findings sub extra media or amend report.
- Inform Medical Staff.

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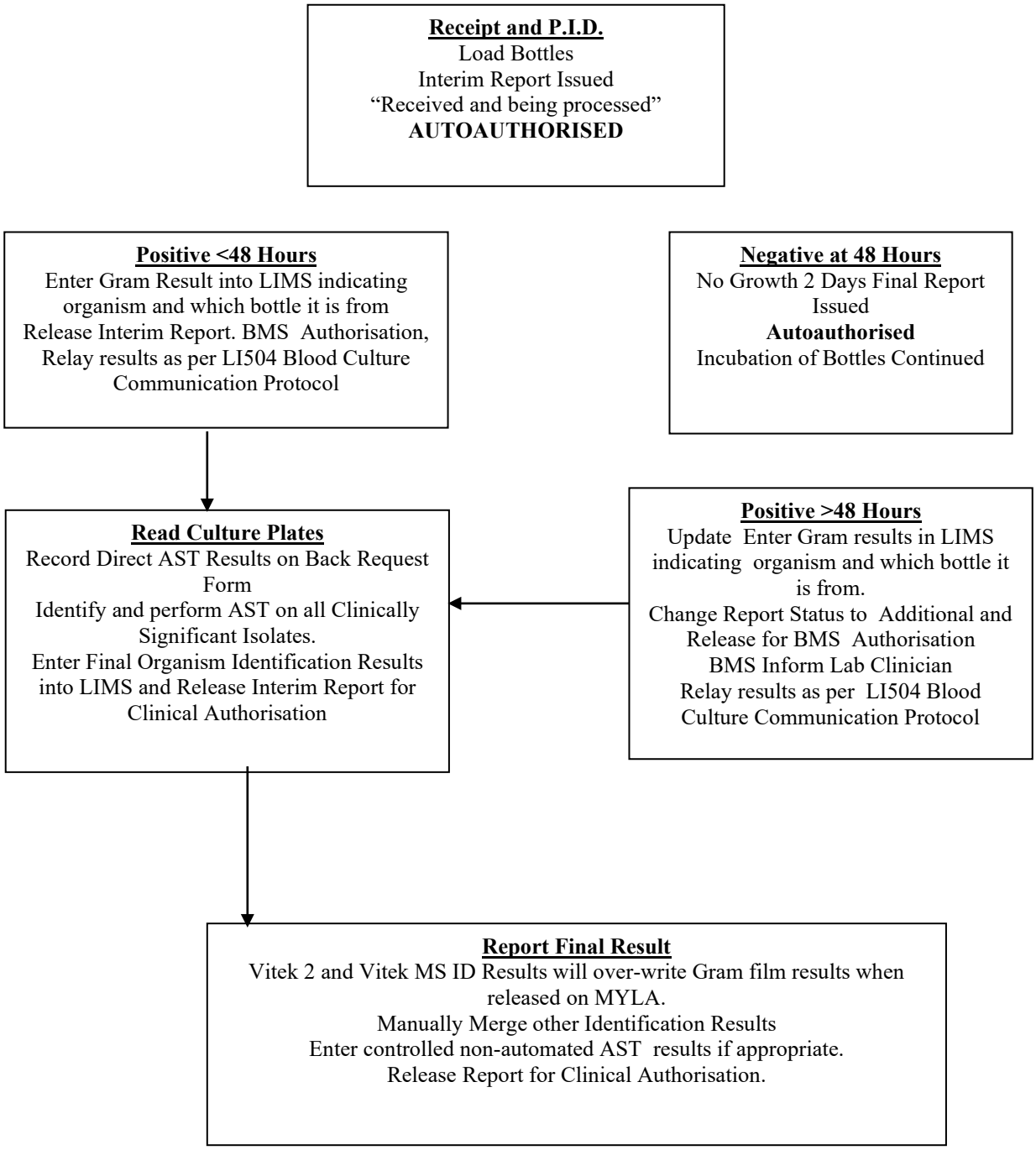
BLOOD CULTURE REPORTING FLOW CHART



10.3 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	Vitek2 or API	Disk Diffusion	-80C, 1year Except Cl.perfringins.	
Staph aureus	SAID agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Clinically Significant Coag Negative Staphylococcus	SAID agar Staph Latex Vitek MS	VITEK 2	VITEK 2	-80C, 1year	
Non Significant Coag Negative Staphylococcus	SAID agar Staph Latex	Vitek MS	Not required	Room Temp 1 week	
Micrococcus species	SAID agar, Staph latex, Colonial Morphology	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2	-80C, 1year	EARRS required
α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	API	Disc diffusion or E-Test if clinically significant	Clinically Significant -80C, 1year Non Significant room temp 1 week.	
B-Haemolytic Streps	Strep Group Latex Test	Vitek MS or API	Vitek 2 for Group B. Disk Diffusion all others	-80C, 1year	Group A typing required.
Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2	-80C, 1year	
Propionibacterium spp.	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ E-Test	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Reference Lab.	Disk Diffusion	-80C, 1year	If appropriate
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/ E-Test	-80C, 1year	If appropriate
Yeast	Colonial Morphology Vitek MS	Mycology Section	Sensititre YO10	-80C, 1year	If appropriate

Positive Blood Culture Day 2 Work Flow

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. Direct sensitivity results and preliminary identification results recorded on request form to medical staff. (by 10:00am if possible)
2. MALDI-TOF identification initiated ASAP and results authorised in LIMS as soon as available.
3. Vitek sensitivity results reviewed, transferred and authorised in LIMS.
4. Isolate storage, diffusion sensitivity tests set up, isolates referral.

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.
- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.
- **Isolate Storage****
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001,SCHEDULE 5
  see Q-Pulse for guidance.
- Positive blood culture bottles should be discarded one week after they become positive.

11.Quality Control Procedures

BacT/ALERT® 3D performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are indicated by instrument fault code 60 "Cell Failed QC". See **9.Calibration Procedures** above.
Temperatures of incubator modules are recorded daily.
Annual service performed by Biomerieux.

12. Interferences

Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

A sample is determined to be positive if there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2 as indicated by the colour of the sensor in the bottom of each culture bottle and measured by a photodetector.

14. Biological reference intervals.

Not applicable.

15.Reportable interval of examination results.

Not applicable.

16.Instruction for determining quantitative results when a result is not within the measurement interval.

Not applicable.

17. Alert/critical values.

Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19.Potential sources of variation.

False positives may be encountered due to the biological nature of the samples, i.e. high white blood cell count or turbid body fluid. False positives may also occur if the bottles are filled with more than 10 ml of blood.
The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20.References.

BacT/ALERT® 3D user manual, Q-Pulse [ED505]
UK Standards for Microbiology Investigations Investigation of Blood Cultures (for Organisms other than Mycobacterium species) Bacteriology B37 .

INVESTIGATION OF BLOOD CULTURES USING BacT/ALERT 3D

LABORATORY PROCEDURE

NUMBER / VERSION	2
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AUTHORISED BY	Pam Saunders
AUTHOR	Jane McCulloch
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DOCUMENT REVIEW HISTORY
All review / revision details are available in Q-Pulse

Date	Amendment	Initials

1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood

is essential for microbiological diagnosis of bacteraemia, fungaemia, infective endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (eg joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a lighter colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO2 is generated, more light is reflected. This information is compared to the initial CO2 level in the bottle. If there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® 3D has been performed see **[T040]** Validation of BacT/ALERT" in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

7. Required Equipment and Reagents

- BacT/ALERT 3D Incubator Modules
- BacT/ALERT 3D Control Module
- BacT/ALERT Reflectance Standards
- Digital Thermometers
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes
- Glass Microscope Slides
- Solid Culture Media as required

8. Environmental and Safety Controls

Risk assessment	Reference	Hazard	Precautions
Safety Manual	**Refer to local Safety Manual**	General	Good lab practice
Blood culture analysis by automated system	**R508**	Biological	Good lab practice
Handling Cat 2 organisms	**R529**	Biological	Good lab practice. Hand washing etc Harvest *Neisseria sp* in Microbiological Safety Cabinet
Handling ACDP Cat 3 organisms	**R058**	Biological	Good lab practice

- **Laboratory lab coats (Howie style coats) must be worn (correctly fastened) in all laboratory areas**
- **All staff must adhere to the GG&C Glove Policy ED533**
- **Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.**

9.Calibration Procedures

A cell which has failed the automatic internal diagnostic check no longer records bottle readings and will not be indicated as available when loading bottles. The cell must be calibrated or disabled.

See “Calibrating an Instrument Cell” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

Temperature calibration is required: “Anytime the optimal (setpoint) temperature is changed; or anytime the periodic check of the Reference Thermometer reveals that the temperature is more than 0.5°C above or below the module's setpoint”.

See “Calibrating an Incubation Module Temperature” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

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10.Procedural Steps

Overview

The BacT/ALERT® 3D Microbial Detection System is a totally automated test system capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once placed in the unit, handling of a specimen bottle is not required until a result is obtained. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep. If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle – No growth 2 days (Telepath code N2D)
Anaerobic Bottle – No growth 2 days (Telepath code N2D)
OR
Paediatric Bottle – No growth 2 days (Telepath code N2D)

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added:
'Culture will be continued, no further report will be issued unless this becomes positive'

Authorised by Automatic release by system
Final report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative. The system will also indicate the negative samples that are ready for removal from the system when prompted.
Because the system handles bottles individually, testing of new specimens may begin at any time. The system also utilises barcode technology to assist in specimen and data tracking.

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8
- Follow routine reception rejection criteria
- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol. If no bacteria seen on microscopy then load bottle into BacT/ALERT 3D.
- Examine for any external contamination of bottles and disinfect if appropriate.
- Label bottle(s) and request form with laboratory barcode number labels.
- Remove bottle bar code labels and attach to request form, if unavailable, manually transcribe the alpha numeric details onto the request form.
- Enter PID/sample details into LIMS.
- Load bottles as soon as possible into the BacT/ALERT 3D.
- Electronic report to user confirming receipt of sample issued automatically by LIMS.
- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 Positive Bottles (either on receipt or flagged by BacT/ALERT)

Note : Results must be accessible at all stages for laboratory clinical staff as soon as they become available.
See "Blood Culture Communication Protocol" LI504

- Confirm bottle and request form details match.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment
- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- Disinfect rubber septum with alcohol wipe.
- Using a sterile sub-vent unit, prepare a film for Gram stain and inoculate media. **(See Table 1)**
- Record Gram film result on request form.
- Enter Gram film results into Telepath indicating which bottle.
- Release interim report.
- BMS Authorises.
- Inform Clinical Staff.

Table 1

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

<u>If no bacteria seen check growth curve for bottle on Bact/Alert.</u>
If curve not suggestive of bacterial activity i.e. no steep climb. Return bottle to Bact/Alert. Examine growth curve and record appearance.
Enter in LIMS Comment Code BCFP "No Organisms Seen Bottle Reloaded"

Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation.
If no growth return bottle status in Telepath to Negative.

If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve .
Re-film bottle, if no organism seen, seek second opinion of microscopy if possible.
Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
Inform clinical staff of findings.
Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer Table 2

If bacteria seen.

- Set up direct sensitivities and any appropriate additional tests and media e.g set up AZT/CNA plate for grams suggestive of coliform and Staph/strep
- For coliform like organisms dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.
  (See Table 2)

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Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity.	Await results of solid media subculture.	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C. Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, Abiotrophia species.
No bacteria seen and growth curve not suggestive of bacterial activity.	Await results of solid media subculture	Return bottle to system.
GPC (? Strep)	GP1 and GP2 Set on BMH Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	If GPC in clusters inoculate Chromogenic Staph agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	BC2 Set on BMH CO2	
GPB (large, square-ended)	BC1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	BCGNB1 and BCGNB2 on MH Dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.	CLED agar
GNB ? *Haemophilus*	Haemophilus/Moraxella Set BCGNB1 and BCGNB2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

When organisms seen and no growth on solid media excluding Propionibacteria:

- Refilm bottle
- Check growth curve
- Depending on findings sub extra media or amend report.
- Inform Medical Staff.

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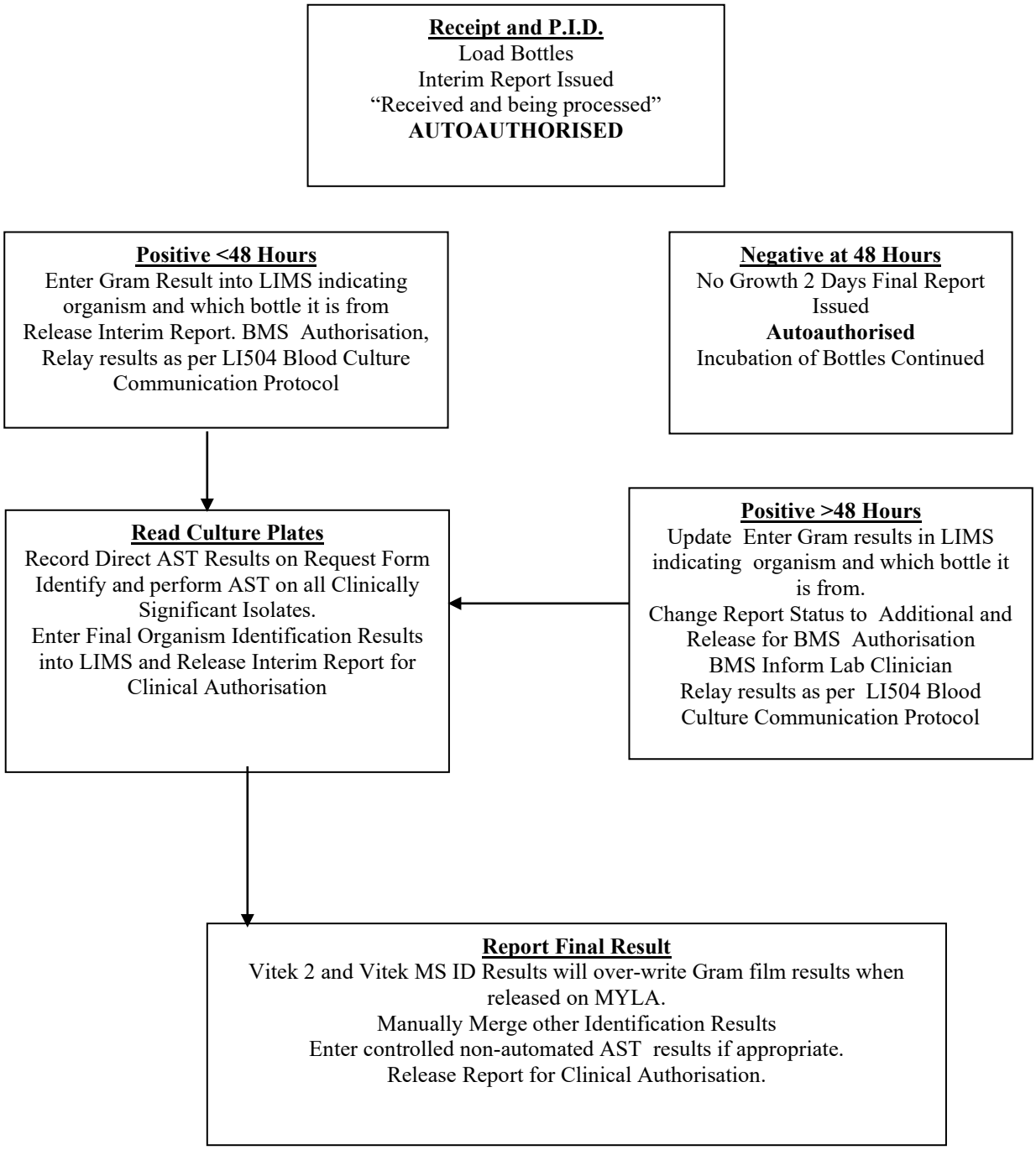
BLOOD CULTURE REPORTING FLOW CHART



10.3 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	Vitek2 or API	Etest/MICE	-80C, 1year Except Cl.perfringins.	
Staph aureus	Chromogenic Staph agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Clinically Significant Coag Negative Staphylococcus	SAID agar Staph Latex Vitek MS	VITEK 2	VITEK 2	-80C, 1year	
Non Significant Coag Negative Staphylococcus	Chromogenic Staph agar Staph Latex	Vitek MS	Not required	Room Temp 1 week	
Micrococcus species	Chromogenic Staph agar Staph latex, Colonial Morphology	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2 (STO1)	-80C, 1year	EARRS required
α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	VITEK 2	If Clinically significant Vitek2 STO1) and MICE/ E-Test as directed by Microbiologist	Clinically significant -80C, 1year Non Significant room temp 1 week.	
B-Haemolytic Streps	Strep Group Latex Test	Vitek MS or API	Vitek2 (STO1)	-80C, 1year	Group A typing required.
Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2 (P607)	-80C, 1year	
Propionibacterium spp.	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/E-Test	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Reference Lab.	Etest/MICE (Cipro and Vanc) Interpretated with CLSI	-80C, 1year	If appropriate
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/E-Test/MICE	-80C, 1year	If appropriate
Yeast	Colonial Morphology Vitek MS	Mycology Section	Sensititre YO10	-80C, 1year	If appropriate

Positive Blood Culture Day 2 Work Flow
Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. Direct sensitivity results and preliminary identification results recorded on request form to medical staff. (by 10:00am if possible)
2. MALDI-TOF identification initiated ASAP and results authorised in LIMS as soon as available.
3. Vitek sensitivity results reviewed, transferred and authorised in LIMS.
4. Isolate storage, diffusion sensitivity tests set up, isolates referral.

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.
- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.
- **Isolate Storage****
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001,SCHEDULE 5
  see Q-Pulse for guidance – ED552
- Positive blood culture bottles should be discarded one week after they become positive.

11.Quality Control Procedures

- BacT/ALERT® 3D performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are indicated by instrument fault code 60 "Cell Failed QC". See **9.Calibration Procedures** above
- Temperatures of incubator modules are recorded daily.
- Annual service performed by Biomerieux.

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

- Initial verification T040
- Quality of sample and sample preparation cannot be measured directly but is vital to whole process
- Refer to QP512

14. Biological reference intervals.

Not applicable.

15. Reportable interval of examination results.
Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.
Refer to QP512 – Measurement of Uncertainty SOP

17. Alert/critical values.
Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples, i.e. High white blood cell count or turbid body fluid.
- False positives may also occur if the bottles are filled with more than 10 ml of blood.

The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20. References.

BacT/ALERT® 3D user manual, Q-Pulse [ED505]
UK Standards for Microbiology Investigations Investigation of Blood Cultures (for Organisms other than Mycobacterium species) Bacteriology B37 .

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Investigation of Blood Cultures Using BacT/ALERT 3D	
LOCATION OF COPIES	Clyde BC Section North BC Section South BC Section

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

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2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a yellow colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO2 is generated, more light is reflected. This information is compared to the initial CO2 level in the bottle. If there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® 3D has been performed see **[T040]** Validation of BacT/ALERT” in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

7. Required Equipment and Reagents

- BacT/ALERT 3D Incubator Modules
- BacT/ALERT 3D Control Module
- BacT/ALERT Reflectance Standards
- Digital Thermometers
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes
- Glass Microscope Slides
- Solid Culture Media as required

8. Environmental and Safety Controls

Risk assessment	Reference	Hazard	Precautions
Safety Manual	**Refer to local Safety Manual**	General	Good lab practice
Blood culture analysis by automated system	**R508**	Biological	Good lab practice
Handling Cat 2 organisms	**R529**	Biological	Good lab practice. Hand washing etc Harvest *Neisseria sp* in Microbiological Safety Cabinet
Handling ACDP Cat 3 organisms	**R058**	Biological	Good lab practice

- **Laboratory lab coats (Howie style coats) must be worn (correctly fastened) in all laboratory areas**
- **All staff must adhere to the GG&C Glove Policy ED533**
- **Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.**

9.Calibration Procedures

A cell which has failed the automatic internal diagnostic check no longer records bottle readings and will not be indicated as available when loading bottles. The cell must be calibrated or disabled.

See “Calibrating an Instrument Cell” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

Temperature calibration is required: “Anytime the optimal (setpoint) temperature is changed; or anytime the periodic check of the Reference Thermometer reveals that the temperature is more than 0.5°C above or below the module's setpoint”.

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10.Procedural Steps

Overview

The BacT/ALERT® 3D Microbial Detection System is a totally automated test system capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once placed in the unit, handling of a specimen bottle is not required until a result is obtained. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep. If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle – No growth 2 days (Telepath code N2D)
Anaerobic Bottle – No growth 2 days (Telepath code N2D)
OR
Paediatric Bottle – No growth 2 days (Telepath code N2D)

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added:
'Culture will be continued, no further report will be issued unless this becomes positive'

Authorised by Automatic release by system
Final report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative. The system will also indicate the negative samples that are ready for removal from the system when prompted.
Because the system handles bottles individually, testing of new specimens may begin at any time. The system also utilises barcode technology to assist in specimen and data tracking.

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8
- Follow routine reception rejection criteria
- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol. If no bacteria seen on microscopy then load bottle into BacT/ALERT 3D.
- Examine for any external contamination of bottles and disinfect if appropriate.
- Label bottle(s) and request form with laboratory barcode number labels.
- Remove bottle bar code labels and attach to request form, if unavailable, manually transcribe the alpha numeric details onto the request form.
- Enter PID/sample details into LIMS.
- Load bottles as soon as possible into the BacT/ALERT 3D.
- Electronic report to user confirming receipt of sample issued automatically by LIMS.
- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 Positive Bottles (either on receipt or flagged by BacT/ALERT)

Note : Results must be accessible at all stages for laboratory clinical staff as soon as they become available.
See "Blood Culture Communication Protocol" LI504

- Confirm bottle and request form details match.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment
- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- Disinfect rubber septum with alcohol wipe.
- Using a sterile sub-vent unit, prepare a film for Gram stain and inoculate media. **(See Table 1)**
- Record Gram film result on request form.
- Enter Gram film results into Telepath indicating which bottle.
- Release interim report.
- BMS Authorises.
- Inform Clinical Staff.

Table 1

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

<u>If no bacteria seen check growth curve for bottle on Bact/Alert.</u>

If curve not suggestive of bacterial activity i.e. no steep climb. Examine growth curve and record appearance or print graph.
Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C
Return bottle to Bact/Alert..
Enter in LIMS Comment Code BCFP "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
The report is released as Next Save Quit NSQ.
Examine culture plates the following day and if no growth then re-incubate for a further 24 hours. i.e. 48 hours total incubation. Update bottle status at 24hrs.
If no growth at 24hrs return bottle status in LIMS to negative by removing Positive and replacing with NY or N2D.

If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve .
Re-film bottle, if no organism seen, seek second opinion of microscopy if possible.
Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
Inform clinical staff of findings.
Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer Table 2

If bacteria seen.

- Set up direct sensitivities and any appropriate additional tests and media e.g set up AZT/CNA plate for grams suggestive of coliform and Staph/strep
- For coliform like organisms dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.
  (See Table 2)

Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity.	Await results of solid media subculture.	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C. Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, Abiotrophia species.
No bacteria seen and growth curve not suggestive of bacterial activity.	Await results of solid media subculture	Return bottle to system. Chocolate Agar incubate in 5-10% CO^2 at 34-36°C.
GPC (? Strep)	GP1 and GP2 Set on BMH Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	If GPC in clusters inoculate Chromogenic Staph agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	GP2 Set on BMH CO2	
GPB (large, square-ended)	GP1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	GN1 and GN2 on MH Dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.	CLED agar
GNB ? *Haemophilus*	Haemophilus/Moraxella Set GN1 and GN2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

When organisms seen and no growth on solid media excluding Propionibacteria:

- Refilm bottle
- Check growth curve
- Depending on findings sub extra media or amend report.
- Inform Medical Staff.

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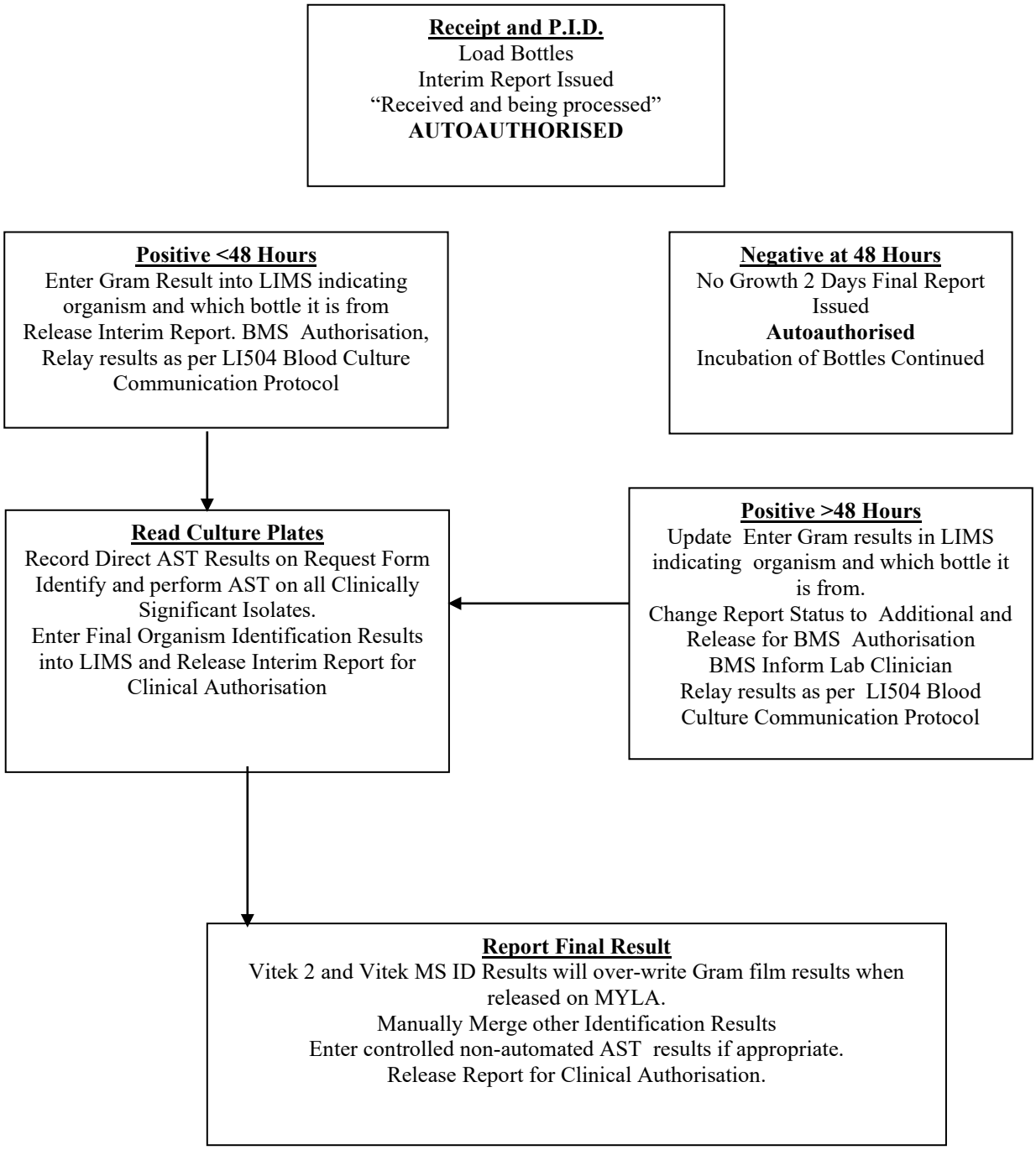
Issued: 29/11/2016	Author: Jane McCulloch

BLOOD CULTURE REPORTING FLOW CHART



10.3 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.
Salmonella typhi/paratyphi (*Salmonella typhi* - serology 'O' antigen 9, vi and 'H' antigen d)	Vitek MS	Serology	Etests ciprofloxacin and azithromycin (both Etest results to be suppressed and cipro S/R to be entered according to MIC) Standardised Discs GN1 and 2 (rule out ESBL and for AST)	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Salmonella species (non typhi/non paratyphi)	Vitek MS	Serology	Etests ciprofloxacin and azithromycin (both E-test results to be suppressed and cipro S/R to be entered according to MIC) as per GNB / Coliform	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	Vitek2 or API	Etest/MICE	-80C, 1year Except Cl.perfringens.	
Staph aureus	Chromogenic Staph agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Coagulase Negative Staphylococcus (pure growth)	Chromogenic staph agar Staph Latex Vitek MS	VITEK 2	VITEK 2 As directed by Microbiologist.	-80C, 1year	
Mixed strains Coagulase Negative staphylococci	No further work unless directed by Microbiologist	N/A	N/A	Room Temp 1 week	
Micrococcus species	Chromogenic Staph agar Staph latex, Colonial Morphology	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2 (STO1)	-80C, 1year	EARRS required
Primary plate cultural findings	**Primary ID Method**	**Secondary ID Method***	**AST Method**	**Isolate Storage****	**Send to Ref.Lab*****
B-Haemolytic Streps[1]	Strep Group Latex Test	Vitek MS or API	Vitek2 (STO1)	-80C, 1year	Group A typing required.

Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2 (P607)	-80C, 1year	
Propionibacterium spp.	Colonial Morphology Vitek MS	N/A	MICE / E-TESTS as directed by Microbiologist.	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/E-Test	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Reference Lab.	Etest/MICE (Cipro and Vanc)	-80C, 1year	If appropriate
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/E-Test/MICE	-80C, 1year	If appropriate
Yeast[2]	Colonial Morphology Vitek MS	Mycology Section	Sensititre YO10	-80C, 1year	If appropriate

Notes:

- [1]*Streptococcus pyogenes* – a small number of Strep group As when typed by reference laboratory will be emm type (STG62647) *Strep dysgalactiae subsp. Equisimilis* isolates possessing Lancefield's group A.
  Report such strains as *Strep dysgalactiae* - issue an amended report and notify Consultant Microbiologist
- [2] where clinically indicated, blood cultures may require an extended incubation of up to three weeks for *Cryptococcus* species and up to six weeks for *Histoplasma* species (SMI B37 Issue No 8)

Positive Blood Culture Day 2 Work Flow

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. Direct sensitivity results and preliminary identification results recorded on request form to medical staff. (by 10:00am if possible)
2. MALDI-TOF identification initiated ASAP and results authorised in LIMS as soon as available.
3. Vitek sensitivity results reviewed, transferred and authorised in LIMS.
4. Isolate storage, diffusion sensitivity tests set up, isolates referral.

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.
- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.
- **Isolate Storage****
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001,SCHEDULE 5
  see Q-Pulse for guidance – ED552

- Positive blood culture bottles should be discarded one week after they become positive.

11.Quality Control Procedures

- BacT/ALERT® 3D performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are indicated by instrument fault code 60 "Cell Failed QC". See **9.Calibration Procedures** above
- Temperatures of incubator modules are recorded daily.
- Annual service performed by Biomerieux.

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

- Initial verification T040
- Quality of sample and sample preparation cannot be measured directly but is vital to whole process
- Refer to QP512

14. Biological reference intervals.

Not applicable.

15. Reportable interval of examination results.

Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.

Refer to QP512 – Measurement of Uncertainty SOP

17. Alert/critical values.

Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples, i.e. High white blood cell count or turbid body fluid.
- False positives may also occur if the bottles are filled with more than 10 ml of blood.

The positive status of a sample is always confirmed by one or more of the

following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20. References.

- BacT/ALERT® 3D user manual, Q-Pulse [ED505]
- SMI B37 Issue No 8 – Investigation of Blood Cultures (for Organisms other than Mycobacterium species)

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	Authorised by: Linda Stewart
Issued: 20/11/2017	Author: Linda Stewart

Investigation of Blood Cultures Using BacT/ALERT 3D	
LOCATION OF COPIES	Clyde BC Section North BC Section South BC Section

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood is essential for microbiological diagnosis of bacteraemia, fungaemia, infective

endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (eg joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a yellow colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO2 is generated, more light is reflected. This information is compared to the initial CO2 level in the bottle. If there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® 3D has been performed see **[T040]** Validation of BacT/ALERT" in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate, heart blood)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

7. Required Equipment and Reagents
Common lab consumables, equipment and the following

- BacT/ALERT 3D Incubator Modules
- BacT/ALERT 3D Control Module
- BacT/ALERT Reflectance Standards
- Digital Thermometers
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes

8. Environmental and Safety Controls

Risk assessment	Reference	Hazard	Precautions
Safety Manual	**Refer to local Safety Manual**	General	Good lab practice
Blood culture analysis by automated system	**R508**	Biological	Good lab practice
Handling Cat 2 organisms	**R529**	Biological	Good lab practice. Hand washing etc Harvest *Neisseria sp* in Microbiological Safety Cabinet
Handling ACDP Cat 3 organisms	**R531**	Biological	Good lab practice
Handling of Suspected Viral Haemorrhagic Fever Samples	**LP508**	Biological	Good lab practice Use of Microbiological Safety cabinet
VHF	**LI564**	Biological	Good lab practice Use of Microbiological Safety cabinet

- **Laboratory lab coats (Howie style coats) must be worn (correctly fastened) in all laboratory areas**

- **All staff must adhere to the GG&C Glove Policy ED533**

- **Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.**

9.Calibration Procedures

A cell which has failed the automatic internal diagnostic check no longer records bottle readings and will not be indicated as available when loading bottles. The cell must be calibrated or disabled.
See “Calibrating an Instrument Cell” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.
Temperature calibration is required: “Anytime the optimal (setpoint) temperature is changed; or anytime the periodic check of the Reference Thermometer reveals that the temperature is more than 0.5°C above or below the module's setpoint”.
See “Calibrating an Incubation Module Temperature” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

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10.Procedural Steps

Overview
The BacT/ALERT® 3D Microbial Detection System is a totally automated test system capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once placed in the unit, handling of a specimen bottle is not required until a result is obtained. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep. If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle - No growth 2 days (Telepath code N2D)
Anaerobic Bottle - No growth 2 days (Telepath code N2D)
OR
Paediatric Bottle - No growth 2 days (Telepath code N2D)

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added this expands to:
'Culture will be continued, no further report will be issued unless this becomes positive'

Authorised by Automatic release by system
Final report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative. The system will also indicate the negative samples that are ready for removal from the system when prompted.
Because the system handles bottles individually, testing of new specimens may begin at any time. The system also utilises barcode technology to assist in specimen and data tracking.

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8.

- If sample not labelled/ incorrectly labelled - contact the ward to alert medical staff and ask for a repeat where possible, or to confirm sample belongs to correct patient - note on request form the name and designation of person spoken to.
- Follow routine Specimen Reception Rejection Instructions (LI555)
- Add code **RRR** – "requester takes full responsibility for results".

- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol.
- Examine for any external contamination of bottles and disinfect if appropriate using 10% Distel.
- Label bottle(s) and request form with laboratory barcode number labels.
- Remove bottle bar code labels and attach to front of request form, if unavailable, manually transcribe the alpha numeric details onto the request form.
- Enter PID/sample details into LIMS
- Care must be taken to input barcodes correctly at PID ensuring they are in capitals and within the correct field.
- Load bottles as soon as possible into the BacT/ALERT 3D.

- Electronic report to user confirming receipt of sample issued automatically by LIMS as follows:

Aerobic Bottle – Received and being processed (Telepath code NY)
Anaerobic Bottle – Received and being processed (Telepath code NY)
OR
Paediatric Bottle – Received and being processed (Telepath code NY)

- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 Positive Bottles (either on receipt or flagged by BacT/ALERT)

- Unload positive bottles from Bac-T-Alert 3D and place in to rack leaving 2 spaces at the start of the rack.
- Leaving 2 spaces at the start of the rack holding the bottles allows for identifying stage of process if interrupted.
- Confirm bottle and request form details match.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment.
- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- If >10 positive bottles, batch samples to avoid a bottle neck at the processing step, this allows the medical staff to report small groups of results at a time avoiding extra pressure being placed on the BMS staff when medical staff are waiting for results.

- Additional BMS may be required to help when there are large numbers of positives.
- Label primary culture plates (Table 1), Microscope slide and Saline tube prior to inoculation.
- Disinfect rubber septum with alcohol wipe and allow to dry.
- Using a sterile sub-vent unit, inoculate media (See Table 1), prepare a film for Gram stain and add 1-2 drops to saline tube (for use if GNB seen on Gram film).
- Ensure each Gram film is inoculated individually minimising all risk of contamination.
- Place blood film on hotplate for fixing.
- Gram stain fixed slides along with Gram positive and Gram negative control slides and Daily Gram Film QC (QF542) completed.
- Examine each Gram film and record Gram film result on request form and then if additional work required process one sample at a time.

- If unsure of morphology/ uniformity of a Gram film, repeat the slide and if still unsure check with a colleague or senior member of staff.
- Enter Gram film results into Telepath.
- Release interim/additional report.
- BMS Authorises from site-specific BCP1 list - this must be done prior to giving request forms to medical staff.
- Inform medical staff.

Note: Results must be accessible at all stages for laboratory clinical staff as soon as they become available.

Table 1 Primary Culture Media

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

<u>If no bacteria seen from Gram film check growth curve for bottle on BacT/Alert 3D</u>

If curve **NOT** suggestive of bacterial activity:

- If curve not suggestive of bacterial activity i.e. no steep climb. Record appearance on request form and/or print graph (if available)
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
- Re-load bottle to BacT/Alert.
- Enter in LIMS Comment Code **BCFP** "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
- Release report as Next Save Quit - **NSQ**.
- Examine culture plates the following day and if no growth then re-incubate for a further 24 hours. i.e. 48 hours total incubation.

- Update bottle status at 24hrs - If no growth at 24hrs return bottle status in LIMS to negative by removing Positive and replacing with NY or N2D.

If curve **suggestive** of bacterial activity:

- If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve. Record appearance on request form and/or print graph.
- Re-film bottle.
- If no organism seen, seek second opinion of microscopy if possible.
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C.
- **Inform clinical staff of findings.**
- Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer to Table 2 and discuss with medical staff.
- Medical staff may request an aliquot of sample be sent for PCR.

If bacteria seen

- Set up direct sensitivities and any appropriate additional tests and media as per Table 2.
- Staphylococcus/Streptococcus direct sensitivities can be made direct from the bottle.
- For coliform- like organisms dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests. See Table 2.
- Mixed cultures: set up AZT/CNA plate for Grams suggestive of coliform and Staph/strep.

When organisms seen and no growth on solid media excluding Propionibacteria:

- Refilm bottle.
- Check growth curve.
- Depending on findings sub extra media or amend report.
- Inform Medical Staff.

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Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity	Await results of solid media subculture	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, *Abiotrophia* species
No bacteria seen and growth curve not suggestive of bacterial activity	Await results of solid media subculture	Return bottle to system. Chocolate Agar incubate in 5-10% CO^2 at 34-36°C
GPC (? Strep)	GP1 and GP2 Set on BMH Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	If GPC in clusters inoculate Chromogenic Staph agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	GP2 Set on BMH CO2	
GPB (large, square-ended)	GP1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	GN1 and GN2 on MH Dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.	CLED agar
GNB? *Haemophilus*	Haemophilus/Moraxella Set GN1 and GN2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

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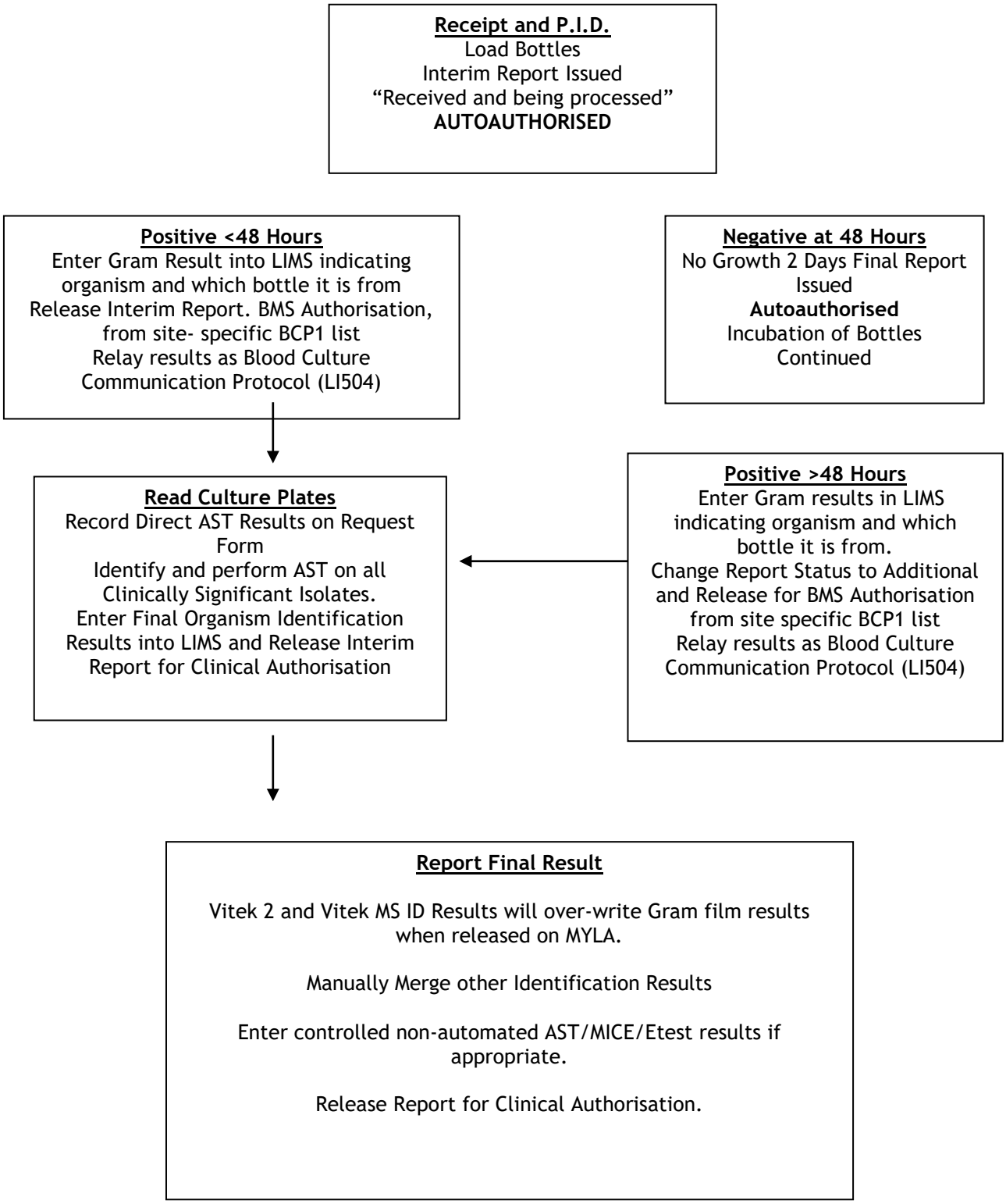
BLOOD CULTURE REPORTING FLOW CHART



Blood Culture Communication Procedure

Refer to Consultant/Medic Rota for Duty Consultant/Medic

DAY 1

- When there are more than 10 positive blood cultures, these should be batched and prioritised appropriately (e.g <48 hour positives processed first).
- Inform medical staff when there is a large number of positive blood cultures (as there may be a delay).
- Where appropriate, 2 members of BMS staff may be required to assist with Day 1 blood culture processing.
- BMS to inform Medic / Clinical Scientist when Gram stains have been reported and ready for phoning (by 10am if possible).

DAY 2 +

- BMS to inform Medic / Clinical Scientist when identification and direct sensitivities available.
- Medic / Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.
- All ongoing request forms should be shown to medical staff daily irrespective of the stage of reporting.

Out of Hours

After 5pm on weekdays

- QEUH: BMS to batch positive BCs and call first on call medic at 7pm
- ***RAH: BMS to batch positive BCs and call first on call medic at 8:00pm – until January 2018 only***
- GRI: BMS to batch positive BCs and call first on call medic at 8pm
- RHC positive BCs: phone Consultant Microbiologist on call

Weekends

- All BCs will be phoned by first on call Microbiologist; all RHC positives to consultant on call
- After medic has left lab / off site, BMS to batch positive BCs and phone as above at 4:30pm

10.3 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.

Salmonella typhi/paratyphi (*Salmonella typhi* – serology 'O' antigen 9, vi and 'H' antigen d)	Vitek MS	Serology	MICE/Etest Ciprofloxacin, Azithromycin and Ceftriaxone (Etest/MICE results to be suppressed and cipro S/R to be entered according to MIC) Standardised Discs GN1 and 2 (rule out ESBL and for AST)	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Salmonella species (non typhi/non paratyphi)	Vitek MS	Serology	MICE/Etest Ciprofloxacin and Azithromycin (both E-test results to be suppressed and cipro S/R to be entered according to MIC) as per GNB / Coliform	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	API Referral to Anaerobic reference lab, Cardiff, if directed by medical staff	Etest/MICE	-80C, 1year Except Cl.perfringens.	
Staph aureus	Chromogenic Staph agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Coagulase Negative Staphylococcus (pure growth)	Chromogenic staph agar Staph Latex Vitek MS	VITEK 2	VITEK 2 As directed by Microbiologist.	-80C, 1year	
Mixed strains Coagulase Negative staphylococci	No further work unless directed by Microbiologist	N/A	N/A	Room Temp 1 week	
Micrococcus species	Chromogenic Staph agar,Staph latex, Colonial, Morphology, NG An02, Pigmentation	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2 (ST03)	-80C, 1year	EARRS required

α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	Vitek2 or API	Vitek2 (ST03)	-80C, 1year	
ß-Haemolytic Streps[1]	Strep Group Latex Test	Vitek MS or API	Vitek2 (ST03)	-80C, 1year	Group A typing required.
Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2 (P607)	-80C, 1year	
Propionibacterium spp.	Colonial Morphology Vitek MS	N/A	MICE / Etest as directed by Microbiologist.	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/Etest as directed by Microbiologist.	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Refer to Colindale reference laboratory on medic request	MICE/Etest (Cipro and Vanc)	-80C, 1year	If appropriate and directed by Microbiolgist
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/E-Test/MICE	-80C, 1year	*Neisseria meningitidis* typing required
Yeast[2]	Colonial Morphology Candida Chromogenic Agar Vitek MS	Refer to Mycology Reference Laboratory, PHE, Bristol on medic request	Sensititre YO10	-80C, 1year	If appropriate and directed by Microbiolgist

Notes:

- [1]*Streptococcus pyogenes* – a small number of Strep group A when typed by reference laboratory will be emm type (STG62647) *Strep dysgalactiae subsp. Equisimilis* isolates possessing Lancefield's group A.
  Report such strains as *Strep dysgalactiae* – issue an amended report and notify Consultant Microbiologist.
- [2]where clinically indicated, blood cultures may require an extended incubation of up to three weeks for *Cryptococcus* species and up to six weeks for *Histoplasma* species (SMI B37 Issue No 8).

Positive Blood Culture Day 2 Work Flow

Medic / Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. When there are numerous positives, prioritise and batch request forms for medical staff. In particular, those which the medics have not seen any direct sensitivities, culture results for, e.g Day 1 Blood cultures.

2. Direct sensitivity results and preliminary identification results recorded on request form for medical staff.

3. MALDI-TOF identification initiated ASAP.

4. MALDI-TOF results transferred from MYLA and interim report issued (results authorised by Medical staff).

   If MALDI-TOF fails to identify organism, inform medical staff so they are aware of delay in reporting – further discussion required E.g. repeat MALDI-TOF, use secondary method of ID as per Table 10.3*

5. Vitek sensitivity results reviewed from previous day transferred and reported in LIMS as Final/Additional. The results are then authorised by medical staff from the appropriate list.

6. Further Vitek sensitivities, MICE/Etest, diffusion sensitivity tests set up, isolate referral and storage can then be completed.

7. Isolate referral – complete LF515.

8. Work outstanding/In progress Daily Check- Complete LF524 -- this must be completed daily and any anomalies/missing numbers investigated.

9. Blood culture orphan bottles/load list to be checked daily - this list is generated via MYLA home page.

   *Monitoring of the orphan bottles on the Bac-T-alert 3D should ensure bottles which have no associated laboratory numbers are paired up to their corresponding blood culture bottles. To be monitored daily by BMS on Blood culture bench

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.

- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.

- **Isolate Storage****
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001, SCHEDULE 5
  See Q-Pulse for guidance – ED552.

- **Discard of blood positive blood culture bottles**

  Positive blood culture bottles should be discarded one week after they become positive.

  Prior to discard each bottle should be checked for report status to ensure there are no in-progress reports which may not be detected from LF524 – Work outstanding/In progress Daily Check.

  Discard blood culture bottles as per H&SP504.

11. Quality Control Procedures

- BacT/ALERT® 3D performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are indicated by instrument fault code 60 "Cell Failed QC". See **9.Calibration Procedures** above
- Temperatures of incubator modules are recorded daily.
- Annual service performed by Biomerieux.

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

- Initial verification T040
- Quality of sample and sample preparation cannot be measured directly but is vital to whole process
- Refer to QP512

14. Biological reference intervals.

Not applicable.

15. Reportable interval of examination results.

Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.

Refer to QP512 – Measurement of Uncertainty SOP

17. Alert/critical values.

Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples, i.e. High white blood cell count or turbid body fluid.
- False positives may also occur if the bottles are filled with more than 10 ml of blood.

The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20. References.

- BacT/ALERT® 3D user manual, Q-Pulse [ED505]
- SMI B37 Issue No 8 – Investigation of Blood Cultures (for Organisms other than Mycobacterium species)

Investigation of Blood Cultures Using BacT/ALERT 3D	
LOCATION OF COPIES	North BC Section South BC Section

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

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	Authorised by: John Bingham
Issued: 24/05/2019	Author: John Bingham

1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood is essential for microbiological diagnosis of bacteraemia, fungaemia, infective endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (e.g. joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium.
When growth of the microorganisms produces CO2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a yellow colour.
A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO2 is generated, more light is reflected. This information is compared to the initial CO2 level in the bottle. If there is a high initial CO2 content, an unusually high rate of CO2 production, and/or a sustained production of CO2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® 3D has been performed see **[T040]** Validation of BacT/ALERT” in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate, heart blood)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

7. Required Equipment and Reagents
Common lab consumables, equipment and the following

- BacT/ALERT 3D Incubator Modules
- BacT/ALERT 3D Control Module
- BacT/ALERT Reflectance Standards
- Digital Thermometers
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes

8. Environmental and Safety Controls

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Hazard Group3 organisms	R531	Biological	Work in CL3
Handling Cat 2 Organisms	R529	Biological	Good lab Practice
Gram Staining	R506 &COSHH511	Chemical	Wear PPE
Blood culture analysis by automated system	R508	Biological	Good lab practice

- **Laboratory lab coats (Howie style coats) must be worn (correctly fastened) in all laboratory areas**

- **All staff must adhere to the GG&C Glove Policy ED502**

- **Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.**

9. Calibration Procedures

- A cell which has failed the automatic internal diagnostic check no longer records bottle readings and will not be indicated as available when loading bottles. The cell must be calibrated or disabled.
  See “Calibrating an Instrument Cell” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.
- Temperature calibration is required: “Anytime the optimal (setpoint) temperature is changed; or anytime the periodic check of the Reference Thermometer reveals that the temperature is more than 0.5°C above or below the module's setpoint”.

See “Calibrating an Incubation Module Temperature” 8-25 BacT/ALERT® 3D user manual [ED505] or instrument PC desktop.

10. Procedural Steps

Overview
The BacT/ALERT® 3D Microbial Detection System is a totally automated test system capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once placed in the unit, handling of a specimen bottle is not required until a result is obtained. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep.

If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle - No growth 2 days (Telepath code N2D)
Anaerobic Bottle - No growth 2 days (Telepath code N2D)
OR
Paediatric Bottle - No growth 2 days (Telepath code N2D)

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added this expands to:

‘Culture will be continued, no further report will be issued unless this becomes positive’

48 hour negative samples are authorised by automatic release by system as a final report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative. The system will also indicate the negative samples that are ready for removal from the system when prompted.
Because the system handles bottles individually, testing of new specimens may begin at any time. The system also utilises barcode technology to assist in specimen and data tracking.

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8.
- If sample not labelled/ incorrectly labelled - contact the ward to alert medical staff and ask for a repeat where possible, or to confirm sample belongs to correct patient - note on request form the name and designation of person spoken to.
- Follow routine Specimen Reception Rejection Instructions (LI555)
- Add code **RRR** – "requester takes full responsibility for results".
- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol.
- Examine for any external contamination of bottles and disinfect if appropriate using 10% Distel.
- Label bottle(s) and request form with laboratory barcode number labels.
- Remove bottle bar code labels and attach to front of request form, if unavailable, manually transcribe the alpha numeric details onto the request form.
- Enter PID/sample details into LIMS
- Care must be taken to input barcodes correctly at PID ensuring they are in capitals and within the correct field.
- Load bottles as soon as possible into the BacT/ALERT 3D.
- Electronic report to user confirming receipt of sample issued automatically by LIMS as follows:

  Aerobic Bottle – Received and being processed (Telepath code NY)
  Anaerobic Bottle - Received and being processed (Telepath code NY)
  OR
  Paediatric Bottle - Received and being processed (Telepath code NY)

- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 Positive Bottles (either on receipt or flagged by BacT/ALERT)

- Unload positive bottles from Bac-T-Alert 3D and place in to rack leaving 2 spaces at the start of the rack.
- Leaving 2 spaces at the start of the rack holding the bottles allows for identifying stage of process if interrupted.
- Confirm bottle and request form details match. It is good practice to cross check the specimen number, patient name and CHI and also the bottle barcode.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment.
- Any sub-dural or brain abscess pus samples which have been incubated in blood culture bottles should be transferred for processing in CL3 if the flag positive. This will be carried out by the CL3 BMS and tied in with the direct culture results for that specimen.

- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- If more than a single rack of positive bottles, batch samples to avoid a bottle neck at the processing step, this allows the medical staff to report small groups of results at a time avoiding extra pressure being placed on the BMS staff when medical staff are waiting for results.
- Additional BMS may be required to help when there are large numbers of positives.
- Label primary culture plates (Table 1), Microscope slide and Saline tube prior to inoculation.
- Disinfect rubber septum with alcohol wipe and allow to dry.
- Using a sterile sub-vent unit, inoculate media (See Table 1), prepare a film for Gram stain and add 1-2 drops to saline tube (for use if GNB seen on Gram film).
  N.B. the tops of the subculture vents are not sharp needles and it has been deemed safe to re-sheath these if required. Subculture vents may be re-sheathed and left in bottles for further processing i.e. set up of direct sensitivity plates. Alternatively the venting units may be removed, disposed of in sharps discard bin and fresh venting units used for any further processing as required.

- Ensure each Gram film is inoculated individually minimising all risk of contamination.
- Place blood film on hotplate for fixing.
- Gram stain fixed slides along with Gram positive and Gram negative control slides and Daily Gram Film QC (QF542) completed.
- Examine each Gram film and record Gram film result on request form and then if additional work required process one sample at a time.
- If unsure of morphology/ uniformity of a Gram film, repeat the slide and if still unsure check with a colleague or senior member of staff.
- Enter Gram film results into Telepath.
- Release interim/additional report.
- BMS Authorises from site-specific BCP1 list - this must be done prior to giving request forms to medical staff.
- Inform medical staff.

  Note: Results must be accessible at all stages for laboratory clinical staff as soon as they become available.

Table 1 Primary Culture Media

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

If no bacteria seen from Gram film

Repeat gram film to check and check growth curve for bottle on BacT/Alert 3D

If curve **NOT** suggestive of bacterial activity:

- If curve not suggestive of bacterial activity i.e. no steep climb. Record appearance on request form and/or print graph (if available)
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C.
- Re-load bottle to BacT/Alert.
- Enter in LIMS Comment Code **BCFP** "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
- Save report by Next Save Quit - **NSQ**.
- Examine culture plates the following day and if no growth then re-incubate for a further 24 hours. i.e. 48 hours total incubation.
- Update bottle status at 48hrs - If no growth at 48hrs return bottle status in LIMS to negative by removing Positive and replacing with N2D.
- Set report to Final
- Release 48 hour negative report by Next Release Quit – **NRQ**
- **These will list on the negative blood culture list for authorisation**

If curve **suggestive** of bacterial activity:

- If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve. Record appearance on request form and/or print graph.
- If no organism seen, seek second opinion of microscopy if possible.
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C.
- **Inform clinical staff of findings.**
- Consider additional culture media (i.e. campylobacter plate) or staining (i.e. ZN / auramine) based on clinical details.
- Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer to Table 2 and discuss with medical staff.
- Medical staff may request an aliquot of sample be sent for PCR.

If bacteria seen in Gram film

- Set up direct sensitivities and any appropriate additional tests and media as per Table 2.
- Staphylococcus/Streptococcus direct sensitivities can be made direct from the bottle.
- For coliform- like organisms dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests. See Table 2.
- Mixed cultures: set up AZT/CNA plate for Grams suggestive of coliform and Staph/strep.

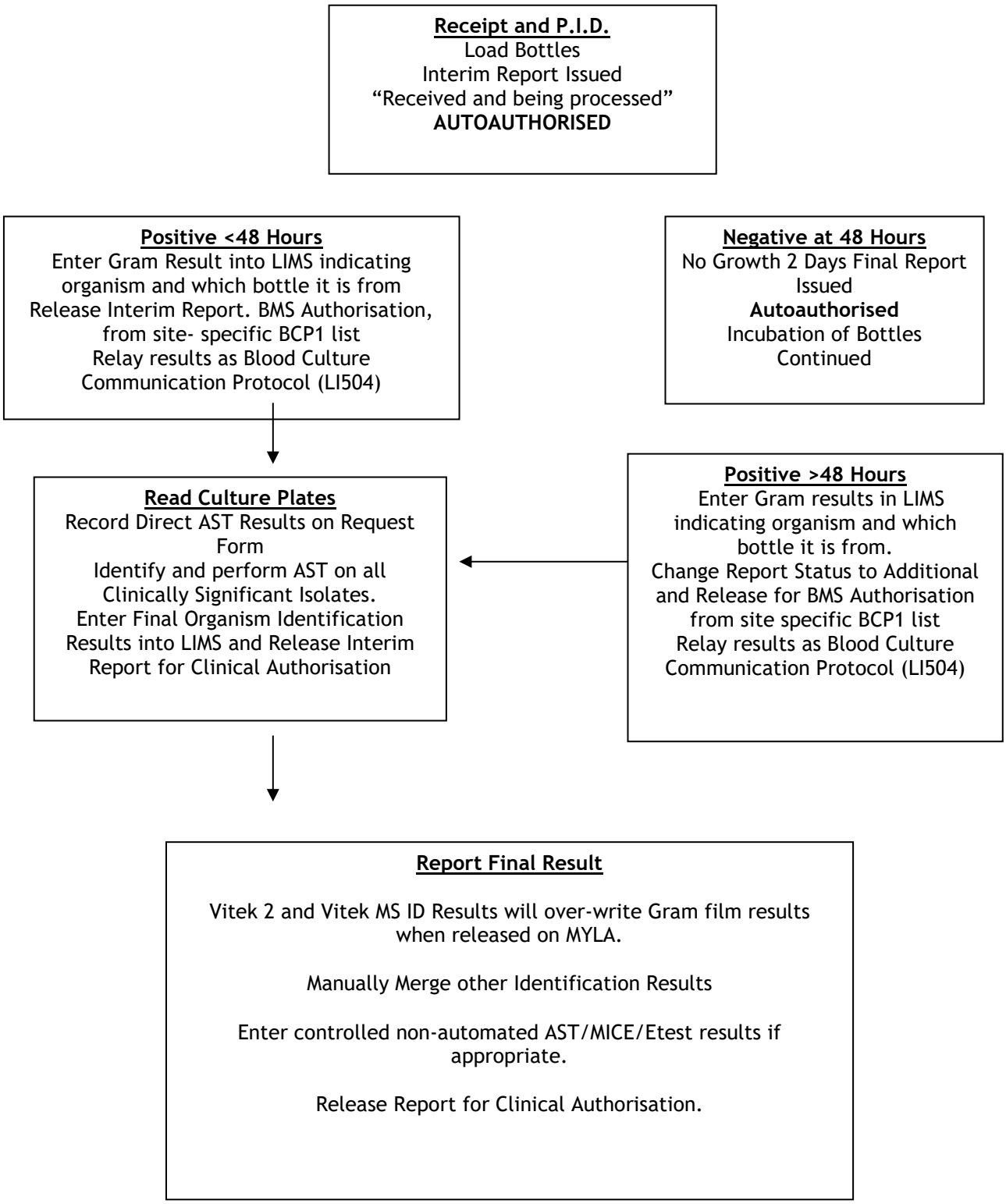
When organisms seen and no growth on solid media excluding Propionibacteria:

- Refilm bottle.
- Check growth curve.
- Depending on findings sub extra media or amend report.
- If Gram positive bacilli (excluding typical Propionibacteria) were seen on film but grow poorly on sub-culture consider possibility of Mycobacteria species and possible need for additional auramine / ZN staining.
- Inform Medical Staff.

Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity	Await results of solid media subculture	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, *Abiotrophia* species. Consider Auramine / ZN staining.
No bacteria seen and growth curve not suggestive of bacterial activity	Await results of solid media subculture	Return bottle to system. Chocolate Agar incubate in 5-10% CO^2 at 34-36°C
GPC (? Strep)	GP1 and GP2 Set on BMH Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	If GPC in clusters inoculate Chromogenic Staph agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	GP2 Set on BMH CO2	
GPB (large, square-ended)	GP1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	GN1 and GN2 on MH Dilute 1-2 drops of positive broth in 5ml saline to perform sensitivity tests.	CLED agar
GNB? *Haemophilus*	Haemophilus/Moraxella Set GN1 and GN2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

BLOOD CULTURE REPORTING FLOW CHART



Blood Culture Communication Procedure

Refer to Consultant/Medic Rota for Duty Consultant/Medic

DAY 1

- When there are large numbers (i.e. more than one rack of positive blood cultures), these should be batched and prioritised appropriately (e.g <48 hour positives processed first).
- Inform medical staff when there is a large number of positive blood cultures (as there may be a delay).
- Where appropriate, 2 members of BMS staff may be required to assist with Day 1 blood culture processing.
- BMS to inform Medic / Clinical Scientist when Gram stains have been reported and ready for phoning (by 10am if possible).

DAY 2 +

- BMS to inform Medic / Clinical Scientist when identification and direct sensitivities available.
- Medic / Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.
- All ongoing request forms should be shown to medical staff daily irrespective of the stage of reporting.

Out of Hours

After 5pm on weekdays

- QEUH: BMS to batch positive BCs and call first on call medic at 7pm
- GRI: BMS to batch positive BCs and call first on call medic at 8pm
- RHC positive BCs: phone Consultant Microbiologist on call

Weekends

- All BCs will be phoned by first on call Microbiologist; all RHC positives to consultant on call
- After medic has left lab / off site, BMS to batch positive BCs and phone as above at 4:30pm

10.3 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.
Salmonella typhi/paratyphi (*Salmonella typhi* – serology 'O' antigen 9, vi and 'H' antigen d)	Vitek MS	Serology	MICE/Etest Ciprofloxacin, Azithromycin and Ceftriaxone (Etest/MICE results to be suppressed and cipro S/R to be entered according to MIC) Standardised Discs GN1 and 2 (rule out ESBL and for AST)	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Salmonella species (non typhi/non paratyphi)	Vitek MS	Serology	MICE/Etest Ciprofloxacin and Azithromycin (both E-test results to be suppressed and cipro S/R to be entered according to MIC) as per GNB / Coliform	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	API Referral to Anaerobic reference lab, Cardiff, if directed by medical staff	Etest/MICE	-80C, 1year Except Cl.perfringens.	
Staph aureus	Chromogenic Staph agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Coagulase Negative Staphylococcus (pure growth)	Chromogenic staph agar Staph Latex Vitek MS	VITEK 2	VITEK 2 As directed by Microbiologist.	-80C, 1year	
Mixed strains Coagulase Negative staphylococci	No further work unless directed by Microbiologist	N/A	N/A	Room Temp 1 week if no further work up -80C, 1 year if further ID and workup required	
Micrococcus species	Chromogenic agar, Colonial morphology and pigment, strict aerobe	Vitek MS	Not required	Room Temp 1 week	

Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2 (ST03)	-80C, 1year	EARRS required
α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	Vitek2 or API	Vitek2 (ST03)	-80C, 1year	
ß-Haemolytic Streps[1]	Strep Group Latex Test	Vitek MS or API	Vitek2 (ST03)	-80C, 1year	Group A typing required.
Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2 (P607)	-80C, 1year	
Propionibacterium spp.	Colonial Morphology Vitek MS	N/A	MICE / Etest as directed by Microbiologist.	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/Etest as directed by Microbiologist.	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Refer to Colindale reference laboratory on medic request	MICE/Etest (Cipro and Vanc)	-80C, 1year	If appropriate and directed by Microbiolgist
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/E-Test/MICE	-80C, 1year	*Neisseria meningitidis* typing required
Yeast[2]	Colonial Morphology Candida Chromogenic Agar Vitek MS	Refer to Mycology Reference Laboratory, PHE, Bristol on medic request	Sensititre YO10	-80C, 1year	If appropriate and directed by Microbiolgist

Notes:

- [1]*Streptococcus pyogenes* – a small number of Strep group A when typed by reference laboratory will be emm type (STG62647) *Strep dysgalactiae subsp. Equisimilis* isolates possessing Lancefield's group A.
  Report such strains as *Strep dysgalactiae* - issue an amended report and notify Consultant Microbiologist.
- [2] where clinically indicated, blood cultures may require an extended incubation of up to three weeks for *Cryptococcus* species and up to six weeks for *Histoplasma* species (SMI B37).

10.4 Positive Blood Culture Day 2 Work Flow

Medic / Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. When there are numerous positives, prioritise and batch request forms for medical staff. In particular, those which the medics have not seen any direct sensitivities, culture results for, e.g Day 1 Blood cultures.

2. Direct sensitivity results and preliminary identification results recorded on request form for medical staff.

3. MALDI-TOF identification initiated ASAP.

4. MALDI-TOF results transferred from MYLA and interim report issued (results authorised by Medical staff).

   If MALDI-TOF fails to identify organism, inform medical staff so they are aware of delay in reporting – further discussion required E.g. repeat MALDI-TOF, use secondary method of ID as per Table 10.3*

5. Vitek sensitivity results reviewed from previous day transferred and reported in LIMS as Final/Additional. The results are then authorised by medical staff from the appropriate list.

6. Further Vitek sensitivities, MICE/Etest, diffusion sensitivity tests set up, isolate referral and storage can then be completed.

7. Isolate referral – complete LF515.

8. Work outstanding/In progress Daily Check- Complete LF524 -- this must be completed daily and any anomalies/missing numbers investigated.

9. Blood culture orphan bottles/load list to be checked daily - this list is generated via MYLA home page.

   *Monitoring of the orphan bottles on the Bac-T-alert 3D should ensure bottles which have no associated laboratory numbers are paired up to their corresponding blood culture bottles. To be monitored daily by BMS on Blood culture bench. For further guidance on process refer to LI616

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.

- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.

- **Isolate Storage****
  All significant isolates which receive identification and sensitivity testing should be stored at -80C using cryovials, as per above table (10.3 of this document).
  The exception to this are organisms covered by the
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001, SCHEDULE 5
  See Q-Pulse for guidance – ED552.
  Refer to MP512 – control of clinical material.

- **Discard of blood positive blood culture bottles**

  Positive blood culture bottles should be discarded one week after they become positive.

  Prior to discard each bottle should be checked for report status to ensure there are no in-progress reports which may not be detected from LF524 – Work outstanding/In progress Daily Check.

  Discard blood culture bottles as per H&SP504.

11. Quality Control Procedures

- BacT/ALERT® 3D performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are indicated by instrument fault code 60 “Cell Failed QC”. See 9.Calibration Procedures above
- Temperatures of incubator modules are recorded daily.
- Annual service performed by Biomerieux.

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

- Initial verification T040
- Quality of sample and sample preparation cannot be measured directly but is vital to whole process
- Refer to QP512

14. Biological reference intervals.

- Not applicable.

15. Reportable interval of examination results.

- Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.

- Refer to QP512 – Measurement of Uncertainty SOP

17. Alert/critical values.

- Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples, i.e. High white blood cell count or turbid body fluid.
- False positives may also occur if the bottles are filled with more than 10 ml of blood.
- The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20. References.

- BacT/ALERT® 3D user manual, Q-Pulse [ED505]
- National Standard method B37 Investigation of Blood Cultures (for Organisms other than Mycobacterium species), Issue date 07.08.2018

Investigation of Blood Cultures Using BACT/ALERT® VIRTUO	
LOCATION OF COPIES	North BC Section South BC Section

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood is essential for microbiological diagnosis of bacteraemia, fungaemia, infective endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (e.g. joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO_2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a yellow colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO_2 is generated, more light is reflected. This information is compared to the initial CO_2 level in the bottle. If there is a high initial CO_2 content, an unusually high rate of CO_2 production, and/or a sustained production of CO_2, the sample is determined to be positive.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® VIRTUO has been performed see VR599 (GRI) and VR600 (QUEH) Validation of BacT/ALERT® Virtuo in Q-Pulse.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (eg splenic aspirate, heart blood)

5. Patient Preparation

Not applicable

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

Microbiology GG&C	LP509v6
	Authorised by: John Bingham
Issued: 07/02/2020	Author: Mary Ann Ritchie

7. Required Equipment and Reagents
Common lab consumables, equipment and the following

- BacT/ALERT® Virtuo Incubator Modules (B modules)
- BacT/ALERT® Virtuo Control Module (A module)
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes

8. Environmental and Safety Controls

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Hazard Group 2 and 3 organisms	R531	Biological	Work in CL3
Gram Staining	R506 &COSHH511	Chemical	Wear PPE
Blood culture analysis by automated system	R508	Biological	Good lab practice

- **Laboratory lab coats (Howie style coats) must be worn (correctly fastened) in all laboratory areas**

- **All staff must adhere to the GG&C Glove Policy ED502**

- **Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.**

- **Any specimen where VHF is suspected should be processed as per LP508. Incubation time on the BacT/alert® Virtuo should be increased to 10 days to prevent automatic discard of bottles, allowing manual unload for discard as category A waste. The form should be held for these by the blood culture bench.**

9. Calibration Procedures
The Virtuo analyser automatically performs a calibration on every cell when unloaded. This is initially carried out with calibrator 3; if this passes the cell goes back into use. If calibrator 3 fails then the full calibration is carried out on the cell. If this fails the cell is put out of use.
Internal temperature checks are performed hourly and can be viewed on temperature reports as readings or graphs.

10. Procedural Steps

Overview
The BacT/ALERT® Virtuo Microbial Detection System is a totally automated test system capable of loading, incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteremia or fungemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF Plus Bottle. Once loaded on to the unit, handling of a specimen bottle is not required unless the bottle flags positive or requires to be manually retrieved. The instrument automatically unloads and discards negative bottles following the set incubation time. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep.

If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

> **Aerobic Bottle – No growth 2 days (Telepath code N2D)**
> **Anaerobic Bottle – No growth 2 days (Telepath code N2D)**
> **OR**
> **Paediatric Bottle – No growth 2 days (Telepath code N2D)**

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added this expands to:

> 'Culture will be continued, no further report will be issued unless this becomes positive'

48 hour negative samples are authorised by automatic release by system as a final report

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative.
The system utilises barcode technology and imaging to assist in specimen and data tracking. A full 360º image of the bottle label and barcode(s) is obtained upon loading. The Virtuo stores the bottle data including bottle barcode, laboratory accession number, media lot number and expiry date. The analyser also detects the fill level of sample within the bottle and alert any out with set parameters.

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8.
- If sample not labelled/ incorrectly labelled - contact the ward to alert medical staff and ask for a repeat where possible, or to confirm sample belongs to correct patient - note on request form the name and designation of person spoken to.
- Follow routine Specimen Reception Rejection Instructions (LI555)
- Add code **RRR** – "requester takes full responsibility for results".
- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol.
- Examine for any external contamination of bottles and disinfect if appropriate using 10% Distel.
- Label bottle(s) and request form with laboratory barcode number labels. Ensure label is placed vertically on blood culture bottle and do not cover the bottle barcode or 2D barcode.
- Remove bottle bar code labels and attach to front of request form, if unavailable, use zebra printer to create replacement barcode labels and affix to form.
- Enter PID/sample details into LIMS
- Care must be taken to input barcodes correctly at PID ensuring they are in capitals and within the correct field.
- Load bottles as soon as possible into the BacT/ALERT Virtuo.
- If Virtuo alerts problem reading barcode, press the alert icon and resolve by checking details on image, locating the correct request form and scanning required lab number.
- Electronic report to user confirming receipt of sample issued automatically by LIMS as follows:

  Aerobic Bottle – Received and being processed (Telepath code NY)
  Anaerobic Bottle – Received and being processed (Telepath code NY)
  OR
  Paediatric Bottle – Received and being processed (Telepath code NY)

- Standard incubation period is set at 5 days. If clinically requested extend incubation time.

10.2 Deviating Bottles

- On occasion when patients have been transferred from other hospitals we may receive bottles which are not compatible with the BactAlert® Virtuo analyser e.g.BD bottles. Perform PID and manually release interim report stating "received and being processed" with comment code SPDEV "Deviating specimen". These should have gram stain and sub culture performed on arrival, the bottle should then be incubated in the hot room with a further gram stain and sub culture being performed at day 2 and day 5 before being reported as negative.
- The Bact/Alert® Virtuo analyser will detect and alert lab staff to those bottles out with fill level parameters or bottles which have reached expiry.

These bottles should have a further interim report issued with comment codes as below:

- **BCFILL**

Inadequately filled bottle(s)
Interpret negative results with caution.

- **BCEXP**

Blood culture bottles expired, please check stocks
Interpret negative results with caution.

10.3 Positive Bottles (either on receipt or flagged by BacT/ALERT)

- Unload positive bottles from BacT-Alert Virtuo by pressing unload button. Bottles will be unloaded via retrieval chute and should then be placed in to rack.
- Confirm bottle and request form details match. It is good practice to cross check the specimen number, patient name and CHI and also the bottle barcode. Record on form that the demographic details have been checked.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment. Any indication of recent foreign travel to high risk areas should result in the bottle being taken to CL3 for sub-culture. Clinical history suggestive of enteric fever, query typhoid or any other hazard group 3 organisms should also be processed in CL3.
- Any sub-dural or brain abscess pus samples which have been incubated in blood culture bottles should be transferred for processing in CL3 if they flag positive. This will be carried out by the CL3 BMS and tied in with the direct culture results for that specimen.
- Any pleural fluid samples which are positive should be taken to CL3 for subculture and processing and will remain the responsibility of the blood culture bench.
- All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.
- If more than a single rack of positive bottles, batch samples to avoid a bottle neck at the processing step, this allows the medical staff to report small groups of results at a time avoiding extra pressure being placed on the BMS staff when medical staff are waiting for results.
- Additional BMS may be required to help when there are large numbers of positives.
- Label primary culture plates (Table 1) and microscope slide prior to inoculation.
- Disinfect rubber septum with alcohol wipe and allow to dry.
- Using a sterile sub-vent unit, inoculate media (See Table 1) and prepare a film for Gram stain.

N.B. the tops of the subculture vents are not sharp needles and it has been deemed safe to re-sheath these if required. Subculture vents may be re-sheathed and left in bottles for further processing i.e. set up of direct sensitivity plates. Alternatively the venting units may be removed, disposed of in sharps discard bin and fresh venting units used for any further processing as required.

- Ensure each Gram film is inoculated individually minimising all risk of contamination.
- Place blood film on hotplate for fixing.
- Perform gram stain of fixed slides along with Gram control slide and complete Daily Gram Film QC (QF542).
- Examine each Gram film and record result on request form.
- If unsure of morphology/ uniformity of a Gram film, repeat the slide and if still unsure check with a colleague or senior member of staff.
- Enter Gram film results into Telepath using barcode scripts.
- Release interim/additional report.
- BMS Authorises from site-specific BCP1 list - this must be done prior to giving request forms to medical staff.
- Inform medical staff.

Note: Results must be accessible at all stages for laboratory clinical staff as soon as they become available.

Table 1 Primary Culture Media

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36˚C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36˚C	18-48hr

<u>If no bacteria seen from Gram film</u>

Repeat gram film to check and check growth curve for bottle on BacT/Alert Virtuo

Paediatric bottles:

- All paediatric bottles regardless of graph shape should have gram repeated using a centrifuged deposit and be subcultured to both Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C and Sabouraud Agar for incubation in O^2 at 34-36˚C.
- If culture is from a paediatric patient RHC ensure that clinical staff at QEUH are informed of positive blood culture and that it is a suspected false positive regardless of graph shape.

If curve **NOT** suggestive of bacterial activity:

- If curve not suggestive of bacterial activity i.e. no steep climb. Record appearance on request form and/or print graph (if available)
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C.
- Re-load bottle to BacT/Alert.
- Enter in LIMS Comment Code **BCFP** "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
- If report status is interim save report by Next Save Quit - **NSQ**.

- Examine culture plates the following day and if no growth then re-incubate for a further 24 hours *i.e.* 48 hours total incubation.
- If growth on plates process as in 10.3 of this SOP. Retrieve bottle from Virtuo to avoid automatic discard in case of further work required.
- If report status is interim update bottle status at 48hrs - If no growth at 48hrs return bottle status in LIMS to negative by removing Positive and replacing with N2D.
- If appropriate set report to Final and release by Next Release Quit – **NRQ**
- **These will list on the negative blood culture list for authorisation**

If curve **suggestive** of bacterial activity:

- If curve suggestive of bacterial activity i.e. a steep climb or S -Shape curve. Record appearance on request form and/or print graph.
- If no organism seen, seek second opinion of microscopy if possible and consider centrifuging an aliquot of blood culture broth to repeat the gram film from the spun deposit as this may allow organisms to be seen.
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
- **Inform clinical staff of findings.**
- Consider additional culture media (i.e. campylobacter plate) or staining (i.e. ZN / auramine) based on clinical details.
- Attempt to reload bottle on to Virtuo. If analyser unloads as positive immediately then place in incubator for continued incubation.
- Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer to Table 2 and discuss with medical staff.
- If growth on plates process as in 10.3 of this SOP.
- Retrieve bottle from Virtuo to avoid automatic discard in case of further work required e.g. medical staff may request an aliquot of sample be sent for PCR.

<u>If bacteria seen in Gram film</u>

- Set up direct sensitivities and any appropriate additional tests and media as per Table 2.
- Staphylococcus/Streptococcus direct sensitivities can be made direct from the bottle.
- For coliform- like organisms dilute 1-2 drops of positive broth in saline to perform sensitivity tests. See Table 2.
- Mixed cultures: set up AZT plate for Grams suggestive of coliform and Staph/strep.

<u>When organisms seen and no growth on solid media excluding Propionibacteria</u>:

- Refilm bottle.
- Check growth curve.
- Depending on findings sub extra media or amend report.
- If Gram positive bacilli (excluding typical Propionibacteria) were seen on film but grow poorly on sub-culture consider possibility of Mycobacteria species and possible need for additional auramine / ZN staining.
- Inform Medical Staff.

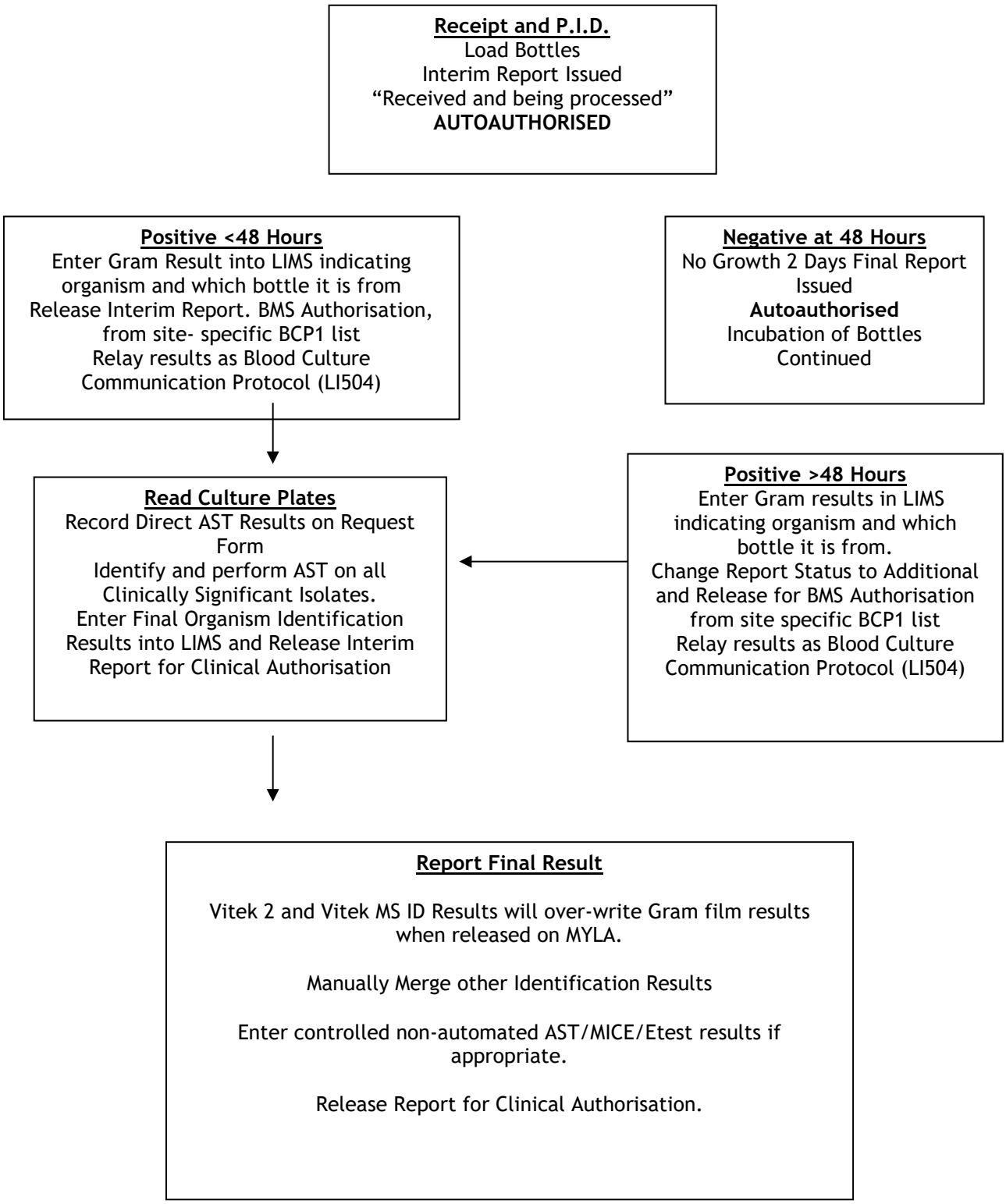
Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity	Await results of solid media subculture	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C Consider referral for Pneumococcal PCR or culture for Campylobacter species, Helicobacter species, Cysteine-dependent organisms, *Abiotrophia* species. Consider Auramine / ZN staining.
No bacteria seen and growth curve not suggestive of bacterial activity	Await results of solid media subculture	Return bottle to system. Chocolate Agar incubate in 5-10% CO^2 at 34-36°C
GPC (? Strep)	GP1 and GP2 Set on BMH Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	If GPC in clusters inoculate Chromogenic Staph agar
GPB (Propionibacterium-like)	No direct sensitivities	
GPB Diphthroid like	GP2 Set on BMH CO2	
GPB (large, square-ended)	GP1 Set on MH Agar O2	Penicillin & Metronidazole on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	GN1 and GN2 on MH Dilute 1-2 drops of positive broth in saline to perform sensitivity tests.	CLED agar
GNB? *Haemophilus*	EHI and EMCAT CO2 on BMH GN1 and GN2 CO2 on BMH	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar

No sensitivities are required on post mortem samples unless specifically requested by medical staff.

Microbiology GG&C	LP509v6
	Authorised by: John Bingham
Issued: 07/02/2020	Author: Mary Ann Ritchie

BLOOD CULTURE REPORTING FLOW CHART



Blood Culture Communication Procedure

Refer to Consultant/Medic Rota for Duty Consultant/Medic

DAY 1

- When there are large numbers (i.e. more than one rack of positive blood cultures), these should be batched and prioritised appropriately (e.g <48 hour positives processed first).
- Inform medical staff when there is a large number of positive blood cultures (as there may be a delay).
- Where appropriate, 2 members of BMS staff may be required to assist with Day 1 blood culture processing.
- BMS to inform Medic / Clinical Scientist when Gram stains have been reported and ready for phoning (by 10am if possible).

DAY 2 +

- BMS to inform Medic / Clinical Scientist when identification and direct sensitivities available.
- Medic / Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.
- All ongoing request forms should be shown to medical staff daily irrespective of the stage of reporting.

Out of Hours

After 5pm on weekdays

- QEUH: BMS to batch positive BCs and call first on call medic at 7pm
- GRI: BMS to batch positive BCs and call first on call medic at 8pm
- RHC positive BCs: phone Consultant Microbiologist on call

Weekends

- All BCs will be phoned by first on call Microbiologist; all RHC positives to consultant on call
- After medic has left lab / off site, BMS to batch positive BCs and phone as above at 4:30pm

10.4 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C,1year	If appropriate.
Salmonella typhi/paratyphi (*Salmonella typhi* – serology 'O' antigen 9, vi and 'H' antigen d Salmonella paratyphi A – serology 'O antigen 2, 'H' antigen a Salmonella paratyphi B – serology 'O antigen 4, 'H' antigen b Salmonella paratyphi C – serology 'O antigen 6:7, Vi and 'H' antigen c)	Vitek MS	Serology	MICE/Etest Ciprofloxacin, Azithromycin, Amoxicillin and Ceftriaxone (Etest/MICE results to be entered and suppressed as comment codes and cipro S/R to be entered according to MIC) Standardised Discs GN1 and 2 (rule out ESBL and for AST)	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Salmonella species (non typhi/non paratyphi)	Vitek MS	Serology	MICE/Etest Ciprofloxacin and Azithromycin (both E-test results to be entered as comment code and suppressed, cipro S/R to be entered according to MIC) Plus Vitek2 AST as per GNB / Coliform	Retain for 7 days after receipt of report from reference laboratory	Send to reference lab on nutrient agar slope for Confirmation and typing
Haemophilus	Vitek MS	X + V discs	Disk Diffusion	-80C, 1year	Typing required
Anaerobe	Vitek MS	ANC/API Referral to Anaerobic reference lab, Cardiff, if directed by medical staff	Etest/MICE - see LI590	-80C, 1year Except Cl.perfringens.	
Staph aureus	Chromogenic Staph agar Staph Latex	Vitek MS or Vitek 2,	Vitek2	-80C, 1year	EARSS required
Coagulase Negative Staphylococcus (pure growth)	Chromogenic staph agar Staph Latex Vitek MS	VITEK 2	VITEK 2 As directed by Microbiologist.	-80C, 1year	
Mixed strains Coagulase Negative staphylococci	No further work unless directed by Microbiologist	N/A	N/A	Room Temp 1 week if no further work up -80C, 1 year if further ID and workup required	

Micrococcus species	Chromogenic agar, Colonial morphology and pigment, strict aerobe	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Optochin Disk Colonial Morphology	Vitek MS	Vitek2 (ST03)	-80C, 1year	EARRS required
α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	Vitek2 or API	Vitek2 (ST03)	-80C, 1year	
ß-Haemolytic Streps[1]	Strep Group Latex Test	Vitek MS or API	Vitek2 (ST03) Group C and G require manual D test	-80C, 1year	Group A typing required.
Enterococci species	Strep Group Latex Test and Vitek MS	Vitek2 ID	Vitek 2 (P662)	-80C, 1year	
Propionibacterium spp.	Colonial Morphology Vitek MS	N/A	MICE / Etest as directed by Microbiologist.	Room Temp 1 week	
Clinically Significant Diphtheroids, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/Etest as directed by Microbiologist.	-80C, 1year	
Non Significant Diphtheroids	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Refer to Colindale reference laboratory on medic request	MICE/Etest (Cipro and Vanc)	-80C, 1year	If appropriate and directed by Microbiolgist
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Neisseria +Moraxella	Vitek MS	API	Disk Diffusion/E-Test/MICE	-80C, 1year	*Neisseria meningitidis* typing required
Yeast[2]	Colonial Morphology Candida Chromogenic Agar Vitek MS	Refer to Mycology Reference Laboratory, PHE, Bristol on medic request	Sensititre YO10	-80C, 1year	If appropriate and directed by Microbiolgist

Notes:

- [1]*Streptococcus pyogenes* – a small number of Strep group A when typed by reference laboratory will be emm type (STG62647) *Strep dysgalactiae subsp. Equisimilis* isolates possessing Lancefield's group A.
  Report such strains as *Strep dysgalactiae* – issue an amended report and notify Consultant Microbiologist.
- [2]where clinically indicated, blood cultures may require an extended incubation of up to three weeks for *Cryptococcus* species and up to six weeks for *Histoplasma* species (SMI B37).

10.5 Positive Blood Culture Day 2 Work Flow

Medic/Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. When there are numerous positives, prioritise and batch request forms for medical staff. In particular, those which the medics have not seen any direct sensitivities, culture results for, e.g Day 1 Blood cultures.

2. Direct sensitivity results and preliminary identification results recorded on request form for medical staff.

3. MALDI-TOF identification initiated ASAP.

4. MALDI-TOF results transferred from MYLA and interim report issued (results authorised by Medical staff).

   If MALDI-TOF fails to identify organism, inform medical staff so they are aware of delay in reporting – further discussion required E.g. repeat MALDI-TOF, use secondary method of ID as per Table 10.3*

5. Vitek sensitivity results reviewed from previous day transferred and reported in LIMS as Final/Additional. The results are then authorised by medical staff from the appropriate list.

6. Further Vitek sensitivities, MICE/Etest, diffusion sensitivity tests set up, isolate referral and storage can then be completed.

7. Isolate referral – complete LF515.

8. Work outstanding/In progress Daily Check- Complete LF524 -- this must be completed daily and any anomalies/missing numbers investigated.

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.
- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.

- **Isolate Storage****
  All significant isolates which receive identification and sensitivity testing should be stored at -80C using cryovials, as per above table (10.3 of this document).
  The exception to this are organisms covered by the
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001, SCHEDULE 5
  See Q-Pulse for guidance – ED552.
  Refer to MP512 – control of clinical material.

- **Discard of blood positive blood culture bottles**

  Positive blood culture bottles should be discarded one week after they become positive.

  Prior to discard each bottle should be checked for report status to ensure there are no in-progress reports which may not be detected from LF524 – Work outstanding/In progress Daily Check.
  Discard blood culture bottles as per H&SP504.

10.6 Catastrophic failure of BacT/ALERT® VIRTUO
In the event of complete analyser failure, direction should be taken from Biomerieux and the Service Manager/Operational Managers. A discussion should take place regarding transfer of new specimens to the other GGC Microbiology site should capacity permit this. There is a protocol for analyser failure within the BacT/ALERT® VIRTUO.

11. Quality Control Procedures

- BacT/ALERT® VIRTUO performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are then taken out of use by the analyser.
- Temperatures are recorded hourly for each instrument and data can be seen on instrument reports.
- Annual service performed by Biomerieux.

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as Pseudomonas aeruginosa, Streptococcus species and yeasts in continuous monitoring blood culture systems.
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated.

13. Principle of procedure for calculating results.

- Initial verification VR599 (GRI) and VR600 (QEUH)
- Quality of sample and sample preparation cannot be measured directly but is vital to whole process
- Refer to QP512

14. Biological reference intervals.

- Not applicable.

15. Reportable interval of examination results.

- Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.

- Refer to QP512 – Measurement of Uncertainty SOP

17. Alert/critical values.

- Bottle flag positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of bottle flag positive and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by laboratory clinical staff.

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples *i.e.* high white blood cell count or turbid body fluid.
- False positives may also occur if the bottles are filled with more than 10 ml of blood.
- The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.

20. References.

- BacT/ALERT® VIRTUO user manual held on Q-Pulse (ED652) & internal PC on VIRTUO.
- National Standard method B37 Investigation of Blood Cultures (for Organisms other than Mycobacterium species), Issue date 05.09.2019

Microbiology GG&C	LP509v7
	Authorised by: Margaret De Smith
Issued: 13/03/2023	Author: Karen Cullen

Investigation of Blood Cultures Using BACT/ALERT® VIRTUO Including specimens other than Blood	
LOCATION OF COPIES	North BC Section South BC Section

DOCUMENT REVIEW HISTORY
All document details are available in Q-Pulse

Date	Amendment	Initials
Further Amendments will require the document to be updated to the next version, incorporating all previous listed amendments		

Microbiology GG&C	LP509v7
	Authorised by: Margaret De Smith
Issued: 13/03/2023	Author: Karen Cullen

1. Purpose of examination

Blood culture is considered to be the ‘‘gold standard’’ investigation for the detection of micro-organisms in blood. The culture of micro-organisms from blood is essential for microbiological diagnosis of bacteraemia, fungaemia, infective endocarditis, and many infective conditions associated with a clinical presentation of pyrexia of unknown origin (PUO). It is also an important component of the diagnosis of prosthetic material infections (e.g. joints and vascular grafts) and intravascular line-associated sepsis. Blood cultures may also detect bloodstream infection in association with other infectious diseases such as pneumonia, septic arthritis and osteomyelitis.

2. Principle and method of the procedure

If microorganisms are present in the test sample, carbon dioxide is produced as the microorganisms metabolise the substrates in the culture medium. When growth of the microorganisms produces CO_2, the colour of the sensor in the bottom of each culture bottle changes from blue-green to a yellow colour. A light-emitting diode (LED) projects light onto the sensor. The light reflected is measured by a photodetector. As more CO_2 is generated, more light is reflected. This information is compared to the initial CO_2 level in the bottle. If there is a high initial CO_2 content, an unusually high rate of CO_2 production, and/or a sustained production of CO_2, the sample is determined to be positive.

The BacT/ALERT® Virtuo Microbial Detection System is a totally automated test system capable of loading, incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens suspected of having bacteraemia or fungaemia. Adult requests consist of a pair of bottles, BacT/ALERT SA Bottle and BacT/ALERT SN Bottle. Paediatric requests consist of a single BacT/ALERT PF plus Bottle. Once loaded on to the unit, handling of a specimen bottle is not required unless the bottle flags positive or requires to be manually retrieved. The instrument automatically unloads and discards negative bottles following the set incubation time. Immediately upon detection, positive flagged bottles are indicated visually on the unit's monitor and by an audible beep.

If no microbial growth is present after a specified time (normally 5 days), a specimen is determined to be negative.
The system utilises barcode technology and imaging to assist in specimen and data tracking. A full 360º image of the bottle label and barcode(s) is obtained upon loading. The Virtuo stores the bottle data including bottle barcode, laboratory accession number, media lot number and expiry date. The analyser also detects the fill level of sample within the bottle and alert any out with set parameters.

3. Performance Characteristics

Laboratory verification of BacT/ALERT® BACT/ALERT VIRTUO has been performed, see VR609 for GRI/QEUH combined validation of BacT/ALERT® BacT/Alert Virtuo in Q-Pulse.
VR629 Ongoing validation of direct Lancefield grouping to aid rapid identification of Streptococci from blood cultures. GGC study on the benefits of direct grouping from blood culture to aid patient treatment and management.

4. Type of Sample

- Blood (Adult and paediatric)
- Stem Cell Blood Products
- Ascitic Fluid
- Joint Fluid
- Pleural fluid/Aspirate
- Post mortem aspirate (e.g. splenic aspirate, heart blood)
- Brain aspirate/Pus

5. Patient Preparation

The GGC Microbiology website details instruction for collection on blood cultures: Tests Offered [redacted]

6. Type of Container and Additives

- BacT/ALERT SN Bottle
- BacT/ALERT SA Bottle
- BacT/ALERT PF Plus

7. Required Equipment and Reagents

Common lab consumables, equipment and the following

- BacT/ALERT® BacT/Alert Virtuo Incubator Modules (B modules)
- BacT/ALERT® BacT/Alert Virtuo Control Module (A module)
- Biomerieux Sterile Airway Needle/Subculture Units
- 70% IPA Alcohol Wipes

8. Environmental and Safety Controls

Risk assessment / SOP	Reference	Hazard	Precautions
Safety Manual	See local safety manual	Biological	General lab practice
GGC Waste SOP	H&SP506	Biological Chemical	Good lab practice, Wear PPE
GGC Waste Risk Assessment	R542	Biological Chemical	Good lab practice, Wear PPE
Hazard Group 2 & 3 Pathogens: Microbiological Manipulations & Exposure	R531	Biological	Work in CL3
Gram Staining	R506 &COSHH511	Chemical	Wear PPE
Blood culture analysis by automated system	R508	Biological	Good lab practice
Handling of Suspected Viral Haemorrhagic fever samples	LP508	Biological	Good lab practice
Viral Haemorrhagic Fever (VHF) Sample handling	R500	Biological	Good lab practice

- Laboratory lab coats (Howie style coats) must be worn (fully fastened) in all laboratory areas

- All staff must adhere to the GG&C Glove Policy ED502

- Gloves must be worn when opening sample bags, opening samples and processing samples and when dealing with waste.

- Any specimen where **VHF** is suspected should be processed as per LP508. Incubation time on the Virtuo should be increased to 10 days to prevent automatic discard of bottles, allowing manual unload for discard as Category A waste. The form should be held for these by the Blood culture bench.

- Any **COVID** positive sample should have incubation time on the Virtuo increased to 10 days to prevent automatic discard of bottle, allowing manual unload for discard as Category A waste. The form should be held for these by the Blood culture bench.

- Any specimen where **Brucellosis** is suspected should have incubation time on Virtuo increased to 10 days (see appendix 3), and a terminal subculture performed following 10 days incubation. Following identification of *Brucella spp*, all work must be relocated to CL3.

- If clinically indicated, blood cultures may require an extended incubation of up to three weeks for *Cryptococcus* species and up to six weeks for *Histoplasma* species (SMI B37).

- To check if a bottle is still in Virtuo – use the Request form tab on Virtuo "Negative to date "option and if > 5 days unload from Virtuo if required. N.B. do not remove bottles that require longer incubation until ready to be unloaded

9. Calibration Procedures

The Virtuo analyser automatically performs a calibration on every cell when unloaded. This is initially carried out with calibrator 3; if this passes the cell goes back into use. If calibrator 3 fails then the full calibration is carried out on the cell. If this fails the cell is put out of use.

Internal temperature checks are performed hourly and can be viewed on temperature reports as readings or graphs.

10. Procedural Steps

10.1 Receipt, Booking In and Incubation of Bottles

- Wear appropriate PPE – refer to section 8.
- If sample not labelled/ incorrectly labelled - contact the ward to alert Medical Microbiologist and ask for a repeat where possible, or to confirm sample belongs to correct patient - note on request form the name and designation of person spoken to.
- Follow Specimen Reception Rejection Instructions (LI555)
- Add code **RRR** – "requester takes full responsibility for results".
- Examine bottle(s) visually for positive indicator on arrival. If indicator positive (yellow colour), process according to positive protocol.
- Examine for any external contamination of bottles and disinfect if appropriate using 10% Chemgene
- Label bottle(s) and request form with laboratory barcode number labels. Ensure label is placed vertically on blood culture bottle and do not cover the bottle barcode, fill lines or 2D barcode.
- Remove bottle bar code labels and attach to front of request form, if unavailable, use zebra printer to create replacement barcode labels and affix to form.
- Enter PID/sample details into LIMS
- Care must be taken to input barcodes correctly at PID ensuring they are in capitals and within the correct field.
- Load bottles as soon as possible into the Virtuo.
- Electronic report to user confirming receipt of sample issued automatically by LIMS as follows:

On receipt of samples an initial report of NY- "received and being processed is issued"

Aerobic Bottle - Received and being processed (LIMS code NY)
Anaerobic Bottle - Received and being processed (LIMS code NY)
OR
Paediatric Bottle - Received and being processed (LIMS code NY)

- Standard incubation period is set at 5 days. If clinically requested extend incubation time (see appendix 3)

10.2 Deviating Bottles

Bottles non compatible with BacT/Alert Virtuo

On occasion when patients have been transferred from other hospitals we may receive bottles which are not compatible with the Virtuo analyser e.g.BD bottles.

- Perform PID and manually release interim report stating "received and being processed" with comment code **SPDEV "Deviating specimen".**
- Gram stain and sub culture performed on arrival, the bottle should then be incubated in the hot room with a further gram stain and sub culture being performed at day 2 and day 5 before being reported as negative.

Under-filled /Expired Blood culture bottles- Refer to Appendix 1

- The Virtuo analyser will detect and alert laboratory staff to those bottles out with fill level parameters or bottles which have reached expiry date and should have a report issued.

Anonymous Bottles

- If the Virtuo is unable to detect the bottle ID, refer to appendix 2 for instruction.

10.3 Negative Bottles

If a sample is negative after 48hours a report is issued. A negative report is generated from the LIMS via the interface with the BacT/ALERT which states:

Aerobic Bottle – No growth 2 days (LIMS code N2D)
Anaerobic Bottle – No growth 2 days (LIMS code N2D)
OR
Paediatric Bottle – No growth 2 days (LIMS code N2D)

For all Negative blood cultures an Auto comment (Neg1, Neg2) is added this expands to:

'Culture will be continued, no further report will be issued unless this becomes positive'

48 hour negative samples are authorised by automatic release by system as a final report

10.4 Positive Bottles (either on receipt or determined positive by Virtuo)

- Unload positive bottles from Virtuo by pressing unload button. Bottles will be unloaded via retrieval chute and should then be placed in to rack.
- Confirm bottle and request form details match. Check the specimen number, patient name and CHI and also the bottle barcode. Record on form that the demographic details have been checked.
- From patient history determine if bottle is safe to be sub-cultured out with CL3 containment. **Any indication of recent foreign travel to high risk areas should result in the bottle being taken to CL3 for sub-culture**. Clinical history suggestive of **enteric fever, query Typhoid, COVID positive or any other hazard group 3** organisms should also be processed in **CL3**.
- Any Sub-Dural or brain abscess pus samples which have been incubated in blood culture bottles should be transferred for processing in CL3 if they are determined to be positive . This will be carried out by the CL3 BMS and tied in with the direct culture results for that specimen.
- Any pleural fluid samples which are positive should be taken to CL3 for subculture and processing and will remain the responsibility of the blood culture bench.
- **All bottles to be sub-cultured in a Class 1/Class 2 Microbiological Safety Cabinet.**
- If more than a single rack of positive bottles, batch samples to avoid a bottle neck at the processing step. This allows the medical microbiologist to report small groups of results at a time avoiding extra pressure being placed on the BMS staff when medical microbiologists are waiting for results.

- Label primary culture plates (Table 1) by scanning Laboratory number from the bottle and printing a label from Zebra printer .Handwrite the microscope slide with full Laboratory number including check digit prior to inoculation
- Disinfect rubber septum with alcohol wipe and allow to dry.
- Using a sterile sub-vent unit, inoculate media (See Table 1) and prepare a film for Gram stain.

  N.B. the tops of the subculture vents are not sharp needles and it has been deemed safe to re-sheath these if required. Subculture vents may be re-sheathed and left in bottles for further processing i.e. set up of direct sensitivity plates. Alternatively the venting units may be removed, disposed of in sharps discard bin and fresh venting units used for any further processing as required.
- Place blood film on hotplate for fixing.
- Perform Gram stain of fixed slides along with Gram control slide and complete Daily Gram Film QC (QF542).
- Examine each Gram film and record result on request form.
- If unsure of morphology/ uniformity of a Gram film, repeat the slide in the first instance and if necessary take advice if required.
- Enter Gram film results into LIMS using barcode scripts.
- Release Interim/Additional report.
- BMS Authorises from site-specific BCP1 list - this must be done prior to giving request forms to the medical microbiologist.
- Inform medical microbiologist.

Table 1 Primary Culture Media

MEDIA	ATMOSPHERE	TEMPERATURE	INCUBATION TIME
Blood agar	5-10% CO^2	34-36°C	18-48hr
Fastidious anaerobe agar	Anaerobic with MTZ5 disc	34-36°C	18-48hr

If no bacteria seen from Gram film

Repeat gram film and check growth curve for bottle on Virtuo
Paediatric bottles:

- All paediatric bottles regardless of graph shape should have gram repeated using a centrifuged deposit and be subcultured to both Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C and Saboraud Agar for incubation in O^2 at 34-36°C.
- If culture is from a paediatric patient RHC ensure that clinical staff at QEUH are informed of positive blood culture and that it is a suspected false positive regardless of graph shape.

If curve **NOT** suggestive of bacterial activity:

- If curve not suggestive of bacterial activity i.e. no steep climb. Record appearance on request form and/or print graph (if available)
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36°C.
- Check the Sample received date for all NBS prior to re loading to Virtuo, attempt re load
- **If < 4 days** and Virtuo **unloads as positive immediately** then place in rack in Hot room/incubator and do Terminal sub at 5 days CBA & 5-10% CO^2 at 34-36°C, FHB anaerobically with MTZ5 disc 34- 36°C
- **If >4days do not reload bottle** ,do Terminal sub at 5 days onto CBA & 5-10% CO^2 at 34-36°C, FHB anaerobically with MTZ5 disc 34- 36°C, place bottle in rack in Hot room/incubator once subbed
- If Interim Report use Barcode label scripts LI669 and change status of bottle to positive
- Enter in LIMS Comment Code **BCFP** "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
- If report status is **Interim** save report by Next Save Quit - **NSQ**.
- If report is at **Final** – report Comment code **BCFP** "No Organisms Seen Bottle Reloaded". Enter into LIMS Next Save Quit -**NSQ Do not change status of bottle**.
- Examine culture plates the following day and if no growth then re-incubate for a further 24 hours *i.e.* 48 hours total incubation.
- If growth on plates process as in 10.4 of this SOP. Retrieve bottle from Virtuo to avoid automatic discard in case of further work required.
- If report status is interim update bottle status at 48hrs - If no growth at 48hrs return bottle status in LIMS to negative by removing Positive and replacing with N2D.
- If appropriate set report to Final and release by Next Release Quit – **NRQ**
- **These will list on the negative blood culture list for authorisation**

If curve **suggestive** of bacterial activity:

- If curve suggestive of bacterial activity i.e. exponential or S -Shape curve. Record appearance on request form and/or print graph.
- If no organism seen, seek second opinion of microscopy if possible. Centrifuge an aliquot of blood culture broth and repeat the gram film from the spun deposit.
- Further subculture bottle to Chocolate Agar for incubation in 5-10% CO^2 at 34-36˚C.
- **Inform medical microbiologist of findings.**
- Consider additional culture media (i.e. Campylobacter plate)
- Consider ZN/Auramine staining at Microbiology consultant request, with all laboratory results recorded on back of specimen request form.
- **If < 4 days incubation** attempt to reload bottle on to Virtuo .If analyser unloads as positive immediately then place in incubator for continued incubation before Terminal sub is done at Day 5.
- **If >4days do not reload bottle**. Perform terminal subculture at 5 days onto CBA 5-10% CO^2 at 34-36˚C, FHB anaerobically with MTZ5 disc 34- 36˚C. Place bottle in rack in Hot room/incubator once subbed
- Enter in LIMS Comment Code **BCFP** "No Organisms Seen Bottle Reloaded". This is a suppressed comment.
- If report status is **Interim** save report by Next Save Quit - **NSQ**.
- If report is at **Final** – report Comment code **BCFP** "No Organisms Seen Bottle Reloaded". Enter into LIMS Next Save Quit –**NSQ - do not change status of bottle**.
- Examine culture plates the following day and re-incubate for 24 hours if no growth i.e. 48 hours total incubation – refer to Table 2 and discuss with a medical microbiologist.
- If growth on plates process as in 10.4 of this SOP.
- At the request of medical microbiologist, extend incubation to 10 days in total, remove bottle to blood culture section at 5 days when incubation is complete. This avoids automatic discard in case further work is required e.g. a medical microbiologist may request an aliquot of sample be sent for PCR.

<u>If bacteria seen in Gram film</u>

- Set up direct sensitivities and any appropriate additional tests and media as per Table 2.
- Staphylococcus/Streptococcus direct sensitivities can be made direct from the bottle.
- For coliform- like organisms dilute 1-2 drops of positive broth in saline to perform direct sensitivity tests. See Table 2.
- Mixed cultures: set up an additional AZT plate for Gram films suggestive of coliform and Staph/strep. Set up appropriate direct sensitivities for each respective organism.

When organisms seen and no growth on solid media after 48hrs excluding Propionibacterium (Cutibacterium):

- Re-film bottle.
- Check growth curve.
- Depending on findings sub extra media or amend report .Re-incubate media for 48hrs
- Inform medical microbiologist
- If Gram positive bacilli (excluding typical Propionibacterium (Cutibacterium) were seen on film, but grow poorly on sub-culture, consider possibility of Mycobacteria species and possible need for additional Auramine / ZN staining. This must be highlighted to the medical microbiologist, and auramine/ZN stain performed upon Microbiology consultant request only.

Table 2

MORPHOLOGY	DIRECT SENSITIVITIES	ADDITIONAL DIRECT TESTS
No bacteria seen but growth curve suggestive of bacterial activity	Await results of solid media subculture	Chocolate Agar incubate in 5-10% CO^2 at 34-36°C Consider referral for Pneumococcal PCR or culture for Campylobacter species at 34-36°C microaerophilic, Helicobacter species 34-36°C microaerophilic, Cysteine-dependent organisms, *Abiotrophia* species. Consider Auramine/ZN staining (at Micro consultant request only).
No bacteria seen and growth curve not suggestive of bacterial activity	Await results of solid media subculture	Return bottle to system. Chocolate Agar incubate in 5-10% CO^2 at 34-36°C
GPC (? Strep)	GP1 and GP2 Set on MHB Agar CO2	
GPC (? Staph)	GP1 and GP2 Set on MH Agar O2	
GPB Propionibacterium-like (Cutibacterium)	No direct sensitivities	
GPB Diphtheroid like	GP2 Set on MHB CO2	
GPB (large, square-ended)	GP1 Set on MH Agar O2	Penicillin & Metronidazole discs on blood agar ANO2 if Clostridium suspected
GNB "coliform like"	GN1 and GN2 on MH Agar O2. Dilute 1-2 drops of positive broth in saline to perform sensitivity tests.	CLED agar
GNB? *Haemophilus*	EHI ,EH2 and EMCAT CO2 on MHB GN1 and GN2 CO2 on MHB	Chocolate agar 5% CO2
GNC/GNCB	Ceftriaxone, Penicillin	Chocolate agar 5% CO2
YEAST	N/A	SAB, Candida Chromogenic Agar
Mixed culture	Respective susceptibilities	Blood agar with Aztreonam if Gram positive and Gram negative organisms seen

No sensitivities are required on post mortem samples unless specifically requested by a medical microbiologist.

Streptococcal grouping from blood cultures may be performed at the request of a consultant microbiologist only. Grouping results must be recorded on back of request form only.

Streptococcal grouping latex may be requested on positive blood culture specimens which have streptococci identified on Gram stain. In this incidence, the strep grouping can be performed directly from blood culture. To perform test:

- Add 1 drop of Extraction Reagent 1 to test tube
- Add 1-2 drops of positive blood culture to tube
- Add 1 drop of Extraction Reagent 2 to tube
- Add 5 drops of Extraction Reagent 3 to tube and mix gently
- Dispense one drop of each group latex reagent onto separate circles on test card and, using Pasteur pipette, place one drop of extract beside latex reagents and mix

N.B. Confirmation streptococcal grouping must also be performed following 18-24 hour culture.

Bench process for 6 hour ID /AST & ongoing verification

To improve the blood culture pathway, cultures which flag positive and are processed in the **first batch of the morning daily, undergo a 6 hour ID and are set-up for a 6 hour Vitek AST**. Specimens flagging later in the day are NOT to be included at present in this protocol.

- Follow 10.4 Positive Bottles flagged by BacT/ALERT)

It is important that plates inoculated from the day's first batch of blood culture positives are incubated as soon as possible to enhance growth and allow 6 hour incubation to begin.

- Use 6 hour MS ID Record sheet LF599 to record Laboratory number, aerobic , anaerobic or Paediatric bottle and Gram result -GRI only
- Do not ID Yeasts or? Diphtheroid
- Do not ID Mixed Gram results
- **After 6 hours**, plates are removed from the CO_2 incubator to examine for any growth. (Unlikely to have growth on FHB if an anaerobe so leave these undisturbed).
- If sufficient growth is present **perform MS ID on subculture** from **EACH** bottle which has been **determined positive.**
- The MS ID should be checked to ensure it correlates with the Gram film appearance.

- Record MS ID result and AST set up if required on 6hr MSID Record sheet LF599 for GRI or on reverse of request form at QEUH
- This is transferred to LIMS and then reported as Interim. If a pair of bottles (aerobic and anaerobic) both yield the same organism ID then delete the second result, transfer one result and Interim the report with that organism isolated from both bottles.
- If organisms differ between bottles then issue result in LIMS as belonging to the aerobic or anaerobic bottle, providing Gram and MSID correlate.
- Paediatric bottles organism ID - transfer result to LIMS and issue report as Interim.

If the same MS ID is obtained for both bottles in a set, then all further AST work should only be done on one of the bottles.

- **N.B Inform** medical microbiologist **of any Salmonella species at 6 hrs but do not report in LIMS until serology is complete. At this stage prepare nutrient agar slope and nutrient agar plate from 6hr plate.**
- If **MSID does not match Gram**, Re film media plate, check original Gram and if required repeat film from bottle. Inform TM/medical microbiologist of any anomalies and update LIMS if required. Do not release any dubious ID.

All GNB and? Streptococci/Enterococci should have an appropriate AST card set up, if sufficient growth at 6 hours.

N.B. *Staphylococci*, Gram-positive bacilli and other less frequently encountered and more fastidious organisms do not require 6 hour AST.

- ***E.coli*** The **check digit letter should be added** to these on **Vitek** and results transferred to LIMS and reported with no need to repeat.
- All **other GNB** and **Streptococci/Enterococci** 6 hour Vitek AST tests should be set up with the **Lab number MINUS the check digit** (i.e. 20.1411234). This is to ensure the results do not accidentally transfer to LIMS and get reported.

The following day, after ensuring purity of cultures

- ***E.coli*** AST results may be accepted and reported.
- **ESBL can be confirmed by direct susceptibility if Vitek AST correlate**
- **For all other isolates, the 6 hour AST results MUST NOT be reported. They should be printed and retained for the ongoing validation. These do NOT need to be given to a medical microbiologist.**
- **AST should be repeated from the 18-24 hour culture plate and reported as per current process.**

Note: Results must be accessible at all stages for medical microbiologists as soon as they become available.

MS Failure- MS ID can guide choice of appropriate AST card or process to follow

- **GNBs** - If **MS fails** then oxidase (GNBs)-Oxidase Negative set up AST N381, Oxidase Positive set up AST N383
- **GPC? Streptococci**-If **MS fails**-Examine preliminary growth around penicillin disc. If zone to Penicillin disc visible set up AST ST03 , if No zone to Penicillin set up AST P662
- **GPC? Staph**- If **MSID fails** then re incubate plates for 18-24hrs
- **Other organisms e.g. GNC/GNCB If MSID fails then re incubate plates for 18-24hrs**

Microbiology GG&C	LP509v7
	Authorised by: Margaret De Smith
Issued: 13/03/2023	Author: Karen Cullen

BLOOD CULTURE REPORTING FLOW CHART

Receipt and P.I.D.
Load Bottles
Interim Report Issued
"Received and being processed"
AUTOAUTHORISED

Positive <48 Hours
Enter Gram Result into LIMS indicating organism and which bottle it is from
Release Interim Report. BMS Authorisation, from site- specific BCP1 list
Relay results as Blood Culture Communication Protocol. **Perform and release 6hr ID/ and set-up 6hr AST* if positive determined is within morning batch of positive blood cultures**

Negative at 48 Hours
No Growth 2 Days Final Report Issued
Auto authorised
Incubation of Bottles Continued

Positive >48 Hours
Enter Gram results in LIMS indicating organism and which bottle it is from.
Change Report Status to Additional and Release for BMS Authorisation from site specific BCP1 list
Relay results as Blood Culture Communication Protocol. **Perform and release 6hr ID/set-up 6hr AST* if positive determined is within morning batch of positive blood cultures**

Read Culture Plates
Record Direct AST Results on form but report out all E. coli AST set up at 6hrs
Identify and perform AST on all other Clinically Significant Isolates. (Non E. coli 6 hour AST results are not released but are kept as part of ongoing validation)
Enter Final Organism Identification Results into LIMS and Release Interim Report for Clinical Authorisation

Report Final Result

Vitek 2 and Vitek MS ID Results will over-write Gram film results when released on MYLA.

Manually Merge other Identification Results

Enter controlled non-automated AST/MICE/E test results if appropriate.

Release Report for Clinical Authorisation.

* ***Staphylococci*, gram-positive bacilli and other less frequently encountered and more fastidious organisms do not require 6 hour AST.**

Blood Culture Communication Procedure

DAY 1

- When there are large numbers these should be batched appropriately.
- BMS to inform medical microbiologist / Clinical Scientist when Gram stains have been reported and are ready for phoning (by 10am if possible).
- Inform medical microbiologist of all RHC paediatric false positive findings
- Perform 6 hour IDs on morning batch of positives and inform medical microbiologist of results regardless of whether isolate identifies or not - issue 6 hour ID result as interim report.

DAY 2 +

- BMS to inform Medical Microbiologist / Clinical Scientist when identification and direct sensitivities available.
- All ongoing request forms should be shown to medical microbiologist daily, irrespective of the stage of reporting
- GRI- Final reports should be placed in appropriate clip at bench
  QEUH- Final reports must be taken to medical microbiologist

Out of Hours

After 5pm on weekdays

- QEUH: BMS to batch positive BCs and call first on-call Medical Microbiologist at 7pm
- GRI: BMS to batch positive BCs and call first on-call Medical Microbiologist at 8pm
- RHC positive BCs: phone Microbiology consultant on call at 7pm

Record all the above information on request form and Patient Note pad in LIMS as per MP509

Weekends

- All BCs will be phoned by first on call Medical Microbiologist; all RHC positives to Microbiology consultant on call
- After Medical Microbiologist has left lab / off site, BMS to batch positive BCs and phone as above at 4:30pm and 7pm QEUH and 8pm GRI

Record all the above information on request form and Patient Note pad in LIMS as per MP509

10.5 Positive Blood Culture Procedural Table

Primary plate cultural findings	Primary ID Method	Secondary ID Method*	AST Method	Isolate Storage**	Send to Ref.Lab***
GNB/Coliform	Vitek MS	Vitek2 or API	Vitek2	-80C, indefinitely	If appropriate.
Salmonella typhi/paratyphi (*Salmonella typhi* – serology 'O' antigen 9, vi and 'H' antigen d Salmonella paratyphi A – serology 'O antigen 2, 'H' antigen a Salmonella paratyphi B – serology 'O antigen 4, 'H' antigen b Salmonella paratyphi C – serology 'O antigen 6:7, Vi and 'H' antigen c)	Vitek MS	Serology	MICE/E test Ciprofloxacin, Azithromycin, Amoxicillin and Ceftriaxone (E test/MICE results to be entered and suppressed as comment codes and Ciprofloxacin S/R to be entered according to MIC) Standardised Discs GN1 and 2 (rule out ESBL and for AST)	Retain for 7 days after receipt of report from reference laboratory QEUH/GRI - retain in CL3 and record on Schedule 5 sheet	Send to reference lab on nutrient agar slope for Confirmation and typing
Salmonella species (non typhi/non paratyphi)	Vitek MS	Serology	MICE/E test Ciprofloxacin and Azithromycin (both E-test results to be entered as comment code and suppressed, Ciprofloxacin S/R to be entered according to MIC) Plus Vitek2 AST as per GNB / Coliform	-80C, indefinitely	Send to reference lab on nutrient agar slope for Confirmation and typing
Haemophilus	Vitek MS	X + V discs	Disc Diffusion	-80C, indefinitely	Typing required
Anaerobe	Vitek MS	ANC/API Referral to Anaerobic reference lab, Cardiff, if directed by medical microbiologist	E test/MICE - see LI590	-80C, indefinitely	
Staph aureus	Chromogenic Staph agar Staph Latex Vitek MS	Vitek 2	Vitek2 P634	-80C, indefinitely	EARSS required

Coagulase Negative Staphylococcus (pure growth)	Chromogenic staph agar Staph Latex Vitek MS	VITEK 2	VITEK 2 P634 As directed by a medical microbiologist. Teicoplanin MIC on Staph. Epidermidis and Staph Haemolyticus at GRI	-80C, indefinitely	
Mixed strains Coagulase Negative staphylococci	No further work unless directed by Microbiologist	N/A	N/A	Room Temp 1 week if no further work up, indefinitely if further ID and workup required	
Micrococcus species	Colonial morphology and pigment, strict aerobe	Vitek MS	Not required	Room Temp 1 week	
Pneumococci	Vitek MS Colonial Morphology	Vitek 2	Vitek 2 ST03	-80C, indefinitely	EARRS required
α-Haemolytic Streps	Optochin Disk Colonial Morphology Vitek MS	Vitek 2 or API	Vitek 2 ST03	-80C, indefinitely	
ß-Haemolytic Streps[1]	Strep Group Latex Test	Vitek MS or API	Vitek 2 ST03 Group C and G require manual D test	-80C, indefinitely	Group A typing required.
Enterococci species	Strep Group Latex Test on BHS only and Vitek MS	Vitek 2 ID	Vitek 2 P662 Daptomycin MICE for VRE	-80C, indefinitely	
Propionibacterium (Cutibacterium) spp.	Colonial Morphology Vitek MS	N/A	MICE / E test as directed by Microbiologist.	Room Temp 1 week	
Clinically Significant Diphtheroid, Listeria, Gram Positive Bacillus.	Colonial Morphology Vitek MS	N/A	Disc Diffusion/ MICE/E test as directed by Microbiologist.	-80C, indefinitely	
Non-Significant Diphtheroid	Colonial Morphology	N/A	N/A	Room Temp 1 week	
Clinically Significant Bacillus species	Colonial Morphology Vitek MS	Refer to Colindale reference laboratory on Medical Micro request	MICE/E test (Ciprofloxacin and Vancomycin)	-80C, indefinitely	If appropriate & directed by Microbiologist
Non-Significant Bacillus species	Colonial Morphology	N/A	N/A	Room Temp 1 week	

Neisseria +Moraxella	Vitek MS	API	Disc Diffusion/E-Test/MICE as directed by Micro medical staff	-80C, indefinitely	*Neisseria meningitidis* typing required
Yeast	Colonial Morphology Candida Chromogenic Agar Vitek MS	Refer to appropriate Mycology Reference Laboratory, as directed by medical microbiologist	Micronaut	-80C, indefinitely	If appropriate and directed by medical microbiologist

Notes:

- [1]*Streptococcus pyogenes* – a small number of Strep group A when typed by reference laboratory will be emm type (STG62647) *Strep dysgalactiae subsp. Equisimilis* isolates possessing Lancefield group A.
  Report such strains as *Strep dysgalactiae* – issue an amended report and notify Microbiology consult.

10.6 Positive Blood Culture Day 2 Work Flow

Medical microbiologist/Clinical Scientist to review plates on BC bench as necessary and to liaise closely with BMS regarding additional work for each sample.

Availability of results for patient management is the priority, to facilitate this, the following prioritisation is suggested.

1. When there are numerous positives, prioritise and batch request forms for the medical microbiologist. In particular, those which the medical microbiologist has not seen any direct sensitivities, culture results for, e.g. Day 1 Blood cultures.

2. Direct sensitivity results and preliminary identification results recorded on request form for the medical microbiologist.

3. MALDI-TOF identification initiated ASAP.

4. MALDI-TOF results transferred from MYLA and interim report issued (results authorised by a medical microbiologist).

   If MALDI-TOF fails to identify organism, inform medical Microbiologist so they are aware of delay in reporting – further discussion required e.g. repeat MALDI-TOF, use secondary method of ID as per Table 10.5*

   If any problems with Myla or Vitek MS inform medical microbiologist

5. Vitek sensitivity results reviewed from previous day transferred and reported in LIMS as Final/Additional. The results are then authorised by the medical microbiologist from the appropriate list.

6. Further Vitek sensitivities, MICE/E Test, diffusion sensitivity tests set up, isolate referral and storage can then be completed.

7. Isolate referral – complete LF515.

8. Work outstanding/in progress Daily Check- Complete LF524 -- this must be completed daily and any anomalies/missing numbers investigated.

9. Check NBL and PBC.1 list daily

Positive Blood Culture Procedural Table serves as a guide for the majority of isolates. More detailed analyses of isolates may be requested depending on patient's clinical history.

- **Secondary ID Method***
  Perform only when primary ID method not available or inconsistent with other findings.
- **Send to Ref. Lab*****
  When unable to ID, epidemiology studies or further AST required.

- **Isolate Storage****
  All isolates which receive identification and sensitivity testing should be stored at -80C using cryovials, as per above table (10.5 of this document).
  The exception to this are organisms covered by the
  ANTI-TERRORISM, CRIME AND SECURITY ACT 2001, SCHEDULE 5
  See Q-Pulse for guidance – ED552.
  Refer to MP512 – control of clinical material.

- **Discard of blood positive blood culture bottles**

  Positive blood culture bottles should be discarded one week after they become positive those which are still in progress, retain until reporting complete.

  Prior to discard each bottle should be checked for report status to ensure there are no in-progress reports which may not be detected from LF524 – Work outstanding/In progress Daily Check.
  Discard blood culture bottles as per H&SP504.

10.7 Catastrophic failure of BacT/ALERT® VIRTUO
In the event of complete analyser failure, direction should be taken from Biomerieux and the Service Manager/Operational Managers. A discussion should take place regarding transfer of new specimens to the other GGC Microbiology site should capacity permit this.

11. Quality Control Procedures

- BacT/ALERT® VIRTUO performs automatic internal diagnostic check on all cells. Cells which fail the automatic internal diagnostic check are then taken out of use by the analyser.
- Temperatures are recorded hourly for each instrument and data can be seen on instrument reports.
- Annual service performed by Biomerieux.
- Participation in EQA schemes – UK NEQAS (various microbiology schemes covering blood culture isolates – identification and susceptibility testing), Lab Quality Blood Culture isolate detection and identification scheme
- GG&C Microbiology IQA/UoM Schedule including blood culture Gram film consistency checks

12. Interferences

- Pre-incubation of inoculated bottles at 35-37°C may be detrimental to the isolation of certain non-fermenting Gram negative bacteria such as *Pseudomonas aeruginosa*, *Streptococcus* species and yeasts in continuous monitoring blood culture systems
- Organisms such as *S.pneumoniae*, may autolyse during incubation therefore, bottles should be subcultured as soon as possible after a positive is indicated
- Refrigeration of Blood cultures in error may be detrimental to isolation of bacteria

13. Principle of procedure for calculating results.

- Previously covered within body of SOP
- Refer to Uncertainty of Measurement SOP QP512

14. Biological reference intervals.

- Not applicable.

15. Reportable interval of examination results.

- Not applicable.

16. Instruction for determining quantitative results when a result is not within the measurement interval.

- Not applicable.

17. Alert/critical values.

- Bottle determined positive and organisms seen in Gram film.

18. Laboratory clinical interpretation.

- Laboratory Clinical Staff informed of positive bottles and organisms seen in Gram film.
- Laboratory Clinical Staff discuss laboratory findings with requesting clinicians.
- Final positive reports authorised by a medical microbiologist

19. Potential sources of variation.

- False positives may be encountered due to the biological nature of the samples *i.e.* high white blood cell count or turbid body fluid.
- Delayed loading of blood culture bottles into the Virtuo machine after collection

- False positives may also occur if the bottles are filled with more than 10 ml of blood.
- The positive status of a sample is always confirmed by one or more of the following, microscopy (Gram stain), isolation of organisms on solid media or the detection of bacterial antigen in the sample.
- 6hr MS ID – ensure ID correlates with Gram finding

  If **MSID does not match Gram**, Re film media plate, check original Gram and if required repeat film from bottle. Inform TM/medical microbiologist of any anomalies and update LIMS if required. Do not release any dubious ID.

- 6hr MSID AST- weight of growth at 6hrs on media

20. References.

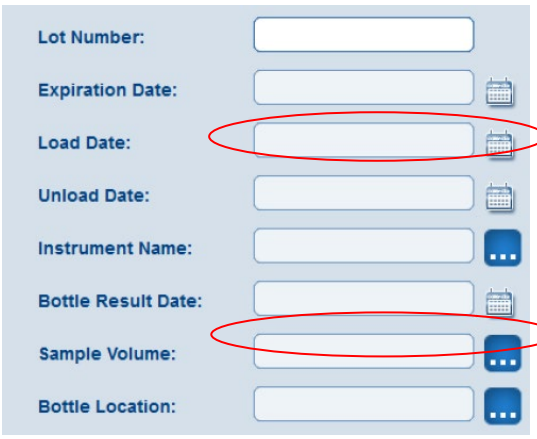
- BacT/ALERT® VIRTUO user manual held on Q-Pulse (ED652) & internal PC on BACT/ALERT VIRTUO.
- UK Health Security Agency (2023) - UK Standards for Microbiology Investigations. S12. Sepsis and systemic or disseminated infections

Appendix 1: Bottle Alerts - Under-filled /Expired blood culture bottles

The BacT/Alert® Virtuo analyser will detect and alert lab staff to those bottles out with fill level parameters or bottles which have reached expiry and should have an Interim report issued

The HCSW on blood cultures can retrieve forms in **one of two ways** for **Under-filled bottles**:

1. Let sample volume alerts build up on Virtuo and then use these to retrieve a batch of forms at a time.
2. Dismiss sample volume alerts and retrieve forms by running Searches for under-filled bottles throughout the day and printing lists of forms to be pulled from filing



Select appropriate range for load date and time

Select under-filled bottles

Searches should be run at the following times:

- 9am – search from 16:00 the previous day onwards
- 12pm – search from 09:00 that day onwards
- 4pm – search from 12:00 that day onwards

NB: specimens which give a volume of "0ml" should be ignored and forms not retrieved for these; they may be flagging due to incorrect label placement and not inadequate volume.

3. Specimen request forms should be given to the BMS on blood cultures or left in appropriate place on BC bench

The BMS should use the barcodes provided on the housekeeping sheet (LI684) to issue interim reports issued with comment code as below:

- **BCFILL**

*****Inadequately filled bottle(s) *****
Interpret negative results with caution.

The comment should only be added to blood culture specimens.
Pleural, Ascitic, synovial and other fluid types should NOT have the BCFILL comment added.

Expired bottles - HCSW can retrieve forms by :

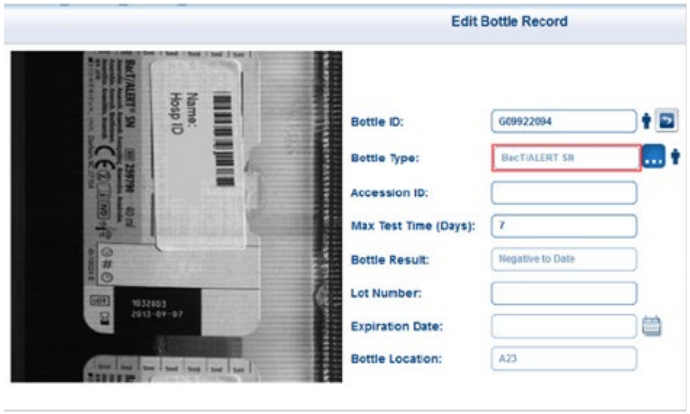
- Select Continue with test on Virtuo screen, retrieve appropriate forms and Issue an Interim report with comment code as below:
  - **BCEXP**

***** Blood culture bottles expired, please check stocks*****
Interpret negative results with caution.

Appendix 2: Anonymous bottles

Follow these steps to identify an anonymous bottle i.e. when the Virtuo alerts problems reading the barcode/where the bioMérieux bottle ID number is not visible from the bottle image. To follow this procedure, use a scannable barcode provided by bioMérieux.

1. Select the Anonymous bottle alert
2. Enter your BioMérieux username and password if not already logged in
3. Select **OK**
4. If you are unable to determine the bottle details from the image, obtain and a generic bottle ID barcode.
5. Enter the bottle ID from the code into the bottle ID field



6. Select **Save**

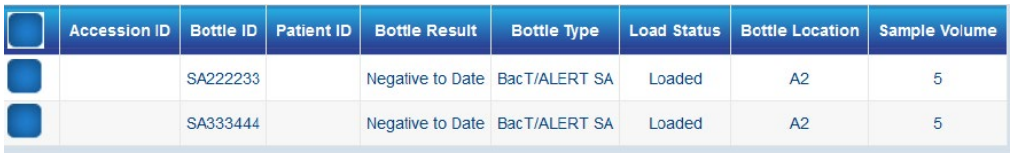
7. Select **Unload** to unload the bottle
8. Reload the bottle and use the form to update the accession number and bottle type. Save and close.

Appendix 3: Extending Bottle Incubation Period on BacT/Alert Virtuo:

In order to extend the incubation period for blood culture bottles:

- From the Home Screen, click on the Search icon:
- In the 'Accession ID' field, scan the lab number for the specimen:

- Click the Search icon:
- From the results screen, click the checkbox on the left of the panel to select the required bottle(s). You can select all by clicking the top left checkbox.



	Accession ID	Bottle ID	Patient ID	Bottle Result	Bottle Type	Load Status	Bottle Location	Sample Volume
		SA222233		Negative to Date	BacT/ALERT SA	Loaded	A2	5
		SA333444		Negative to Date	BacT/ALERT SA	Loaded	A2	5

- Click the View icon to display specimen details:
- Click on the Edit Icon to edit the bottle record:

  N.B You may be asked to enter your BioMérieux username and password at this stage.

- Enter the new total incubation period in the 'Max Test Time' field:

  N.B The Max Test Time must be extended for each individual bottle

- Select Save:
- Close by clicking the blue cross at the top right of the bottle record:
- Return to the Home Screen:

Appendix 4: Stem Cell PID

PID of Blood Culture Bottles from Stem Cell Lab, GGH

Stem cell samples often arrive in sets of three. Please ensure all requests are entered into LIMS correctly, and LIMS entry matches corresponding request form. These samples should be entered into LIMS as follows, to ensure all required information is included within the final report issued to the user:

Patient details:

- Samples will be received with small blue Microbiology forms as they cannot be requested through Trak care and so must be PID out with Trak care.
- Enter patient details in the required fields (non-donor samples – patient details given)
- Where **donor** samples received, enter specimen details in LIMS request entry as follows:
    - CHI = U (unknown)
    - Surname = Donor
    - Forename = unique number as specified (enter 19 digit donor number with no spaces, e.g.1234DKMO12345678910)
    - DOB = 12.12.12
    - These will require allocation of ZM number as no CHI is given
    - Consultant field should be entered using free text with the requestor name (e.g. E Farrell)
    - Location code is GSTCL or can be found with @STEM search

Specimen details:

- Enter lab number, date and time collected and received
- Specimen type should be entered as BC
- Site field: Free text specimen details as specified on the request form, e.g. HPC-A 1 PRE
- Investigation entered as BC
- BC Bottle barcodes should be entered in the appropriate fields

Please note, failing to PID these samples correctly results in the user telephoning the lab to request an amended report.

Objective

To ensure that Infection Prevention and Control Teams in Greater Glasgow & Clyde (GGC) have processes in place to initiate the NHS GG&C Boards Outbreak and Incident Management Plan & Chapter 3 of the National Infection Prevention and Control Policy

[redacted]

This framework applies to all staff employed by NHS Greater Glasgow & Clyde and locum staff on fixed term contracts.

KEY CHANGES FROM THE PREVIOUS VERSION OF THIS DOC

- Update to appendices.
- Scottish Government Standards on Healthcare Associated Infection Indicators (SGHAII) replace LDP.

Document Control Summary

Approved by and date	Board Infection Control Committee 21st February 2024
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Developed by	Infection Prevention and Control Team
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Distribution/ Availability	NHSGGC Infection Prevention and Control web page [redacted]
Lead Manager	Director Infection Prevention and Control
Responsible Director	Executive Lead for Healthcare Associated Infections

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1. Introduction

This IPCT Incident Management Process Framework supports the implementation of the GG&C Outbreak and Incident Management Plan and Chapter 3 of the National Infection Prevention and Control Manual in the context of the Infection Prevention and Control service within NHSGGC.

The purpose of this document is to provide those responsible for responding to incidents and outbreaks within the IPCT, a framework to ensure compliance with both local and national policies and that ward to board governance systems are informed.

This framework should be read in conjunction with other local and national guidance. Training on incident management is available via Public Health for everyone involved in the incident management process.

2. IPCT IMT Process

2.1. Initial Assessment/Problem Assessment Group

An initial assessment is required to determine if an outbreak or incident is taking place. In a hospital, this will be carried out by the IPCT, or through a Problem Assessment Group (PAG).

The initial assessment will be based on available information. It may not be possible to make a decision on the information available immediately and further investigations may be required. A PAG may not always be required, and it is not necessary to hold a PAG prior to activating an Incident Management Team (IMT) meeting.

If an assessment is required or a PAG is held the IPCT will complete a NHS GGC IPC Incident summary (Appendix 1)/or if no further action is required a situation summary will be completed as a record of discussions held. There are normally two potential outcomes to a PAG:

- No significant risk to public health and/or patients; the PAG stood down, but surveillance continues or

- There are some concerns and the situation is assessed using the National Healthcare Infection Incident Assessment Tool (HIIAT) [redacted] all assessments regardless of outcome must be recorded on the Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) Outbreak Reporting Tool (ORT).

2.2. Incident Management Team (IMT)

The IMT is an independent multi-disciplinary group with the responsibility for investigating and managing the incident. Incidents must all be managed as per:

• Chapter 3 of the National Infection Prevention & Control Manual (NIPCM); and NHSGGC Outbreak and Incident Management Plan [redacted]

The documents from all outbreaks and incidents will be held in the NHSGGC IPCT pan Glasgow shared drive. Key documents in bold which must be held in each folder are:

- NHS GGC IPC Incident summary (Appendix 1) or Hot Debrief (Appendix 8);
- GGC IPCT IMT Decision & Improvement Log or minutes (Appendix 2)
- ARHAI SBAR
- Any results from environmental samples if done
- Any typing results if done
- Timeline if done
- Press releases
- Any other relevant reports/items

2.3. IMT Process and Template Agenda's

The IPCT process has a number of agenda templates. These are included in (Appendix 3)

These should ensure that all of the core actions required of an IMT, as detailed in Chapter 3 of the NIPCM and NHSGGC Outbreak and Incident Management Plan, are adhered to.

2.4. Action Log/Minute

Decisions must be clearly documented. The record must include not only the decision made, but if the alternative options have been considered and the rationale for the choice(s) made.

All actions must be concluded at the end of the final IMT or if this is not possible there is a clearly documented account of the actions that are to be included in another process or action plan. If minutes are taken then a separate action log must also accompany minute.

2.5. Environmental Sampling

Environmental sampling should only be undertaken at the instruction of the Infection Control Doctor (ICD). ICDs instructing sampling must ensure that the correct documentation is completed:

- Ad Hoc/Additional Environmental Request Swabs etc Non Water (Appendix 4)
- Ad Hoc/Additional Environmental Request Air & Water (Appendix 5)

And whomever is collecting the sample must ensure that full details are included on the request form, i.e. Room number (both ward ID and estates if applicable);

- Location within the room, e.g. bedroom, en-suite etc;
- Item, e.g. sink, bed table etc and Other relevant details.

2.6. Communicating with Patients, Carers and Families

The primary aim of the documents listed below which were developed by GGC Communications Team, is to set out the key principles which should be adhered to when communicating with patients with infections and their relatives and carers, other cohorts of patients and families, ward staff, NHSGGC staff, and the public during incidents and outbreaks and should guide IMT participants at all times in the decision process with regards to what, whom and when.

- Healthcare Associated Infection Communications Strategy [redacted]

- Communications during an incident or outbreak: Guidance for Problem Assessment Groups and Incident Management Teams [redacted]

A representative from Corporate Communications must be invited to all IMTs.

2.7. Risk Register

At the end of each incident the IMT will discuss if there is the requirement to consider if any actions or risks identified should be included on either the service or IPCT Risk Register.

This will also be considered in the hot debrief or IMT report which will be submitted to the relevant IPC Clinical Governance Committees (see section 3) and the relevant service clinical governance committees.

2.8. Duty of Candour

During an IMT, Clinical Teams may be referred to the NHSGGC Duty of Candour guidance.

Duty of Candour will be considered at the beginning and throughout the incident (Appendix 6) and members of the IMT will be required to follow the Health Boards Duty of Candour Policy:

[redacted]

3. Reporting and Governance

National Reporting

All incidents and outbreaks that are HIIAT assessed are reported to ARHAI via the ARHAI Online Reporting Tool (ORT).

GGC Senior Management Team

A weekly report which contains a brief description of any incidents or outbreaks which are assessed as amber/red is completed and submitted to Board Executive Directors, Service Directors and Heads of Nursing and Medicine. An update on any incidents or outbreaks previously reported is also included until the incident is closed.

Infection Prevention and Control Governance Committees

All Incidents that score red/amber using the ARHAI HIIAT assessment will be reported to the relevant IPC governance groups, i.e. AICC or PICSG. All red/amber incidents will have a hot debrief/incident summary/IMT report completed at the closure of the incident.

A summary of **all** incidents that score amber or red will be presented at all IPC governance groups via the HAIRT

Acute Services

A summary of any incidents or outbreaks which are assessed as HIIAT amber/red will be included in the IPC report which is presented monthly to the Acute Clinical Governance Committee (ACGC).

NHS GGC Clinical & Care Governance Committee

The Healthcare Associated Infection Reporting Template (HAIRT) will include a summary of all incidents including actions taken and lessons learned in order to support immediate or ongoing improvement. The HAIRT will be submitted to all IPC governance groups for information and review and to the Clinical Care Governance Group for Assurance. A summary HAIRT will be submitted to the NHS Board for assurance.

HAIRT – The HAIRT Report is the national mandatory reporting tool and is presented bi-monthly to the NHS Board. This is a requirement by the Scottish Government HAI Task Force and informs NHSGGC of activity and performance against Healthcare Associated Infection Standards and performance measures.

Summary HAIRT – Summary HAIRT will include performance in relation to the Scottish Government Standards on Healthcare Associated Infection Indicators (SGHAII), mandatory surveillance, cleaning and estates audit results. A summary of all incidents and outbreaks that score red or amber will also be included.

Escalation

The IPCT will complete an IPC Incident summary (Appendix 1) for all amber/red incidents and outbreaks.

The IMT may, after consideration, choose to brief the executive team via the existing rapid alert system. IMT Governance and Assurance chart is included in Appendix 7.

Appendix 1

NHSGGC IPC Incident summary

Date reporting / Update no.	
Sector / Hospital	
Ward / departments	
Incident statement	
Patient cases	
Control measures	
Investigations	
Hypothesis	
HIIAT Score	
Patient Services Transmission Public Anxiety	
Communications / next steps	
Press statement	
Date	Incident update

Instruction for completion:

1. *Complete the above following all incident /PAG meetings that require HIIAT assessment.*
2. *Send to DIPC, LICD and ANDIPC (or deputy) for review*
3. *DIPC/ANDIPC will ask IPCT to complete ORT*
4. *IPC Data team will update ORT and sent to ARHAI*

Appendix 2

GGC IPCT IMT Decision Log & Improvement Plan

Meeting:		**Meeting called by:**	
		Date:	
Attendees:		**Apologies**	
Next Meeting:		**Venue:**	

DATE	AGENDA ITEM	WHAT: (Action)	WHEN: (Commit)	WHO: (Owner)	RESULT: (Conclusion)

CLOSED ACTIONS:

DATE	AGENDA ITEM	WHAT: (Action)	WHEN: (Commit)	WHO: (Owner)	RESULT: (Conclusion)

Appendix 3

Template Agenda
(Hospital, ward/dept/organism if applicable)
Date Location

1. Welcome and Introduction (Reminder of confidentiality)
2. Apologies
3. Minutes from the previous meeting
4. Incident summary
    a) General situation statement
    b) Patient report
    c) Microbiology report
    d) Epidemiology
    e) Environmental report
    f) Other relevant reports
5. Case definition
6. Hypothesis
7. Risk Management/Control Measures
    a) General
    b) Patients
    c) Staff
    d) Public Health
8. Further Investigation
    a) Epidemiological
    b) Environmental
    c) Standard Infection Control Precautions Standards (SICPS) audit
    d) HH audit
    e) Route Cause Analysis (RCA)

9. Control measures

10. Healthcare Infection Incident Assessment Tool (HIIAT)

11. Communications
    a) Advice to patients and carers
    b) Advice to public
    c) Advice to Staff
    d) Media (print, radio, TV, websites, social networking sites)
    e) Executive management team/Clinical Governance
    f) Health Protection Scotland (HPS) / Scottish Government (SG) HAI Policy Unit (HIIORT)
    g) Duty of Candour Consideration

12. AOCB

13. Action list with timescale and allocated responsibility

14. Date and time of next meeting

Appendix 4

LF591v2 Ad Hoc/Additional Environmental Request (Swabs etc - Non Water)

For Completion by Infection Control Team				
Date of Issue		**Authorising Infection Control Consultant**		
Expected Sampling Date		**Frequency of Testing (e.g. One Off / Each Thurs)**		
Estimated Numbers		**Location(s) Hospital Site / Ward**		
Sample Type(s)				
Estimation Request & Clinical Rational for Testing	*Example: Specify target organism only / All isolates / GNB only as part of IMT investigation*			
Result Notification	List all staff emails to receive a copy of results as they become available			
ALL SAMPLES TO BE SUBMITTED WITH A COMPLETED ENVIRONMENTAL REQUEST FORM				
For Completion by Laboratory Staff (in conjunction with Infection Control Consultant)				
Booking in PID as per LI720	*Ensure full details of locations are included so all samples are PID'd using the same location for duration if incident*			
Reporting Criteria	*Example: Any isolate present to be reported /Only target organism to be reported*			
Set Up & Reporting Procedure	*Media to be set up*			
	Temperature and atmosphere		*Duration of Incubation & Read Frequency*	
	Reporting Criteria	*Example: NG2D/'Target Organism' Not Detected*		
All reported organisms to be stored in freezer				

Appendix 5

LF592v2 Ad Hoc/Additional Environmental Request (Air & Water)

For Completion by Infection Control Team			
Date of Issue		**Authorising Infection Control Consultant**	
Expected Sampling Date		**Frequency of Testing (e.g. One Off / Each Thurs)**	
Estimated Numbers		**Location(s) Hospital Site / Ward & Contact Details**	
Examination Request & Clinical Rational for Testing	**Air:** */ Air Sampling / Target Organism /Particle Count* **Water:** *Specify if target organism only / TVC / All GNB only as part of IMT investigation*		
Result Notification	*List all staff emails to receive a copy of results as they become available*		
ALL SAMPLES TO BE SUBMITTED WITH A COMPLETED ENVIRONMENTAL REQUEST FORM			
For Completion by Laboratory Staff (in conjunction with Infection Control Consultant)			
Booking in PID as per LI720	*Ensure full details of locations are included so all samples are PID'd using the same location for duration of incident*		
Reporting Criteria	*Example: Any isolate present to be reported /Only target organism to be reported*		
Set Up & Reporting Procedure	**Media to be set up / Additional Plates set up**		
	Reporting Criteria	*Example: Target organism Isolated/'Target Isolate' Not Detected etc*	
	All reported organisms to be stored in freezer		

Appendix 6

Duty of Candour Considerations for Infection Control Incidents

For an infection incident to be considered as a Duty of Candour Event, there are a number of points to consider.

Incident Definition

An 'incident' as defined in NHS GGC Incident Management Policy is any event or circumstance that led to unintended or unexpected harm.

Was the patient harmed?

For the purposes of the Duty of Candour legislation, harm can be:

- death of the person
- a permanent lessening of bodily, sensory, motor, physiologic or intellectual functions
- an increase in the person's treatment
- changes to the structure of the person's body
- the shortening of the life expectancy of the person
- an impairment of the sensory, motor or intellectual functions of the person which has lasted, or is likely to last, for a continuous period of at least 28 days
- the person experiencing pain or psychological harm which has been, or is likely to be, experienced by the person for a continuous period of at least 28 days
- the person requiring treatment by a registered health professional in order to prevent – (i) the death of the person, or (ii) any injury to the person which, if left untreated, would lead to one or more of the outcomes mentioned above

Was the event causing the harm avoidable?

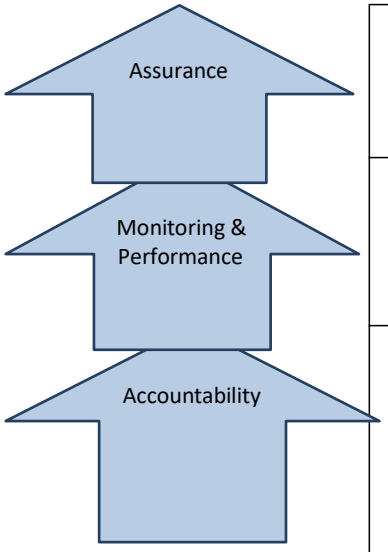
Harm may occur as a result of the natural progression of a disease or is an inherent risk of the treatment given. That harm may be deemed unavoidable, in which case Duty of Candour legislation would not apply. There does however need to be evidence that this was considered. If the incident review process is unable to determine that the harm was avoidable, a SAER should be commissioned to seek to answer this in line with the NHS GGC Management of Significant Adverse Events Policy.

	Was the Patient Harmed?	Yes	No
1	As a result of the infection, did the patient suffer harm (in line with the Organisational Duty of Candour Legislation definitions)? **Was the patient harmed?** For the purposes of the Duty of Candour legislation, harm can be: • death of the person • a permanent lessening of bodily, sensory, motor, physiologic or intellectual functions • an increase in the person's treatment • changes to the structure of the person's body • the shortening of the life expectancy of the person • an impairment of the sensory, motor or intellectual functions of the person which has lasted, or is likely to last, for a continuous period of at least 28 days • the person experiencing pain or psychological harm which has been, or is likely to be, experienced by the person for a continuous period of at least 28 days • the person requiring treatment by a registered health professional in order to prevent – (i) the death of the person, or (ii) any injury to the person which, if left untreated, would lead to one or more of the outcomes mentioned above		

If answered no, this does not meet the threshold for Duty of Candour.			
	On balance of probability was the unintended or unexpected infection incident avoidable?	Yes	No
2	Was there a systems issue e.g. guidance not followed, lack of PPE, poor practice identified?		
If answered no, this does not meet the threshold for Duty of Candour.			
The Clinical Service If you have answered yes to question 1 and 2, the infection incident meets the threshold for Organisational Duty of Candour, pass to service specialty to progress a review in line with the NHS GGC Management of Significant Adverse Event Policy. If the answer is no, a SAER would not normally be required, evidence that the avoidability has been considered should be retained for future evidence.			
SERVICE SPECIALTY (follow local SAER process)			
Ensure Datix has been completed			
Complete briefing note and commission of SAER			
Forward briefing note to clinical.risk[redacted]			

Appendix 7

Governance and Assurance IMT Process

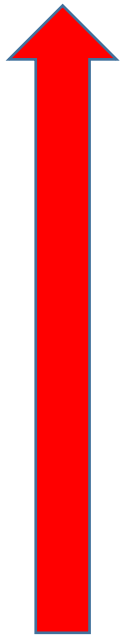


- Summary *HAIRT NHS GGC Board.
- HAIRT to Clinical Care Governance Committee.
- HAIRT Board Clinical Governance Committee.
- HAIRT/Hot Debriefs to Board Infection Control Committee.

- HAIRT/Hot Debriefs & IPC Risk Register to BICC, Acute Infection Control Committee & Partnership Infection Control Support Group.
- Weekly update to Board Executives, Service Directors, Chiefs of Medicine and Chiefs of Nursing re ongoing red/amber incidents.
- Director's dashboard to include details on incident and outbreaks.

- IMT Process and associated paperwork.
- Incident update in monthly sector reports to be tabled at service governance groups. Outstanding actions to be included in monthly report.

Rapid Alert will be completed by chair of IMT if considered that this is required by the IMT.



Appendix 8

Hot Debrief Tool

Hot debriefing document

This is not a mandatory requirement but for the purpose of sharing lessons learned across Scotland particularly for rare or unusual events. The IPCT/HPT or chair of the IMT should complete this immediately following the end of an incident. It may be deemed that a full IMT report is not needed and this document may be sufficient. A full IMT reporting template can be found in the [redacted]

1.Incident reference
Please provide a reference/title for this incident.
2. Details of incident
Please provide a brief summary of incident: Include details of the following where relevant: dates when incident started/ended; case definition; description, number and features of cases; care areas/locations affected; source and modes of cross-transmission/exposure; diagnosis and treatment, any enhanced surveillance of interventions, any hypotheses.
3. What went well?
Please list aspects of the incident considered to have been managed well:

4. What did not go well?	
Please list aspects of the incident considered not to have been managed well:	
5.Lessons Learned	
Please provide details of any learning points or recommendations:	
6. IMT lead details	
Name:	Email:
Job Title:	Address:
Contact number:	Contact number (mobile):
Date:	Signed:
Completed templates to be returned to: NSS.HPSInfectionContro[redacted]	

Glossary

AICC	Acute Infection Control Committee
ARHAI	Antimicrobial Resistance and Healthcare Associated Infection
BICC	Board Infection Control Committee
HIIAT	Healthcare Infection Incident Assessment Tool
ICBEG	Infection Control in the Built Environment Group
ICD	Infection Control Doctor
IMT	Incident Management Team
IPCT	Infection Prevention and Control Team
NIPCM	National Infection Prevention Control Manual
ORT	Online Reporting Tool
PAG	Problem Assessment Group
PICSG	Partnership Infection Control Support Group
SAER	Significant Adverse Event Report
SBAR	Situation Background Assessment Recommendation

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J Antimicrob Chemother 2020; **75**: 1654–1658
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Advance Access publication 12 March 2020

In vitro activity of 20 antibiotics against *Cupriavidus* clinical strains

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Sir,

Cupriavidus are Gram-negative non-lactose-fermenting motile bacilli with peritrichous flagella, a number of which were previously and successively classified in the *Ralstonia* and *Wautersia* genera. Until the recent expansion of MALDI-TOF MS, *Cupriavidus* could be mistaken for *Burkholderia* or *Pseudomonas* species. They are resistant to heavy metals and have been described from environmental (soil and water) samples, as well as from human samples.[1] *Cupriavidus gilardii*, *Cupriavidus pauculus* and *Cupriavidus metallidurans* are involved in invasive human infections, such as bacteraemia and pneumonia, most of which (though not exclusively) occur in immunocompromised patients.[2–4] Additionally, *Cupriavidus* species, *Cupriavidus respiraculi* in particular, are increasingly identified in patients

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with cystic fibrosis (CF).[5] However, their clinical relevance in CF is not established. Due to the rare occurrence of *Cupriavidus* infections, antibiotic susceptibility data are only available from sparse case reports. Therefore, we determined the MICs of 20 antibiotics for a panel of *Cupriavidus* clinical strains, mainly from respiratory samples of CF patients (82%).

Thirty-seven epidemiologically unrelated clinical isolates of *Cupriavidus* obtained from the collection of the French Observatoire *Burkholderia cepacia* and from 11 French hospitals, as well as two type strains from clinical sources, i.e. *C. pauculus* LMG 3244^T and *C. respiraculi* LMG 21510^T (Laboratory of Microbiology, Ghent University, Ghent, Belgium),[1] were included. Isolates were identified by amplified ribosomal DNA restriction analysis (ARDRA)[6] and MALDI-TOF MS (Maldi Biotyper Microflex®, Bruker Daltonics, Bremen, Germany; IVD 7712). The experimental panel thus comprised 18 *C. respiraculi*, 6 *C. gilardii*, 5 *C. pauculus*, 4 *C. metallidurans*, 2 *Cupriavidus necator*, 2 *Cupriavidus taiwanensis*, 1 *Cupriavidus basilensis* and 1 unidentified *Cupriavidus* sp.

The MICs of 20 antibiotics, listed in Table 1, were determined using the broth microdilution method, as recommended by EUCAST (www.eucast.org). Briefly, each strain was inoculated on a blood agar plate (bioMérieux, Marcy-l'Étoile, France) for 16 h at 35°C. Bacterial suspensions in Mueller–Hinton broth (Bio-Rad, Marnes-la-Coquette, France) at concentrations of 5×10^5 cfu/mL were dispensed in 96-well microtitre plates (Dutscher, Brumath, France, 160 μL per well). Antimicrobial agents were added at increasing 2-fold concentrations (40 μL per well). The MICs were determined as the lowest antibiotic concentrations that inhibited visible bacterial growth after an 18 ± 2 h incubation at 35°C in an aerobic atmosphere. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as controls. All MICs were determined in triplicate and replicates never differed by more than 2-fold. For each MIC determination, the median of the replicates was recorded as the MIC. The MICs were interpreted according to *Pseudomonas* EUCAST breakpoints (2019) for colistin, amikacin and tobramycin, according to non-Enterobacteriaceae CLSI breakpoints (2019; https://clsi.org) for minocycline and co-trimoxazole and according to pharmacokinetic/pharmacodynamic (non-species-related) EUCAST breakpoints (2019) for the other antibiotics, except for temocillin, for which the previously proposed breakpoint of 16 mg/L was used.[7] The EUCAST and CLSI breakpoints that could be used to interpret *Cupriavidus* MICs are listed in Table S1 (available as [redacted] at *JAC* Online).

The susceptibility testing results are summarized in Table 1 and the full MIC distributions of each tested antibiotic are available in Figure S1. Since *C. pauculus* and *C. metallidurans* are phylogenetically close species[1] and exhibited the same susceptibility profiles, they were considered as a group. Our collection comprises very few strains of *C. basilensis, C. necator, C. taiwanensis and Cupriavidus sp.* (maximum $n=2$ for each species). Moreover, these species have similar antibiotic susceptibility profiles, that's why they were also considered as a group. The susceptibility testing results interpreted with CLSI breakpoints are available in Table S2. The only significant change in our study between the EUCAST and CLSI interpretations concerned piperacillin/tazobactam. Eleven strains had an MIC of 8 or 16 mg/L (nine *C. respiraculi*). They were classified as susceptible to piperacillin/tazobactam according to CLSI breakpoints, but intermediate according to EUCAST breakpoints.

Nearly all strains were resistant to amoxicillin, amoxicillin/clavulanate, temocillin and aztreonam. Regarding cephalosporins, only 23% of *Cupriavidus* strains were ceftazidime susceptible, whereas 74% and 82% were susceptible to ceftriaxone or cefotaxime, respectively. The ceftolozane/tazobactam combination also demonstrated good activity, except against *C. gilardii.* For most strains, ceftazidime/avibactam MICs were similar compared with ceftazidime alone. Cefepime was the most active β-lactam, with 95% of strains being susceptible, whereas only a few strains were susceptible to meropenem (8%). Interspecies differences were observed for piperacillin/tazobactam and imipenem, since they were less active against *C. respiraculi* and *C. gilardii* than against the other species. Such discrepancies between meropenem and imipenem activities were previously noticed in case reports.[2,3] Similarly to *P. aeruginosa*, they could be due to the overexpression of efflux pumps from the resistance-nodulation-division family.[8] Indeed, a homologue of the MexAB OprM efflux pump that extrudes meropenem in *P. aeruginosa* has been identified in *C. gilardii*.[9]

Aminoglycosides were poorly active, in agreement with case reports,[2,3] probably due to efflux pumps and aminoglycoside-modifying enzymes.[9] Minocycline was the most active antibiotic, with very low MICs and a 100% susceptibility rate. Fluoroquinolones were frequently active, with over 80% of strains being susceptible, except for *C. pauculus and C. metallidurans.* Interspecies discrepancies were also noticed for co-trimoxazole. It was active against approximatively 80% of *C. gilardii* and *C. respiraculi* strains, whereas more than 50% of the strains belonging to other species were resistant.

Over 90% of *C. respiraculi* and 67% of *C. gilardii* strains were susceptible to colistin, while strains from the other species were mostly colistin-resistant. Colistin susceptibility was one of the characteristics of the *Cupriavidus* genus initially described by Vaneechoutte *et al.*[1] However, Petrou *et al.*[10] showed that the expression of ArnT was particularly strong in a strain of *C. metallidurans.* This enzyme catalyses the attachment of the cationic sugar 4-amino-4-deoxy L-arabinose (L-Ara4N) to lipid A phosphate groups. The subsequent reduction of negative membrane charge is responsible for colistin resistance. We detected homologues of *arnT* (CP000353.2: 1481129–1482725) using BLASTn in *C. basilensis*, *C. necator*, *C. pauculus* and *C. taiwanensis* sequenced strains (a query cover >80%, an identity >70% and an E value $<1 \times 10^{-40}$ were chosen as cut-off values for significance), which is in accordance with the high rate of colistin resistance in these species observed in our study. Additionally, *C. gilardii* appears to be the origin of the gene *mcr-5*, which is an emerging plasmid-mediated mechanism of colistin resistance in other environmental species such as *Salmonella* and *Pseudomonas.*[11]

In conclusion, our study showed that minocycline and cefepime exhibited the best *in vitro* activities against *Cupriavidus* strains. Meropenem, aminoglycosides and polymyxins, often considered antibiotics of last resort against infections caused by Gram-negative bacilli, do not have reliable activity against *Cupriavidus*. Perhaps resistance to these agents confers a selective advantage to *Cupriavidus* and therefore it may emerge in clinical scenarios where these agents are used, such as in patients with CF. Imipenem was more active than meropenem and cefotaxime/ceftriaxone was more active than ceftazidime. Ceftolozane/tazobactam had reasonable activity against *Cupriavidus*, whereas the novel inhibitor avibactam does not seem to add to the activity of

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Table 1. MICs of 20 antibiotics for 39 *Cupriavidus* clinical strains, including two type strains, determined by the broth microdilution method

Bacteria (*n*)	Antibiotic	MIC (mg/L)		Percentage susceptible (breakpoint, mg/L)	Percentage resistant (breakpoint, mg/L)
		MIC_{50}	MIC_{90}		
Cupriavidus spp., all isolates (39)	amikacin	64	512	23 (≤8)	72 (>16)
	amoxicillin	512	>512	5 (≤2)	90 (>8)
	amoxicillin/clavulanate	256	>512	8 (≤2)	87 (>8)
	aztreonam	32	256	0 (≤4)	97 (>8)
	cefepime	1	4	95 (≤4)	0 (>8)
	cefotaxime	1	2	82 (≤1)	8 (>2)
	ceftazidime	16	32	23 (≤4)	54 (>8)
	ceftazidime/avibactam	8	32	69 (≤8)	31 (>8)
	ceftolozane/tazobactam	2	8	90 (≤4)	10 (>4)
	ceftriaxone	1	4	74 (≤1)	10 (>2)
	ciprofloxacin	0.125	1	74 (≤0.25)	18 (>0.5)
	colistin	2	16	56 (≤2)	44 (>2)
	co-trimoxazole	1	128	62 (≤2)	38 (>2)
	imipenem	2	8	69 (≤2)	21 (>4)
	levofloxacin	0.25	2	79 (≤0.5)	18 (>1)
	meropenem	32	64	8 (≤2)	74 (>8)
	minocycline	≤0.06	0.5	100 (≤4)	0 (>8)
	piperacillin/tazobactam	8	128	46 (≤4)	26 (>16)
	temocillin	32	512	31 (≤16)	69 (>16)
	tobramycin	256	>256	21 (≤4)	79 (>4)
C. respiraculi (18)	amikacin	128	512	6 (≤8)	89 (>16)
	amoxicillin	512	>512	0 (≤2)	100 (>8)
	amoxicillin/clavulanate	512	>512	0 (≤2)	100 (>8)
	aztreonam	32	32	0 (≤4)	100 (>8)
	cefepime	2	4	89 (≤4)	0 (>8)
	cefotaxime	1	2	78 (≤1)	11 (>2)
	ceftazidime	16	16	6 (≤4)	61 (>8)
	ceftazidime/avibactam	8	16	72 (≤8)	28 (>8)
	ceftolozane/tazobactam	2	4	94 (≤4)	6 (>4)
	ceftriaxone	1	4	67 (≤1)	17 (>2)
	ciprofloxacin	0.06	>16	83 (≤0.25)	17 (>0.5)
	colistin	1	2	94 (≤2)	6 (>2)
	co-trimoxazole	0.5	128	78 (≤2)	22 (>2)
	imipenem	2	8	61 (≤2)	22 (>4)
	levofloxacin	0.125	16	83 (≤0.5)	17 (>1)
	meropenem	64	64	0 (≤2)	83 (>8)
	minocycline	≤0.06	0.125	100 (≤4)	0 (>8)
	piperacillin/tazobactam	8	16	39 (≤4)	11 (>16)
	temocillin	32	32	50 (≤16)	50 (>16)
	tobramycin	>256	>256	6 (≤4)	94 (>4)
C. pauculus (5) and *C. metallidurans* (4)	amikacin	8	128	56 (≤8)	33 (>16)
	amoxicillin	256	512	0 (≤2)	78 (>8)
	amoxicillin/clavulanate	128	256	11 (≤2)	78 (>8)
	aztreonam	256	512	0 (≤4)	100 (>8)
	cefepime	0.5	1	100 (≤4)	0 (>8)
	cefotaxime	1	2	67 (≤1)	11 (>2)
	ceftazidime	8	16	33 (≤4)	44 (>8)
	ceftazidime/avibactam	8	16	78 (≤8)	22 (>8)
	ceftolozane/tazobactam	2	4	100 (≤4)	0 (>4)
	ceftriaxone	1	2	78 (≤1)	0 (>2)
	ciprofloxacin	0.5	1	44 (≤0.25)	44 (>0.5)

Continued

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Table 1. *Continued*

Bacteria (*n*)	Antibiotic	MIC (mg/L)		Percentage susceptible (breakpoint, mg/L)	Percentage resistant (breakpoint, mg/L)
		MIC_{50}	MIC_{90}		
	colistin	16	32	0 (≤2)	100 (>2)
	co-trimoxazole	16	256	22 (≤2)	78 (>2)
	imipenem	0.25	2	100 (≤2)	0 (>4)
	levofloxacin	1	2	44 (≤0.5)	44 (>1)
	meropenem	16	64	11 (≤2)	67 (>8)
	minocycline	0.25	0.5	100 (≤4)	0 (>8)
	piperacillin/tazobactam	2	32	67 (≤4)	22 (>16)
	temocillin	256	512	0 (≤16)	100 (>16)
	tobramycin	64	128	44 (≤4)	56 (>4)
C. gilardii (6)	amikacin	64	128	0 (≤8)	100 (>16)
	amoxicillin	>512	>512	0 (≤2)	100 (>8)
	amoxicillin/clavulanate	>512	>512	0 (≤2)	100 (>8)
	aztreonam	128	128	0 (≤4)	100 (>8)
	cefepime	4	4	100 (≤4)	0 (>8)
	cefotaxime	1	1	100 (≤1)	0 (>2)
	ceftazidime	32	32	17 (≤4)	83 (>8)
	ceftazidime/avibactam	32	32	33 (≤8)	67 (>8)
	ceftolozane/tazobactam	8	16	50 (≤4)	50 (>4)
	ceftriaxone	1	2	67 (≤1)	17 (>2)
	ciprofloxacin	0.25	0.25	83 (≤0.25)	0 (>0.5)
	colistin	1	4	67 (≤2)	33 (>2)
	co-trimoxazole	1	1	83 (≤2)	17 (>2)
	imipenem	8	8	17 (≤2)	67 (>4)
	levofloxacin	0.25	0.25	100 (≤0.5)	0 (>1)
	meropenem	64	64	0 (≤2)	100 (>8)
	minocycline	0.125	0.125	100 (≤4)	0 (>8)
	piperacillin/tazobactam	128	128	0 (≤4)	83 (>16)
	temocillin	512	>512	0 (≤16)	100 (>16)
	tobramycin	256	>256	0 (≤4)	100 (>4)
C. basilensis (1), *C. necator* (2), *C. taiwanensis* (2) and *Cupriavidus* sp. (1)	amikacin	32	64	50 (≤8)	50 (>16)
	amoxicillin	32	256	33 (≤2)	67 (>8)
	amoxicillin/clavulanate	16	64	33 (≤2)	50 (>8)
	aztreonam	64	128	0 (≤4)	83 (>8)
	cefepime	≤0.25	≤0.25	100 (≤4)	0 (>8)
	cefotaxime	0.5	1	100 (≤1)	0 (>2)
	ceftazidime	4	8	67 (≤4)	17 (>8)
	ceftazidime/avibactam	4	8	83 (≤8)	17 (>8)
	ceftolozane/tazobactam	1	1	100 (≤4)	0 (>4)
	ceftriaxone	0.25	1	100 (≤1)	0 (>2)
	ciprofloxacin	0.125	0.25	83 (≤0.25)	0 (>0.5)
	colistin	16	16	17 (≤2)	83 (>2)
	co-trimoxazole	16	64	50 (≤2)	50 (>2)
	imipenem	0.25	2	100 (≤2)	0 (>4)
	levofloxacin	0.125	0.25	100 (≤0.5)	0 (>1)
	meropenem	8	16	33 (≤2)	33 (>8)
	minocycline	≤0.06	0.5	100 (≤4)	0 (>8)
	piperacillin/tazobactam	1	4	83 (≤4)	17 (>16)
	temocillin	128	512	50 (≤16)	50 (>16)
	tobramycin	64	64	50 (≤4)	50 (>4)

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ceftazidime. Interspecies variations were observed, especially concerning colistin, co-trimoxazole, fluoroquinolones and piperacillin/tazobactam. Clinical data is now required to establish the optimal treatment of *Cupriavidus* infections.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online.

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Pharmacokinetics of once-daily doravirine over 72 h following drug cessation

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Sir,

Successful combination ART (cART) relies on daily adherence to cART.[1,2] The 'optimal' adherence pattern may be difficult to adopt as cART is for life and doses can be forgotten or delayed, making antiretrovirals with long half-lives ($t_{1/2}$s) desirable. Such drugs may allow for missed or delayed doses when drug concentrations are maintained at therapeutic levels until the next dose is administered.

Data on drug persistence and terminal $t_{1/2}$ are available for different cARTs and have been useful to advise clinicians and patients on delayed or missed doses.[3] Herein, we investigated the pharmacokinetic (PK) 'forgiveness' of the new NNRTI doravirine. Doravirine was recently approved to treat HIV infection as a single entity (Pifeltro®) and as a fixed-dose combination with tenofovir disoproxil fumarate and lamivudine (Delstrigo®).[4] Since the PK forgiveness of tenofovir disoproxil fumarate and lamivudine has been extensively studied,[5,6] in the present study we characterized the persistence of doravirine in the absence of other agents.

Regulatory and ethical approvals (London Westminster Research Ethics Committee 19/LO/0666) were obtained before initiating the study. Written informed consent was obtained from participants prior to study enrolment. In this Phase I, open-label, PK study, the participants received 100 mg of doravirine once daily for 7 days to

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Global and local selection acting on the pathogen *Stenotrophomonas maltophilia* in the human lung

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Bacterial populations diversify during infection into distinct subpopulations that coexist within the human body. Yet, it is unknown to what extent subpopulations adapt to location-specific selective pressures as they migrate and evolve across space. Here we identify bacterial genes under local and global selection by testing for spatial co-occurrence of adaptive mutations. We sequence 552 genomes of the pathogen *Stenotrophomonas maltophilia* across 23 sites of the lungs from a patient with cystic fibrosis. We show that although genetically close isolates colocalize in space, distant lineages with distinct phenotypes separated by adaptive mutations spread throughout the lung, suggesting global selective pressures. Yet, for one gene (a distant homologue of the *merC* gene implicated in metal resistance), mutations arising independently in two lineages colocalize in space, providing evidence for location-specific selection. Our work presents a general framework for understanding how selection acts upon a pathogen that colonizes and evolves across the complex environment of the human body.

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Bacterial species evolve in the human body, whether within the microbiome[1–4] or over the course of an infection[5–7]. Mutations can sweep through the population[8] or lead to diversification into distinct subpopulations that differ genotypically and phenotypically[5–7,9–15]. Subpopulations within an individual can be spatially segregated across the structured environment of the human body[14,16–19], but it is unknown to what extent such spatial variations reflect local differences in selective pressures. Separating which genes are important for the local versus global adaptation of a pathogen is critical for predicting and possibly manipulating the survival strategy of the pathogen. However, it remains challenging to determine whether a spatially enriched genotype is the result of local selection or physical isolation and drift[14].

Here, to disentangle these confounding effects and identify local selection, we ask whether independently occurring adaptive mutations colocalize in space. We sample and sequence the whole genomes of 552 *Stenotrophomonas maltophilia* isolates from 23 sites across the explanted lungs of a cystic fibrosis (CF) patient; *S. maltophilia* is a Gram-negative bacillus and an emerging pathogen in CF as well as in nosocomial and community-acquired infections[20]. We first determine specific genes that were under selection during diversification by identifying genes with recurrent mutations. We then test whether mutations of a gene under selection that occurred independently in different lineages colocalize to the same set of sites despite sufficient time for dispersal. This approach therefore allows us to separate global and local selective pressures acting at the level of genes in pathogen populations.

Results

Sampling *S. maltophilia* diversity from lung tissue sites. We studied the population diversity of *S. maltophilia* within and between multiple tissue sites sampled across the lungs of a CF patient. The patient was chronically infected with *S. maltophilia* and underwent bilateral lung transplantation. *S. maltophilia* was first detected in the patient 3 years before the transplant and had been the dominant pathogen in the lung. Sputum was sampled from the patient 1 day before the transplant. Hours after the lungs were removed from the patient, they were each cut parasagittally into four cross-sections; the knife was sterilized between slices to minimize contamination. Tissue specimens (31 total, 0.1–1 cm per side) were sampled from different sites in each section (Fig. 1 and Supplementary Table 1). Examination of corresponding histologic sections of tissue specimens confirmed a diversity of anatomic sites (Supplementary Fig. 1). Specimens and sputum were homogenized and plated for single colonies, and 24 *S. maltophilia* isolates were randomly chosen from each sample that exhibited bacterial growth (23 specimens and sputum; total 576 isolates).

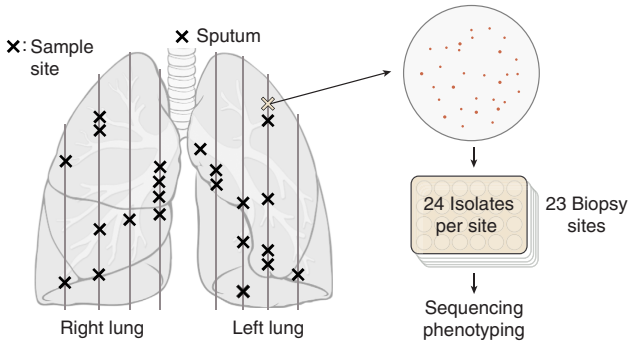

Figure 1 | Sampling *Stenotrophomonas maltophilia* populations across explanted CF lungs. From each lung, we took 4 parasagittal cross-sections along the medial to lateral axis. Each sample was homogenized and plated on MacConkey agar; 24 isolates were randomly selected from 23 tissue samples that exhibited growth. All isolates were whole-genome sequenced and phenotyped. Sample site locations are approximate. Lung figure adapted from original by Patrick J. Lynch and C. Carl Jaffe (goo.gl/iC8AjM), CC-BY-2.5.

Population diversified into multiple coexisting lineages. Whole-genome sequencing and phylogenetic analysis revealed that the pathogen diversified into multiple coexisting lineages. Sequencing the whole genomes of all isolates revealed 334 polymorphic genomic positions (Supplementary Data 1). A parsimony tree exposed several coexisting lineages, including two major lineages A and S, with lineage S exhibiting a small colony variant morphology (Supplementary Fig. 2), and two minor lineages B and C. These lineages were separated by 47 single-nucleotide polymorphisms (SNPs) and had copy number variations in 4 gene regions (Supplementary Fig. 3). However, lineages had very similar genomic architecture (Supplementary Fig. 4) and the extent of population diversity was consistent with the expected, assuming mutations accumulating during the infection period with a molecular clock typical of CF pathogens[21]. Therefore, similar to other pathogens in chronic infections[6,12,13,15–16], these results indicated *de novo* diversification of a single clone into multiple coexisting lineages within the patient.

Identifying genes undergoing adaptive evolution. Lineage differentiation was driven by adaptive selection. Mutations separating lineages showed elevated ratio of nonsynonymous to synonymous substitutions ($dN/dS = 1.9$, $P = 0.027$; Fig. 2c), indicating a signal for positive selection consistent with previous findings in other CF pathogen, *Pseudomonas aeruginosa*[16,22]. Many of these lineage-separating mutations appear in genes known to be important for virulence (Supplementary Table 2): for example, one mutation in a secreted serine protease (StmPr2/AprV2 homologue), recently shown to contribute to toxicity in human epithelial cells as well as degradation of extracellular matrix protein and interleukin-8 (ref. 23), could disrupt a stabilizing disulfide bridge in a disordered region of the protein (Supplementary Fig. 5). In addition to the dN/dS signal, which may be biased because of hitchhiking, we found further support for positive selection by detecting four genes with at least two lineage-separating mutations (Supplementary Fig. 6a and Supplementary Table 2); mutational events in these genes are marked in Fig. 2a. As two of these genes are implicated in antibiotic resistance (DNA topoisomerase IV and an efflux transporter), we measured the resistance of every isolate to three different drug classes administered to the patient early in the infection. Indeed, there was a significant difference in resistance profiles between the lineages (Fig. 2b). Altogether, the signal for positive selection from dN/dS, genes with recurrent mutations and difference in resistance phenotypes support that adaptive selection drove lineage divergence.

Evidence of adaptive evolution was also observed within the lineages. Although mutations within lineages did not show a dN/dS signal for selection as a whole ($dN/dS = 0.93$, 95% confidence interval 0.74–1.20), four genes were mutated at least three or more times, much more than expected by chance (Supplementary Fig. 6b; genes labelled in Fig. 2a, listed in Supplementary Table 3). This suggests positive selection acting upon these genes ($P = 0.004$, a threshold of 3 mutations was chosen to best remove false positives, bootstrapping a random allocation of mutations across the genome; see Methods). The four genes encode a mercury resistance protein homologue

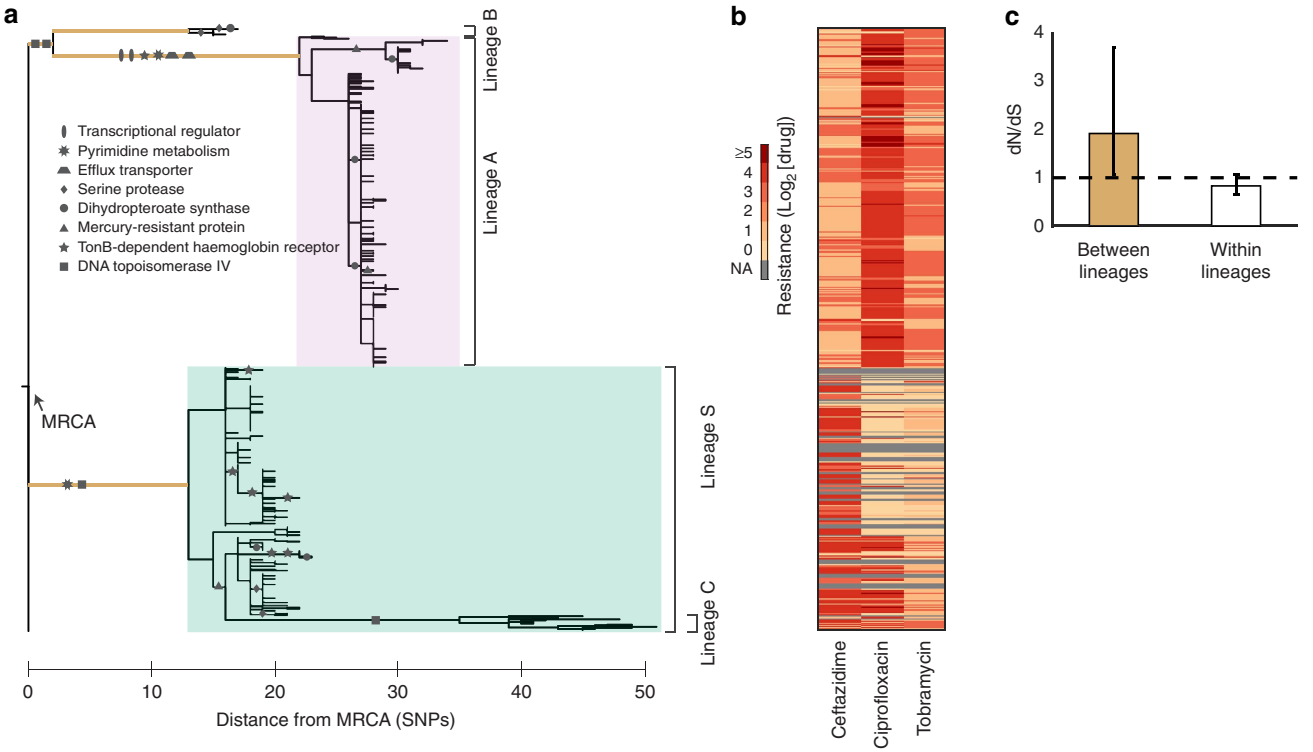

Figure 2 | Positive selection drove differentiation of *S. maltophilia* into distinct coexisting lineages within the patient. (**a**) Parsimony tree of the population constructed from 334 polymorphic mutations. Most recent common ancestor (MRCA) on the left. The mutations of eight genes with recurrent mutations are indicated on the tree, each gene with its own symbol. Lineages A and S (small colony variant) form the majority of the population. Minor lineages B and C are also indicated. (**b**) Resistance profile of all isolates against three antibiotics used in treatment, in twofold drug concentrations. Each row is an isolate aligned to its position on the phylogeny. NA, not available; 0, isolate grew only on no drug plates. The differences in resistance between lineages A and S were highly significant: ceftazidime $P=4\times10^{-39}$, ciprofloxacin $P=3\times10^{-43}$, tobramycin $P=2\times10^{-34}$, Kolmogorov–Smirnov test. (**c**) Lineage-separating mutations are under positive selection (green; dN/dS = 1.9, $P=0.027$, 95% confidence interval (CI) 1.06–3.70), whereas within-lineage mutations are neutral (white; dN/dS = 0.93). Error bars indicate 95% CIs.

(*merC* homologue), dihydropteroate synthase, a TonB-dependent haem/haemoglobin receptor (*shuA* homologue) and a serine protease (*mucD/htrA/degP* homologue). Indeed, all 15 mutations across these multiply mutated genes were nonsynonymous, and many mutations were found clustered together near functional domains of the protein (Supplementary Fig. 7); furthermore, the *shuA* homologue was mutated 6 times within lineage S, but none within lineage A, the ancestor of which carries a mutation in this gene. Together, these results show that different selective pressures can act on the two major lineages during their separation and later on during their diversification.

Major lineages are distributed across most sites. Mapping the spatial distribution of the major lineages revealed that they were not segregated between the two lungs and not even between lobes or sites. Lineages coexisted in most sites, indicating that they dispersed throughout most of the lungs over the 3-year infection (Fig. 3a). Notably, both major lineages but neither minor lineage were represented in the sputum sample, suggesting that clinical samples capture only the gross-level diversity of *S. maltophilia*. Similar to previous results obtained for *P. aeruginosa*[14], the ratio between major lineages A and S across sites significantly deviated from the expectation under a well-mixed model (Fig. 3b; $P<10^{-3}$, χ^2 test). Indeed, some sites were dominated or even purely occupied by only one of the two major lineages; minor lineages B and C were also localized to particular sites. Principal component analysis of β-diversity between site populations via Unifrac[24] did not show any site clustering by lung location (Supplementary Fig. 8), though lineage-enriched sites may be more likely to be found in the peripheral planes of the lung (Supplementary Fig. 9). Although contamination during sampling is possible, there was no evidence that parasagittal slicing of the lungs mixed site populations (Supplementary Fig. 8). Altogether, major lineages dispersed throughout most of the lungs, yet were significantly enriched in some sites.

New mutations disperse rapidly out of a site. Spatial confinement of alleles increased towards younger branches of the phylogeny, providing an estimate of their rate of dispersal. As a new mutation arises, we expect that it will first be confined to its site of origin and gradually spread to other sites, a phenomenon known as 'isolation by distance'[25–27]. Following Croucher *et al.*[28], we defined spatial confinement η as a function of mutational age d by the fraction of all isolate pairs separated by a genetic distance of d or less SNPs that were in the same site, normalized by the expected fraction under a null model where isolates are randomly distributed ($\eta=1$ when there is no spatial confinement; see Methods)[28]. As expected, spatial confinement was high for genetically similar isolates (Fig. 3c, orange line; $\eta>1$ for $d<3$, $P<10^{-3}$). Colocalization was rapidly lost as the genetic distance increased, suggesting that a new mutation disperses out of a site in the time it takes to accumulate only ~3 SNPs, translating to ~4.5 months from an estimate of the molecular clock (see Methods). This relatively rapid dispersion is consistent with the observation that *S. maltophilia* frequently resides in sputum-filled airways[29,30]. However, despite this potential for rapid dispersion, some genotypes maintained confinement longer than expected as evidenced by continued higher spatial confinement than the

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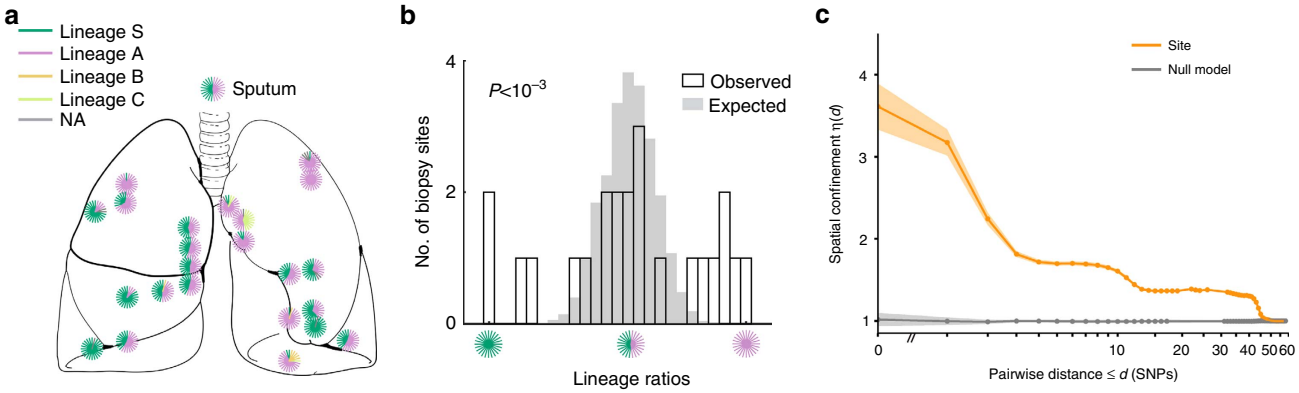

Figure 3 | Mapping the spatial distribution of lineages and estimating the dispersion rate of a mutation. (**a**) The approximate location of each tissue sample is labelled on the lung. Each radial line is coloured by the lineage membership of an isolate with the exception of those that were undetermined because of inadequate sequencing coverage (grey). The sputum population is shown at top. Lung figure adapted from original by Patrick J. Lynch and C. Carl Jaffe (goo.gl/iC8AjM), CC-BY-2.5. (**b**) The observed ratios between lineage A and S across sites are significantly different from the expectation in a well-mixed environment ($P<10^{-3}$, χ^2 test). (**c**) We calculated the likelihood $\eta(d)$ that pairs of isolates separated by d or less SNPs are in the same site (orange line); grey line indicates null model. Shaded error bars indicate 1 s.d. with respect to null model. We inferred a dispersion time of $d\sim3$.

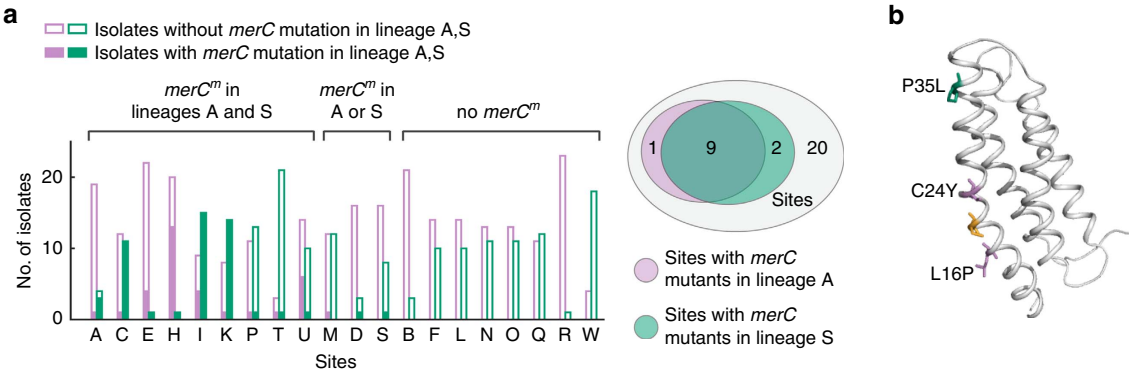

Figure 4 | Location-specific selection of *merC* homologue mutants. (**a**) Independently occurring mutants of the distant *merC* homologue in both lineages A and S colocalize to the same sites ($P=0.005$, Fisher's exact test). (**b**) MerC is an inner membrane protein with four transmembrane domains[45]. The three mutational events in the *S. maltophilia* distant *merC* homologue were mapped to a protein model (generated via EVfold[31,32]). Mutation in lineage S (P35L) is highlighted in teal, whereas mutations in lineage A (C24Y, L16P) are highlighted in pink. The cysteine proposed to form a disulfide bond with C24 is highlighted in orange[45] (C20); C24Y would disrupt this disulfide bond. All three mutations are found on the same side of the first transmembrane domain.

expectation in a null model ($\eta>1$ even at large d). This observation suggested regional isolation or local selection acting on a subset of mutations[14].

Identifying a gene under location-specific selection. Location-specific selection was detected by asking whether independently occurring mutations in a gene under selection colocalized to the same sites. Adaptive lineages can be enriched in a site because of local selective pressures, yet physical segregation could also confine related genotypes to a location (Supplementary Fig. 9). To disentangle the signal for selection from this confounding factor, we focussed on the cases where independent mutations appeared in the same gene and asked whether they colocalized in space. Two genes were mutated in both lineages A and S: *merC* and dihydropteroate synthase. In one of these genes, a distant homologue of the *merC* gene (hereinafter referred to as *merC*), mutations that occurred independently in both lineages proliferated in the same set of sites (Fig. 4a; $P=0.005$, Fisher's exact test). Such location-specific expansion reveals that selection can sort mutations across space even under rapid dispersal. Sites with *merC* mutations were distributed throughout the lung, demonstrating that spatial selection can occur on a small length scale rather than at a gross physiological level such as a lobe or lung. Interestingly, mapping the mutations on a protein model of MerC (generated via EVfold[31,32]) revealed that they were on the same side of the first transmembrane domain (Fig. 4b), suggesting that a modification in this region, and not its absence, is important for survival in these sites. The *merC* gene has been associated with mercury resistance as well as mercury acquisition[33] and found in the *mer* operon[34,35], but in *S. maltophilia* and related species *merC* is found on the chromosome next to a cobalamin synthesis protein (P47K homologue) and near a Co-Zn-Cd metal resistance gene (Supplementary Fig. 10), supporting a divergent role for this homologue[35]. Metal resistance has been associated with increased antibiotic resistance[36], and it is possible that *merC* is important for resistance to antibiotics such as ciprofloxacin (Supplementary Fig. 11). Altogether, the data show that selection for changing the *merC* homologue is location specific and that certain genotypes can locally proliferate because of site-specific selection.

Discussion

In this work we set out to identify genes that are under location-specific selection by testing for colocalization of independently

occurring mutations in the same gene. Although much of the adaptation seems to confer global selective advantage, we identified at least one gene apparently evolving under local selection. Such site-specific selection might contribute to long-term maintenance of diversity in chronic bacterial infections that could impede therapy. It is possible that additional spatial segregation may exist in length scales smaller than the resolution of our tissue samples. Furthermore, some spatial enrichment may be diminished by cross-contamination that could bias our dispersal rate estimator. We demonstrate how a static genotypic distribution of a population can be used to reveal its dynamics and migration rates. This approach can be used to understand location-specific selective forces, and more generally how genetic diversity of bacterial populations evolving in the human body is shaped by selection and migration in structured environments.

Methods

Harvesting tissue from explanted cystic fibrosis lungs. Before lung transplantation, the patient's parents provided informed consent for study of the excised lungs. The study was approved by the Boston Children's Hospital Institutional Review Board. At the time of transplant, the male patient, who has homozygous F508delta *CFTR* mutation, was 10 years old and had been treated for the 3 previous weeks with oral trimethoprim/sulfamethoxazole, oral linezolid and inhaled tobramycin. The patient had also received a 2-week course of intravenous meropenem, intravenous tobramycin, oral minocycline and oral linezolid 4–5 weeks before transplant. Similar courses of antibiotics had been administered 3–4 times per year over the 3 years before transplant. Excised lungs were immediately refrigerated until processed for sample collection that occurred $\sim$8 h following removal. In each of the left and right lung, four parasagittal cross-sections were made with a knife cleaned with ethanol between each slice. Each cross-section of the lung was laid down carefully on a sterile cutting board. Each individual tissue sample was procured with a separate sterile, disposable scalpel. Biopsy samples were placed in 15 ml Falcon tubes with 1 ml phosphate-buffered saline and immediately stored on ice. Adjacent to each site sampled for microbiologic culture, a second sample was procured for histologic examination.

Histologic examination. Tissue sampled for histologic examination was fixed in formalin and processed for paraffin embedding. Paraffin-embedded tissue was sectioned and stained with haematoxylin and eosin, Gram and Steiner stains and examined via light microscopy (Supplementary Fig. 1).

Sequencing *S. maltophilia* populations from biopsy sites. Each sample was homogenized using a tissue grinder (15 ml Covidien Precision Disposable Tissue Grinder) and frozen in 15% glycerol. The sputum samples were homogenized by incubation with 10 mM dithiothreitol and frozen in 15% glycerol. Frozen samples were thawed and plated with beads onto MacConkey II Agar (BD 221270) in 10-fold serial dilutions (10^0 to 10^{-4}). Of the 31 biopsies, 23 exhibited growth; the sputum sample also exhibited growth. For each biopsy/sputum with growth, we chose the dilution level at which we observed 50–300 colonies to ensure diversity while minimizing competition between strains; we then randomly picked 24 colonies into independent wells of 96 deep-well plates (Greiner Bio One 780271 Masterblock 96-well 2 ml sterile V-bottom) filled with 1 ml LB. Cultures were incubated overnight at 37 °C with shaking. Then, 150 µl of saturated cultures were frozen in 15% glycerol and stored at -80 °C, whereas the remaining volume was used to extract whole-genome DNA using Invitrogen PureLink Pro 96 Genomic DNA Purification Kit. Genomic libraries were prepared by diluting Illumina Nextera kit and using custom barcodes for multiplexing, as previously described[37] and sequenced using paired-end 100 bp reads on the Illumina Hi-Seq 2000 platform.

Determining polymorphic mutations from sequence data. FASTQ files were trimmed for adapter sequences with cutadapt[38] v1.8.3 and filtered with sickle2550 (ref. 39) (quality threshold 25, length threshold 50). These reads were then aligned to the reference genome of *S. maltophilia* strain Ab55555 (GenBank accession ALOG01000000; 4,918,929 bp across 6 scaffolds) using bowtie2 (ref. 40) v2.2.4 (paired-end with maximum fragment length 2,000 bp, no-mixed, dovetail, very-sensitive, n-ceil 0, 0.01). All unaligned reads ($\sim$10% per isolate) were pooled across the isolates and used to assemble contigs using velvet[41] v1.2.10 with paired-end reads, a minimum contig length of 1,000 bp and coverage cutoff of 1,000. The resulting 107 contigs (containing 308,871 bp) and the Ab55555 genome were concatenated to create a new master reference genome. All trimmed and filtered reads were realigned to this master reference genome with the same parameters as above; the average alignment rate was 95%. In addition, 98% of isolates (565 of 576) had a coverage mode of 10 reads or higher (Supplementary Fig. 12) with an average mode value of 30, and 99% of isolates (570 of 576) had less than 8% unaligned reads. Only isolates with a coverage mode of >10 were used for the analysis, resulting in 565 isolates used for downstream analysis and 11 isolates being discarded.

For each isolate, we used SAMtools[42] v1.3 to generate candidate mutated positions with respect to the reference genome (FQ < -30). We then combined these positions across all isolates and reduced the list to positions that were polymorphic within the population. Polymorphic positions were determined if any two isolates disagreed in the nucleotide calls with both calls having an FQ score of ≤ -52. With this approached, we obtained 334 SNPs (7 of which were in the contigs). The raw calls for each read aligned at all 334 positions for the 565 isolates (576 sequenced minus 11 isolates without adequate coverage) were used to create a matrix of genotypes. An isolate without adequate coverage (defined as <4 reads) at the position were designated as N; if the difference between the major and minor allele frequencies were $<60\%$ of the major allele frequency, the position was also designated as N. In total, 63 entries from the 334×565 matrix of calls were designated as Ns.

Phylogenetic analysis. All SNPs calls were concatenated as a string for each of the 565 isolates that met the coverage mode threshold to generate an input file for dnapars, part of the PHYLIP[43] v3.696 package. We constructed a parsimony tree using the Ab55555 strain reference genome as the outgroup.

Gene content. Using the coverage at each position of the reference genome given by the DP field of the VCF file, we determined the normalized coverage of each gene for each isolate. DP values are averaged across each coding sequence and divided by the coverage mode for each isolate, resulting in copy numbers in units of isolate mode. To detect genes with copy number changes, for each gene we calculated the difference between the highest and lowest copy number across isolates; a threshold was chosen to find genes with large differences (Supplementary Fig. 3a).

Of the 4,539 coding sequences in the *S. maltophilia* Ab55555 reference genome, 3,972 genes were present in the isolates in this study. Of these 3,972 genes, 47 genes exhibited a significant copy number difference between isolates (Supplementary Fig. 3b); their descriptions are in Supplementary Table 4. Genes that differed in copy number between the lineages included numerous phage proteins and a set of Type II secretion system proteins (Supplementary Fig. 3c).

Calculating dN/dS. Each mutation was determined as nonsynonymous or synonymous according to the annotation of open reading frames in the GenBank file. To calculate the expected value of nonsynonymous to synonymous substitutions in the genome, all intragenic mutations among the lineage-separating SNPs were redistributed across the reference genome in intragenic regions that had sequencing reads; the type of mutation was also preserved (transitions versus transversions). The expected N/S was calculated as the average ratio of nonsynonymous to synonymous substitutions across 1,000 simulations, each conducted for the subset of lineage-separating mutations and the within-lineage mutations. The distribution from this null model was used to calculate the 95% confidence interval. The dN/dS was then calculated as the observed value of N/S divided by its expectation.

Multiply-mutated genes. We created a null model for observing g multiply-mutated genes by randomly distributing the observed mutations across the genome that had coverage among the isolates (genomic positions that had a mean copy number of 1 across the 576 isolates; Supplementary Fig. 4), preserving mutation types. We calculated 1,000 permutations for the null model (Supplementary Fig. 6). We limited our analysis to all coding sequences no longer than 2,000 amino acids, rather than weighting genes by their length.

Measuring antibiotic resistance. We determined the resistance of an isolate as the highest level of antibiotic it could form a colony on solid agar while maintaining similar morphology to the colony on agar with no drug. The isolate library was pinned onto solid MacConkey II agar (BD 281810) poured in Nunc OmniTray Single-Well plates (VWR 62409-600). All isolates were pinned on a total of 16 plates ($3 \times 5 + 1$): 5 concentrations for each of the three antibiotics (with technical duplicates), and one media-only plate. The antibiotic concentrations varied by twofold at each step: ceftazidime 100, 200, 400, 800 and 1,200 µg ml^{-1}; ciprofloxacin 3.75, 7.5, 15, 30 and 60 µg ml^{-1}; and tobramycin 37.5, 75, 150, 300 and 600 µg ml^{-1}. After incubating at 37 °C for $\sim$60 h, all plates were imaged using an in-house system for taking pictures of plates with a Canon EOS Rebel T3i Digital SLR Camera. We analysed the images to determine for each isolate the highest concentration of drug at which growth was detected with a colony morphology similar to that of the no-drug plate.

Comparing lineage distribution across sites with a well-mixed model. We permuted the site locations of all isolates in lineages A and S to construct the null model of a well-mixed environment. For each site, we calculated the ratio between isolates in lineage A and isolates in lineages A or S: A/(A + S). The distribution of

observed ratios across all sites was compared with the expected using the χ^2 goodness of fit.

Spatial confinement. We calculated the fraction of isolate pairs separated by d or less SNPs that were also in the same site. This observed fraction was divided by the expected fraction calculated under a null model where isolates are randomly mixed between the sites, resulting in a likelihood measure. We designated this likelihood as our measure for spatial confinement $\eta(d)$.

$$Q(d) = \frac{\sum D(i,j)_{site} \leq d}{\sum D(i,j) \leq d}$$

$$\eta(d) = \frac{Q^{obs}}{Q^{null}}$$

where $Q(d)$ = the fraction of isolate pairs within genetic distance d that are in the same site and $\eta(d)$ = measure for spatial confinement; compares the observed fraction of isolates in the same site with the expected fraction.

The spatial confinement measure $\eta(d)$ was calculated at d for all integers from 0 to 60. The null model for η was constructed by permuting the site identity of isolates 1,000 times; statistics were calculated from these permutations.

Estimating molecular clock and migration rate. Although a molecular clock is not known for *S. maltophilia* at the time of this work, we inferred a molecular clock from the mean root-to-dip distances over the 3-year infection that was 24 SNPs with 1 s.d. of 5 SNPs (Supplementary Fig. 13). This translated to 8 SNPs per year, with a s.d. of 1.7 SNPs. We used this approximation to convert 3 SNPs to 4.5 months.

Synteny analysis of *merC* homologue. We used the online version of SyntTax[44] to compare the synteny of *merC* homologues within the Xanthomonadales order.

Data availability. The genomic sequence data for the 576 *S. maltophilia* isolates have been deposited in the Sequence Read Archive (SRA) database under accession code SRP090935. The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information files, or from the corresponding author on request.

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Author contributions

T.D.L., G.P.P., A.J.M. and R.K. conceived of the study. S.O.V., K.B.F., T.D.L. and G.P.P. collected the tissue samples. S.O.V. conducted the histologic analysis. H.C. conducted experiments and whole-genome sequencing. H.C. developed and conducted the data analysis. H.C., T.D.L., G.P.P., A.J.M. and R.K. interpreted the results. H.C. and R.K. wrote the manuscript with feedback from all authors.

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CASE REPORT
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Case Report: The Conundrum of What to Pick? Antibiotic Susceptibility Variability in *Burkholderia cenocepacia* in Cystic Fibrosis: Implications for Antibiotic Susceptibility Testing and Treatment

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Within cystic fibrosis microbiology, there is often mismatch between the antibiotic susceptibility result of an isolated bacterial pathogen and the clinical outcome, when the patient is treated with the same antibiotic. The reasoning for this remains largely elusive. Antibiotic susceptibility to four antibiotics (ceftazidime, meropenem, minocycline and trimethoprim-sulfamethoxazole) was determined in consecutive isolates (n = 11) from an adult cystic fibrosis patient, over a 63 month period. Each isolate displayed its own unique resistotype. The first isolate was sensitive to all four antibiotics, in accordance with Clinical and Laboratory Standards Institute methodology and interpretative criteria. Resistance was first detected at four months, showing resistance to ceftazidime and meropenen and intermediate resistance to minocycline and trimethoprim-sulfamethoxazole. Pan resistance was first detected at 18 months (resistotype IV), with three resistotypes (I, II and III) preceding this complete resistotype. The bacterium continued to display further antibiotic susceptibility heterogeneity for the next 45 months, with the description of an additional seven resistotypes (resistotypes V–XI). The Relative Resistance Index of this bacterium over the 63 month period showed no relationship between the development of antibiotic resistance and time. Adoption of mathematical modelling employing multinomial distribution demonstrated that large numbers of individual colony picks (>40/sputum), would be required to be 78% confident of capturing all 11 resistotypes present. Such a requirement for large numbers of colony picks combined with antibiotic susceptibility-related methodological problems creates a conundrum in biomedical science practice, in providing a robust assay that will capture antibiotic susceptibility variation, be pragmatic and cost-effective to deliver as a pathology service, but have the reliability to help clinicians select appropriate antibiotics for their patients. This study represents an advance in biomedical science as it demonstrates potential variability in antibiotic susceptibility testing with *Burkholderia cenocepacia*. Respiratory physicians and paediatricians need to be made

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aware of such variation by biomedical scientists at the bench, so that clinicians can contextualise the significance of the reported susceptibility result, when selecting appropriate antibiotics for their cystic fibrosis patient. Furthermore, consideration needs to be given in providing additional guidance on the laboratory report to highlight this heterogeneity to emphasise the potential for misalignment between susceptibility result and clinical outcome.

Keywords: antibiotic treatment, antibiotic susceptibility, *Burkholderia cenocepacia*, cystic fibrosis, CF, biomedical science, microbiology

INTRODUCTION

There are approximately 1,315 people registered in Ireland with cystic fibrosis (CF) [1] and 10,908 people with CF registered in the United Kingdom [2]. These people are likely to develop lung infections of an acute, intermittent or chronic nature, sometime during their disease journey, which will require clinical intervention in the form of tablet, nebulised or intravenous antibiotics [3]. Lung infections develop in the person with CF due to the underlying pathophysiology of this autosomal recessive life-limiting genetic rare disease, due to an inability of the patient to clear respiratory secretions, often resulting in entrapment of environmental organisms, including bacteria and fungi, with the manifestation of lung infections [3]. For a seminal review of the pathophysiology of CF, please see Shteinberg et al. review [4].

Burkholderia cenocepacia is a clinically significant Gram-negative pathogen isolated from the lungs of people with CF, associated with increased morbidity and mortality [5]. Traditionally, *Burkholderia cenocepacia* organisms in CF have represented some of the most challenging clinical dilemmas in antibiotic management of CF, due to high levels of antibiotic resistance. In Ireland, antibiotic resistance in these organisms has been explained, at least in part, by the presence of class 1 integrons with recombined gene cassettes containing bla-OXA and aac(6′)-1a genes [6].

Antibiotic susceptibility (AS) testing of CF pathogens is important to help guide respiratory physicians and paediatricians in selecting appropriate antibiotics to treat their patients. Antibiotics may be employed in three clinical scenarios, namely, 1) to eradicate CF pathogens on their first isolation, 2) to suppress CF pathogens during chronic infection and 3) to treat serious infections, as part of pulmonary exacerbations. Unlike other infections within clinical microbiology, where there is good alignment between the AS result and the clinical outcome of the patient, CF microbiology has suffered from a relative mismatch between the AS result and the clinical outcome of the patient. For example, this may happen in two contexts, namely, 1) the AS result indicates sensitivity, the clinician treats with that antibiotic, but there is no clinical improvement in the patient and 2) the AS results indicates resistance, the clinician treats with that antibiotic and there is clinical improvement in the patient. Such mismatches have evolved to an extent, which has undermined the value of AS testing (AST) with CF clinicians, leading to some CF clinician abandoning AS testing and opting for empirical antibiotic treatment [7].

Such a situation needs to be revisited by biomedical scientists and microbiologists to help decipher what is going on to have lead to such a lack of confidence of AS testing of CF pathogens. One possibility is that there is greater variability in resistant types (resistotypes) that are present in the lung at any given time, which are difficult to detect phenotypically, due the organisms appearing visibly indistinguishable, leading to the reporting of a narrow and limited range of resistotypes.

To help quantify and add more tools to the AS testing arsenal, in this journal, we have developed the Relative Resistance Index (RRI) [8]. RRI is a simple method where antibiotic susceptibility is quantified by allocating a score of 1 for sensitive, 2 for intermediate resistance and 3 for resistant, for each antibiotic tested. The RRI is the mean value across all antibiotics tested for the isolate and enables quantitative monitoring of antibiotic resistance.

Therefore, the aim of this study was to examine the heterogeneity of resistant phenotypes of an isolate of *Burkholderia cenocepacia* in an adult CF patient over a 63 month period, by examining the antibiotic susceptibility of consecutive isolates to four antibiotics (ceftazidime, meropenem, minocycline and trimethoprim-sulfamethoxazole), in accordance with the Clinical and Laboratory Standards Institute (CLSI) testing methodology and interpretive criteria [9] and to consider the ramifications of antibiotic resistance heterogeneity for routine antibiotic susceptibility testing for the clinical microbiology laboratory and for treatment by the respiratory physician.

Details of the Case

Antibiotic susceptibility tests were performed *in vitro* on a collection of *Burkholderia cenocepacia* isolates (n = 11), from an adult CF patient over a 63 month period from initial isolation, against the following four antibiotics: ceftazidime, meropenem, minocycline and trimethoprim-sulfamethoxazole. Antibiotic susceptibility testing was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) M100-ED33:2023 Performance Standards for Antimicrobial Susceptibility Testing [9], in terms of testing methodology and interpretive criteria. Resulting zone sizes (mm) to disks were recorded as either sensitive (S), intermediate (I) or resistant (R) [9]. Each isolate was categorised into a resistotype, based on the susceptibility to each of the four antibiotics.

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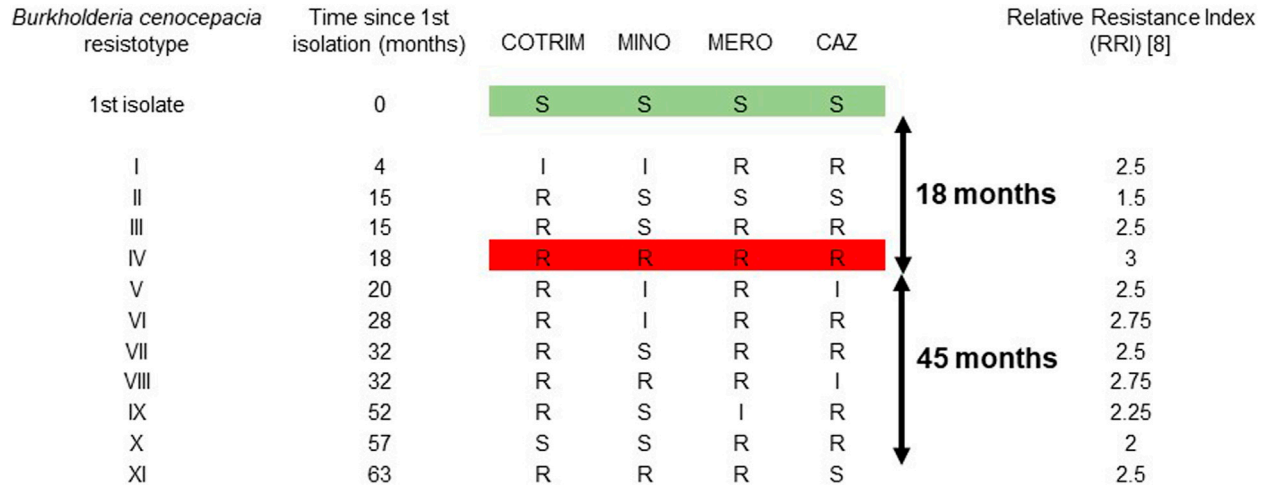

FIGURE 1 | Description of 11 resistotypes of *Burkholderia cenocepacia* isolated from an adult CF patient over a 63 month period. The resistotype profiles are based on susceptibility to four antibiotics (ceftazidime, meropenem, minocycline and trimethoprim-sulfamethoxazole), in accordance with the Clinical and Laboratory Standards Institute (CLSI) testing methodology and interpretive criteria. S, sentive; I, intermediately resistant; R, resistant. CAZ, ceftazidime; COTRIM, trimethoprim-sulfamethoxazole; MERO, meropenem; MINO, minocycline.

The Relative Resistance Index (RRI) was calculated for each isolate, combining the individual susceptibility to each of the four antibiotics, where a mean score = 1, was complete sensitivity to all antibiotics tested and a mean score = 3, represented complete resistance to all antibiotics tested, as previously described [8]. The Relative Resistance Index (RRI) is a simple method where antibiotic susceptibility is quantified by allocating a score of 1 for sensitive, 2 for intermediate resistance and 3 for resistant, for each antibiotic tested. The RRI is the mean value across all antibiotics tested for the isolate and enables quantitative monitoring of antibiotic resistance.

Over the 63 month period, *B. cenocepacia* was isolated from all sputum examined routinely (n = 66), of which 11 isolates yielded a unique resistotype. These isolates were from sputum routinely collected both at routine out-patient clinics, as well as during in-patient stays, as part of the patient's routine care. Strain characterisation/typing was performed which showed the isolates to have a rare phenotypic and molecular uniqueness, namely, cable pilus negative and mucoid, which was present throughout. These eleven isolates represented 11 different resistotypes (**Figure 1**). The first isolate (t = 0 month) was sensitive to all four antibiotics tested, in accordance with CLSI interpretative criteria. Resistance to antibiotics was first detected at 4 months, with an antibiogram showing resistance to ceftazidime and meroperen and intermediate resistance to minocycline and trimethoprim-sulfamethoxazole (R, R, I, I). Pan resistance was first detected at 18 months, with three resistotypes (resistotypes I, II and III) preceding the complete resistotype (resistotype IV). The bacterium continued to display further heterogeneity in its susceptibility profile, for the next 45 months, with the description of an additional seven resistotypes (resistotypes V–XI) (**Figure 1**). The Relative Resistance Index (RRI) of this bacterium over the 63 month period is shown (**Figure 1**). There was no relationship between the development of antibiotic resistance over time (r^2 = 0.0583).

DISCUSSION

Previously, Foweraker et al. demonstrated variability in the reporting of antibiotic susceptibility with *Pseudomonas aeruginosa* from CF aputum [10]. In their study, they showed the co-existence of several resistotypes in a single genotype of *P. aeruginosa*, even when the colony types appeared similar and further showed that the wide range of zone sizes in disc diffusion tests illustrated the variation in susceptibility of 48 colonies from one sputum sample. These authors concluded that the role of conventional antimicrobial susceptibility testing is questionable once *P. aeruginosa* chronically infects the cystic fibrosis lung, where several susceptibility patterns are seen, even within a morphotype and that routine susceptibility often underestimates resistance.

Hypermutation in CF pathogens is regarded as an important mechanism of adaptability of the organism during chronic infection, including a response to challenges to the organism's persistence in the CF lung, including 1) natural survival pressures from the host immune system and 2) imposed survival pressure from antibiotics during patient treatment. Such hypermutational adaptation has been described for CF pathogens including *Pseudomonas aeruginosa* [11], *Staphylococcus aureus* [12] and *Stenotrophomonas maltophilia* [13] A study by Martina et al. showed that the distribution of hypermutator isolates amongst members of the *Burkholderia cepacia* complex, ranged from 0% with *B. vietnamiensis* to 31.3% in *B. cenocepacia* [14]. Interestingly, this study was unable to link the emergence of hypermutators with enhanced antibiotic resistance, with the exception of the fluoroquinolone, ciprofloxacin. Instead, these authors concluded that the high prevalence of hypermutators in

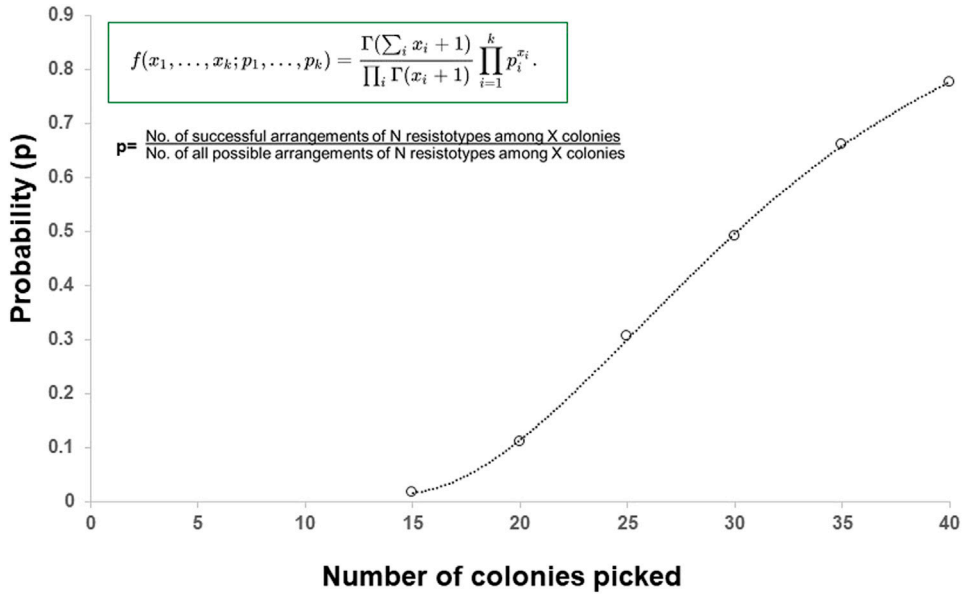

FIGURE 2 | The probability (P) of picking at least one colony of each 11 resistotypes, present at equal frequencies in an infinite population, in a sample of 0–40 colonies randomly picked from the population.

Burkholderia organisms may be explained by their co-selection with other mutations involved in the bacterial pathoadaptability to the CF lung environment [14].

As part of the routine laboratory of workup of *Burkholderia* isolates, biomedical scientists will pick several morphologically similar colonies from the purity plate or non-selective plate, in order to prepare an inoculum for subsequent antibiotic susceptibility testing, in accordance with EUCAST testing methodology [15]. Where different morphotypes are visible, then this will be repeated with each visibly distinct morphotype, and the process repeated so that all visibly distinct morphotypes are covered. This practice will therefore obtain an antibiotic susceptibility result for each morphotype present. Foweraker et al. data showed that the picking of similar morphotypes of *P. aeruginosa* with different susceptibilities can be a chance event, as there may be several hundred different colonies on the plate of the same morphotype, but with several different susceptibility profiles within the one morphotype [10]. Therefore, careful considerations needs to be given to what is picked of the plate for susceptibility testing and how representative these colonies are, in relation to the overall population on the plate? Foweraker et al study demonstrates that the more we pick from the plate, the more representative the range of susceptibility will be detected. However, from a busy hospital microbiology laboratory's perspective, it would not be pragmatic nor cost effective to pursue susceptibility testing in this manner. This, therefore, creates the challenge of what resources to invest in susceptibility testing to achieve a reliable and representative antibiogram, that is meaningful and adds value to the physician in helping to decide upon what antibiotic(s) to prescribe.

In our case, the isolate of *B. cenocepacia* yielded 11 resistotypes, from four antibiotics with three possible susceptibility outcomes per antibiotic, namely, S, I or R. Mathematically, there are a total of 81 possible permutations of these antibiotics/susceptibilities (i.e., $3 \times 3 \times 3 \times 3 = 3^4 = 81$), of which our studies had already identified 11/81 (13.6%) of these resistotypes. One rational approach to help estimate the number of colonies to pick from the plate for susceptibility testing to ensure the equal likelihood of randomly all resistotypes would be to employ a mathematical solution. Previously, such a strategy has been applied to picking morphologically indistinguishable serogroups of *E. coli* from animal and human faecal material by Hedges et al. [16]. Here, the authors based their statistical model on a multinomial distribution, where faecal coliforms constituted an infinitely large population in which N serotypes were present at frequencies p, p1, p2,p3,. . .pi, ... pN. It also assumed that morphologically indistinguishable colonies selected from a culture plate for serotyping were equivalent to a "random sample" drawn from the population. Adoption of this statistical approach and multinomial distribution algorithm to our study of 11 colonially indistinguishable resistotypes of *B. cenocepacia* (**Figure 2**), shows, for example, that 15 individual colonies are picked from a plate of a known population of 11 resistotypes, the probability of equally detecting all resistotypes is 0.018 (1.8%), whereas if 40 individual colonies are picked, the probability rises to 0.776 (77.6%). Adoption of such a mathematical approach offers a structured way of deciding on the required numbers of colonies to pick, based on the expected total number of resistotypes present in the sputum. One potential caveat to this approach however is that this model relies on equal frequencies of the phenotypes present. Presently, we are unsure if the resistotypes present in CF sputum are present in equal frequencies. Nevertheless, such algorithms may aid in the planning of efficient sampling programmes for determining most or all resistotypes present in CF sputum.

Other groups have offered up alternative solutions to address this issue of antibiotic susceptibility variability and how best to efficiently

TABLE 1 | Antimicrobial susceptibility testing of *Burkholderia cepacia* complex (BCC).

Antimicrobial susceptibility testing
It is not currently possible to establish minimum inhibitory concentration (MIC) breakpoints for BCC organisms as: • There is no evidence to describe a relationship between MIC and outcome • BCC is frequently part of a mixed infection • The MIC distribution of BCC for relevant antimicrobials is wide and encompasses the non-species related pharmacodynamic breakpoint. Therefore the epidemiological cut-off value cannot be used to define the wild-type population as either susceptibile or resistant
Susceptibility testing methodology is problematic: • MIC determination by the ISO broth microdilution (BMD) method with Mueller-Hinton broth yields reproducible results • MIC determination by the gradient strip method is less reproducible than BMD • The correlation between the MIC by the ISO BMD method and disk diffusion zone diameters is poor when tested by EUCAST (on Mueller-Hinton agar) or British Society for Antimicrobial Chemotherapy (BSAC) (on Isosensitest agar) methods

Taken from https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/BCC_susceptibility_testing_130719.pdf.

determine this. Zeboud et al. described the Direct sputum antimicrobial susceptibility testing (DSST) method, which involves e-tests being applied directly to a plate inoculated with 1/20 dilution of CF sputum and concluded that the DSST method was an efficient and easy antibiotic susceptibility testing method [17]. A similar approach would be to flood the primary non-selective plate (Columbia Blood Agar plate) to capture all organisms, however EUCAST methodology advises against this on health and safety grounds because pipetting and decanting high concentrations of organisms in suspensions onto the surface of plates, and subsequent removal, carries a high risk of production of aerosols and splashing [15]. Additionally, flooding tends to produce higher density of microorganisms over the agar surface when compared with swabbing [15]. Furthermore, having navigated through a rationale for what gets picked and what does not, the reliably of antibiotic susceptibility testing of *Burkholderia* isolates is complicated [18]. Antimicrobial susceptibility of *Burkholderia cepacia* complex (BCC) organisms, including *Burkholderia cenocepacia*, is problematic for a variety of reasons, as detailed by EUCAST in **Table 1**.

These data have been produced in part under ECDC service contracts and made available by EUCAST at no cost to the user and can be accessed on the EUCAST website. The views and opinions expressed are those of EUCAST at a given point in time. EUCAST recommendations are frequently updated in the latest versions.

Antimicrobial resistance is a major problem in cystic fibrosis [19]. There is controversy that the *in vitro* antibiotic susceptibility of isolates has little impact on clinical outcome. Traditionally, clinicians are informed on the AST of isolates obtained from their patients' sputum and make a clinical management decision on antibiotic choice, based on some form of such AST results. However, there is much debate as to the value of employing AST and how this impacts clinical outcomes. Historically, a study from the UK in 2005 showed that the CF lung may be chronically colonised with several phenotypes of PA, each with their unique susceptibility pattern, even within a single morphotype and concluded that routine antibiotic susceptibility testing is not reproducible and underestimates resistance [10]. Previously, VanDevanter et al. examined the relationship between antibiotic susceptibility and clinical parameters, including change in weight and lung function (ppFEV1) and were unable to demonstrate superior clinical improvements based on antibiotic susceptibility guided therapy. These authors concluded that AST may be of little utility in choosing antimicrobial therapy for *Pseudomonas aeruginosa*-related pulmonary exacerbations [20]. One possibility for such poor alignment between AST results and clinical outcome may be inadequate reporting of the diversity of all resistance types at the laboratory level, thereby placing more importance in developing and adopting suitable AST methodologies that address such AMR diversity, to help guide appropriate antibiotic prescribing.

In the current study, *B. cenocepacia* isolates showed increased resistance over time, suggesting that exposure to antibiotics may possibly drive antimicrobial resistance (AMR) development. Whilst it was not the aim of this case report, it would be interesting in future studies to attempt to correlate antibiotic usage with AMR development, with a larger cohort of CF patients, examining several organisms and involving several classes of antibiotics.

For this heterogeneity of resistance types in *B. cenocepacia*, antibiotic susceptibility testing of this species still remains problematic and a conundrum, which ultimately agrees with the EUCAST guidelines and lack of established breakpoints. The continued absence of such breakpoints and clarity from antibiotic susceptibility guidelines creates a dilemma for the CF clinician, when attempting to pick optimal antibiotics for treatment. Therefore, we hope that this case report re-ignites discussion amongst biomedical scientists and other key stakeholders, towards describing an optimal method for reliable and robust antibiotic susceptibility testing of this problematic pathogen in CF.

This study represents an advance in biomedical science as it demonstrates potential variability in AST with *Burkholderia cenocepacia*. Respiratory physicians and paediatricians need to be made aware of such variation by biomedical scientists at the bench, so that clinicians can contextualise the significance of the reported susceptibility result, when selecting appropriate antibiotics for their CF patient. Furthermore, consideration needs to be given in providing additional guidance on the laboratory report to highlight this heterogeneity to emphasise the potential for misalignment between susceptibility result and clinical outcome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical approval was not required as no research was performed on any human or animal subject. All bacterial isolates were collected with patient consent, as part of the patient's routine clinical care, including for the purposes of antibiotic susceptibility testing. No other clinical information or correlates are discussed, nor can the patient be identified.

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AUTHOR CONTRIBUTIONS

JEM: Conceptualization, formal analysis, methodology, roles/writing—original draft, writing—review and editing. JM: Methodology, writing—review and editing. JR: Writing—review and editing. BM: Conceptualization, formal analysis, methodology, roles/writing—original draft, writing—review and editing. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH ARTICLE

Selective Sweeps and Parallel Pathoadaptation Drive *Pseudomonas aeruginosa* Evolution in the Cystic Fibrosis Lung

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ABSTRACT **Pulmonary infections caused by *Pseudomonas aeruginosa* are a recalcitrant problem in cystic fibrosis (CF) patients. While the clinical implications and long-term evolutionary patterns of these infections are well studied, we know little about the short-term population dynamics that enable this pathogen to persist despite aggressive antimicrobial therapy. Here, we describe a short-term population genomic analysis of 233 *P. aeruginosa* isolates collected from 12 sputum specimens obtained over a 1-year period from a single patient. Whole-genome sequencing and antimicrobial susceptibility profiling identified the expansion of two clonal lineages. The first lineage originated from the coalescence of the entire sample less than 3 years before the end of the study and gave rise to a high-diversity ancestral population. The second expansion occurred 2 years later and gave rise to a derived population with a strong signal of positive selection. These events show characteristics consistent with recurrent selective sweeps. While we cannot identify the specific mutations responsible for the origins of the clonal lineages, we find that the majority of mutations occur in loci previously associated with virulence and resistance. Additionally, approximately one-third of all mutations occur in loci that are mutated multiple times, highlighting the importance of parallel pathoadaptation. One such locus is the gene encoding penicillin-binding protein 3, which received three independent mutations. Our functional analysis of these alleles shows that they provide differential fitness benefits dependent on the antibiotic under selection. These data reveal that bacterial populations can undergo extensive and dramatic changes that are not revealed by lower-resolution analyses.**

IMPORTANCE ***Pseudomonas aeruginosa* is a bacterial opportunistic pathogen responsible for significant morbidity and mortality in cystic fibrosis (CF) patients. Once it has colonized the lung in CF, it is highly resilient and rarely eradicated. This study presents a deep sampling examination of the fine-scale evolutionary dynamics of *P. aeruginosa* in the lungs of a chronically infected CF patient. We show that diversity of *P. aeruginosa* is driven by recurrent clonal emergence and expansion within this patient and identify potential adaptive variants associated with these events. This high-resolution sequencing strategy thus reveals important intraspecies dynamics that explain a clinically important phenomenon not evident at a lower-resolution analysis of community structure.**

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Cystic fibrosis (CF) is the most common fatal genetic disease among Caucasians (1, 2) and is characterized by chronic polymicrobial lung infections that are frequently dominated by opportunistic pathogens, such as *Pseudomonas aeruginosa* and species from the *Burkholderia cepacia* complex (3, 4). Polymicrobial communities in adult CF airways appear to be highly resilient and resistant to change, despite the administration of sequential antibiotic regimens targeting these dominant pathogens (5, 6). However, our perception that these communities are largely static is predominantly driven by culture-dependent studies of sputum and bronchoalveolar lavage fluid, microbiome studies using subregions of the 16S rRNA gene, and long-term longitudinal comparative genomics studies (7–9). While these approaches have tremendously informed our understanding of CF lung microbiology, they have not been performed at the temporal and taxonomic resolution required to characterize short-term intraspecies population dynamics. Since selection on microbial communities acts at the level of individual clones over the short temporal scale of antimicrobial treatments and lung disease exacerbations (10), a higher-resolution taxonomic and temporal approach is required to identify, understand, and ultimately manipulate the factors that control the composition and function of polymicrobial communities.

CF lung microbial communities persist in the face of powerful

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and complex pressures imposed by episodic exacerbations of lung inflammation and courses of antimicrobials, which typically include both long-term treatment with maintenance antibiotics as well as short-term, recurrent courses of other antimicrobials administered alone or in combination (11, 12). A number of studies have used comparative genomics to address how CF pathogens and *P. aeruginosa* in particular adapt to this dynamic lung environment. These have shown that airway-adapted isolates display very high levels of phenotypic diversity, strong mutational parallelism (recurrent mutations in the same genes among independent lineages), the emergence of hypermutable strains, mutations in global regulatory genes, and long-term persistence of a small number of clonal lineages (13–29). While these studies have furthered our understanding of the long-term evolutionary dynamics of *P. aeruginosa* in the CF lung, the large majority have been based on isolates collected several years apart—in some cases, 35 or more years apart—and none have focused on the immediate impact of antimicrobial therapies on the overall population of *P. aeruginosa* in the CF lung over a very short time span (19, 21, 24, 26, 27). This is a critical gap in our knowledge since clinical assessments and antimicrobial therapies are often modified as frequently as weekly (30).

A fundamental assumption of antimicrobial therapies is that they select against and thereby drive down the population size of target pathogens. A nearly unavoidable consequence of these therapies is the selection for resistant clones of the pathogen, which may ultimately dominate the population. The application of a selective pressure to microbial populations commonly leads to selective sweeps, which involve the spread of a clone carrying an adaptive mutation through the population and subsequent displacement of other preexisting and now less fit clones (31–34). Selective sweeps will periodically reoccur in a population as new beneficial mutations arise and new selected clones replace ancestral clones. This pattern of recurrent selective sweeps has historically been called periodic selection (35). While selective sweeps have been well documented in microbial populations (particularly with respect to viral populations, as reviewed in reference 36), the presence and significance of periodic selection in human health and infectious diseases are not known (37).

Clonal evolutionary dynamics such as selective sweeps have been largely overlooked in microbiome studies due to the lack of resolution and power. For example, 16S rRNA gene sequencing can only reliably resolve taxa to the genus level (38), while full metagenome sequencing suffers from the inability to phase genetic variation (assign variants into genotypes) (39). These limitations have restricted our ability to observe clonal evolutionary dynamics, identify their drivers, and assess their resulting impact on the overall microbial community as well as the host.

In the case of CF-associated lung infections, longitudinal population sampling (e.g., multiple clones assessed from each clinical specimen) performed over the short term will reveal the impact of intensive antimicrobial therapies on the dynamics of the target pathogen populations. Understanding intraspecies pathogen evolution over this time scale is essential to assess whether current treatment strategies effect desirable change of pathogen populations or if alternative treatment strategies, coupled with a better understanding of pathogen evolutionary potential, could lead to improved treatment outcomes.

In this study, we sequenced the genomes of 233 *P. aeruginosa* isolates from a single CF patient collected from 12 specimens over a 1-year period to assess very-short-term evolutionary dynamics. We identify the occurrence of a very recent common ancestor for the overall population, the emergence of multiple clonal lineages within a population that has remained relatively constant in size, and strong signals of positive selection—genomic features consistent with recurrent selective sweeps or periodic selection. We also find a very strong signal of parallel pathoadaptation at loci associated with virulence and antibiotic resistance and show how some of these variants confer fitness benefits that are dependent on the environment.

RESULTS

This study focuses on patient CF67, a 34-year-old female CF patient (of CF transmembrane conductance regulator [CFTR] genotype ΔF508/del2–3) who had been chronically infected with *P. aeruginosa* for at least 12 years prior to the initial study sample. The patient had advanced lung disease with a forced expiratory volume in 1 s (FEV_1) of 21% of the predicted value throughout the course of the study. She provided 12 expectorated sputum specimens for evaluation over an ~1-year period (with the first specimen collected 350 days prior to the last specimen) (Fig. 1). The specimens were not collected uniformly over the course of the study, with 11 being collected within the last 126 days of the study.

During the study period, the patient was prescribed 28 distinct courses of antibiotics, which were administered by three different routes (oral, intravenous, and inhaled) in addition to continuous oral azithromycin (Fig. 1A; see Table S1 in the supplemental material). Despite this complex antimicrobial regimen, microbiome analysis via Illumina sequencing of the V5 to V7 hypervariable regions of the 16S rRNA gene (38) reveals that *Pseudomonas* remained the dominant genus throughout (Fig. 1B). Quantitative PCR of the 16S rRNA gene was used to quantify the bacterial load from each of the twelve specimens, revealing no appreciable change in the absolute level of bacteria recovered from each specimen over time (mean ± standard deviation [SD], 0.395 ± 0.185 ng/ml; regression slope, $-0.0005x$).

We collected multiple *P. aeruginosa* isolates from each of the 12 specimens (range, 18 to 20 per specimen) in order to obtain a finer-resolution analysis of the population-level dynamics. An effort was made to maintain overall morphotype relative abundance in our sampling as previously described (40).

Genomic diversity and phylogenetic analyses identify two genetically distinct clades. A total of 233 *P. aeruginosa* isolates underwent whole-genome sequencing (7 of the total of 240 isolates were removed from the analysis due to quality control issues), yielding a median coverage depth of 54× (see Fig. S1 in the supplemental material). The genome of one isolate was assembled *de novo* for use as a reference for assembly of the remaining isolates. This assembly had an N_{50} of 329,133 bp and 46 contigs, with the smallest contig being 1,170 bp and a total genome size of 6,367,116 bp. We used a conservative pipeline to call single nucleotide polymorphisms (SNPs) that required the agreement of three algorithmically distinct reference assembly methods and then re-evaluated each SNP position using a more relaxed inspection of the pileup data (see Fig. S2 in the supplemental material). The pipeline identified 107 segregating SNPs among the 233 isolates, of which 54 were phylogenetically informative (SNPs segregating in at least two isolates), while 39 were found in isolates collected from multiple time points (multispecimen SNPs). Additionally, we identified insertion/deletion (indel) variants by realigning the

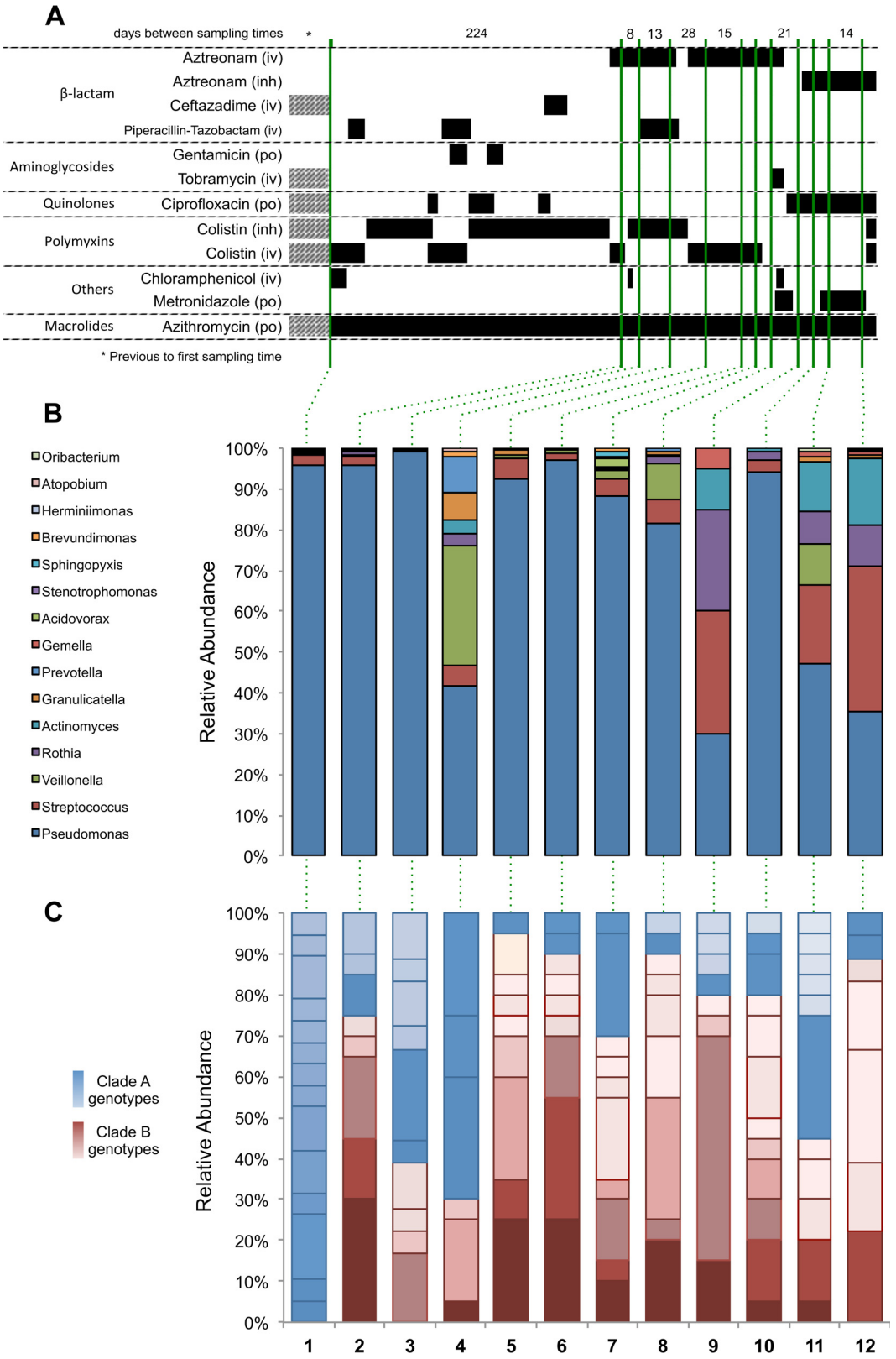

FIG 1 Antibiotic treatment history and the relative abundance of the two clades over time. (A) Black bars indicate antibiotic administration, and hashed bars indicate intermittent exposure in that time block. The method of antibiotic administration is shown as intravenous (iv), inhaled (inh), or oral (po). Sputum samples were collected at the time points indicated by green lines, which extend to panels B and C. (B) Relative abundance at the genus level is shown as a percentage. (C) Relative abundance of clade A (blue shades) and clade B (red shades) genotypes over time. Genotypes are defined as whole-genome sequences differing by one or more SNPs segregating in at least two isolates. The same color and shading are used to identify specific genotypes across specimens.

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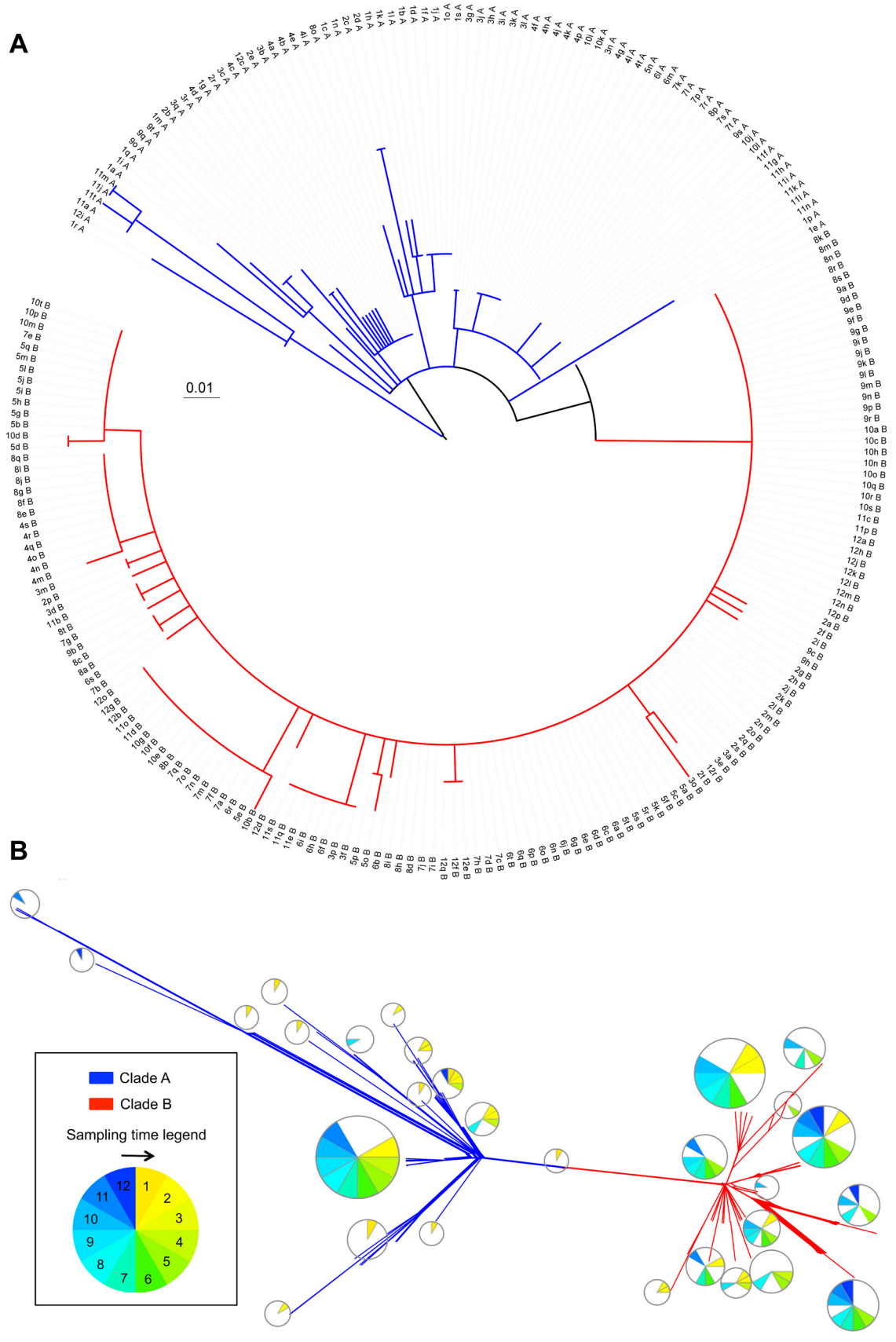
A
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Clade A
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TABLE 1 Genetic diversity

Variable	No. (%)		
	Clade A	Clade B	Total
Isolates	81 (34.8)	152 (65.2)	233
SNPs	72 (67.3)	35 (32.7)	107
Indels	31 (53.4)	28 (48.3)	58[a]
Phylogenetically informative SNPs	36 (64.3)	20 (35.7)	56
Multi-time SNPs	21 (53.8)	18 (46.2)	39 (57.1)
Multi-time indels	13 (46.4)	16 (57.1)	28[a]
Genetic distance, [b] avg ± SD	0.052 ± 0.04	0.029 ± 0.03	0.047 ± 0.04
No. of SNP differences, avg ± SD	4.80 ± 3.97	2.84 ± 1.60	6.49 ± 4.41
No. of indel differences, avg ± SD	4.69 ± 3.11	3.05 ± 1.91	5.03 ± 3.09

[a] One indel segregates in clade A and clade B.
[b] Maximum composite likelihood distance.

sequencing reads to initial candidates and then reviewing all putative indel positions in all isolates. This process revealed 58 indels, of which 28 were found in at least two isolates from different collection time points. Sequencing of the entire population of all *P. aeruginosa* isolates bulk extracted from one specimen did not reveal any mutations that were not present in the individual isolate sequencing, which indicated that our sampling of diversity is close to saturation (see Fig. S3 in the supplemental material).

The data were analyzed by both network-based (neighbor-net) and traditional (maximum likelihood) phylogenetic methods (Fig. 2). For the latter, the position of the root is very similar, regardless of whether it is identified by incorporating *P. aeruginosa* LESB58 into the analysis, selecting it based on the position of the strains isolated in specimen 1, or through midpoint rooting. Visual inspection of both trees revealed an unusual topology. Approximately one-third of the isolates share more structured relationships with relatively long branches, and these are distinctly separated from another set of isolates that have relatively short branches originating from a very recent common ancestor. Based on this observation, we designate two clades: clades A and B. While the clades are discrete and distinct in the network-based analyses, the rooted phylogenetic analysis shows that clade B is nested inside clade A, making clade A technically paraphyletic since it is not monophyletic without the clade B strains. Despite this, we will refer to the two groups as clades for simplicity. Importantly, all analyses comparing the two clades are performed on discrete sets of strains, where all strains are designated as belonging to either clade A or clade B as determined by the network-based phylogenetic analysis.

Clade A has a lower overall relative abundance than clade B (81 isolates versus 152 isolates for clades A and B, respectively [Fig. 1 and Table 1]) but is significantly more genetically diverse, with an average of 4.80 ± 3.97 (mean ± SD) pairwise differences among clade A isolates versus 2.84 ± 1.60 differences for clade B isolates ($P <<< 0.0001$, *t* test) (Fig. 3). Neither clade shows a significant recombination signal, with a four-gamete recombination test identifying a minimum of only one recombination event in the entire sample (41). This is supported by the nearly complete lack of reticulation in the neighbor-net phylogenetic analysis. The relatively uniform distance of clade B strains from their common ancestor seen in the phylogenetic analyses is reminiscent of a "star phylogeny" pattern of divergence, which is often associated with recent purges of genetic variation, such as those seen during population expansions or selective sweeps (42). There are six segregating mutations (four SNPs and two indels) that have variants fixed between the two clades. This is significant since fixed differences reflect population subdivision.

Time to common ancestry. We used the Bayesian approach implemented in BEAST (43, 44) to infer the time to most recent common ancestry (tMRCA) for the total population and each clade individually. The total population of 233 isolates coalesces to an MRCA of 993.24 ± 9.09 days (2.72 years) prior to the last specimen, while clade A isolates coalesce to a tMRCA of 956.75 ± 8.93 days (2.70 years) prior to the last specimen, and clade B isolates coalesce to an tMRCA of 329.42 ± 1.80 days (0.90 years) prior to the last specimen. We compared our isolates to other fully sequenced *P. aeruginosa* isolates (see Fig. S4 in the supplemental material) and found the Liverpool epidemic strain LESB58 to be the closest reference strain, with a tMRCA of 24.06 years prior to the last specimen (45, 46). The probability that our sample of 233 isolates captures the true tMRCA of the population can be calculated by $(n - 1)/(n + 1) = 0.991$, where n is the sample size (47).

Our analysis supports a population model in which clade A is a longer-lived ancestral population that has accumulated more genetic variation than clade B. Clade B, on the other hand, appears to be a more recently emerged population that arose between collection of the first and second specimens. The relatively recent coalescence of the total sample and clade B in particular suggests that we may have captured two periods of clonal emergence, expansion, and replacement. This is supported by the fact that clade A is more abundant in the earlier specimens, while clade B dominates in the vast majority of the later specimens (Fig. 1).

Selection analysis. The emergence and spread of a new clone are called a selective sweep when driven by positive selection;

FIG 2 Maximum likelihood and network-based (neighbor-net) phylogenetic analyses. The phylogenetic structure of 233 *P. aeruginosa* isolates was characterized based on genome-wide single nucleotide polymorphisms (SNPs) using the maximum likelihood (A) and neighbor-net (B) algorithms. The structure of the resulting tree revealed two populations, clades A (blue) and B (red). The 152 isolates in clade B produce a star phylogeny consistent with a recent expansion of a clonal population, while the 81 isolates in clade A show longer branches with more phylogenetic structure. (A) The scale bar shows genetic distance using the maximum composite distance and all segregating SNPs. The strain names shown on the tree are a composite of the specimen number from which the clone was isolated, an arbitrary clone letter, and the clade designation. (B) Individual strain names at the tips of each branch have been replaced with pie charts indicating the distribution of dates during which the strains were sampled (indicated by the circular legend). The scale bar indicates genetic distances.

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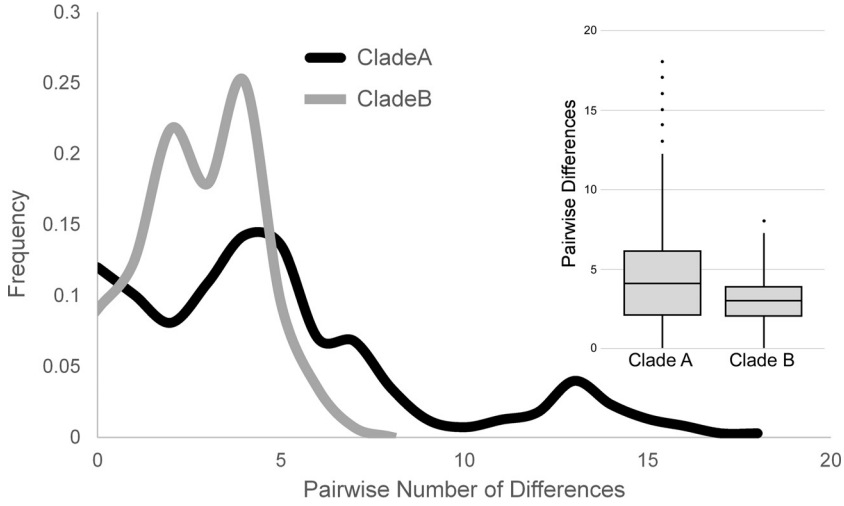

FIG 3 Frequency spectrum of SNPs in clades A and B. (A) The frequencies of the number of SNP differences between isolates within clade A and B reveal different distributions. While in clade A, most pairwise SNP differences occurred evenly, in clade A, pairwise SNP differences lower than 5 are overrepresented. (B) Distribution of pairwise SNP differences within clades A and B shows different profiles ($P <<< 0.0001$, *t* test).

therefore, we sought to identify evidence of selective pressure driving the emergence of clade B. Analysis of the 107 segregating sites in the total population gave a ratio of nonsynonymous to synonymous mutations (*dN*/*dS* ratio) of 1.01, which is consistent with the null model of neutral evolution but which does not rule out more complex selective patterns (Fig. 4 and Table 2). Importantly, this value is also consistent with a nonequilibrium population, in which the short time frame of the study allows us to capture mutations as they arise but before their fate has been determined by natural selection or genetic drift. Consequently, it is probable that some of the observed SNPs are deleterious to some degree and will be culled from the population in the longer term. To minimize this confounding effect, we elected to focus on only those 39 SNPs that segregated over at least two sampling time points (multispecimen SNPs). It would be expected that removing transient deleterious mutations from the analysis should decrease the relative number of nonsynonymous mutations, thus decreasing *dN*/*dS* ratio. Despite this, we find a *dN*/*dS* ratio of 1.43, consistent with weak positive selection acting on mutations maintained over multiple time points. When we split the sample based on the inferred clade structure, we find that the clade A, multispecimen segregating SNPs gave a *dN*/*dS* ratio of 1.04, compared to a *dN*/dS ratio of 2.42 for clade B. Thus, clade B is under greater positive selective pressure than clade A. When we further break down the analysis into the 12 individual specimens, we find a very consistent pattern, with clade B maintaining a higher *dN*/*dS* ratio relative to clade A throughout, with the exception of specimen 9 (Fig. 4). It is also intriguing the *dN*/*dS* ratio observed in clade A starts at 1.3 and then drops to 1.0 or below after specimen 1, once again with the exception of specimen 9. While there are many potential factors that can influence *dN*/*dS* ratios, and their calculation is highly dependent on sampling variance, the overall pattern observed in these data supports stronger and more sustained positive selection in clade B than clade A. Notably, the reversal of the *dN*/*dS* ratio trend noted in specimen 9 coincided with the cessation of aztreonam during that sampling period.

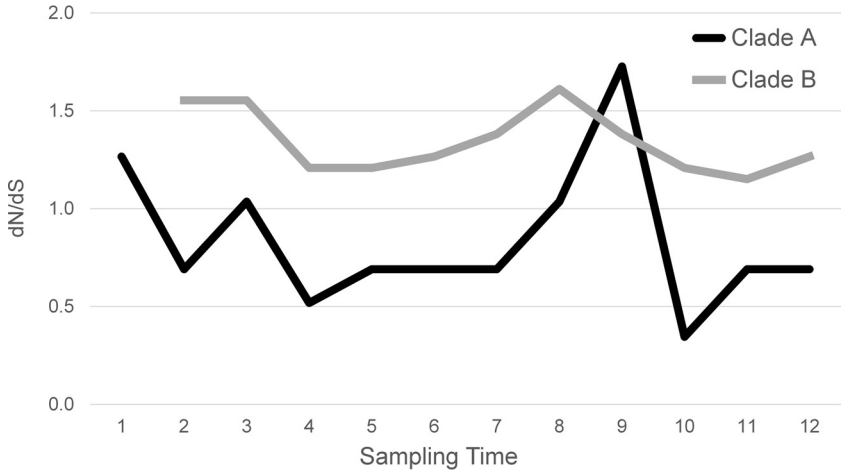

FIG 4 *dN*/*dS* ratio calculation over time. Shown is the nonsynonymous over synonymous substitution rate (*dN*/*dS*) for multispecimen SNPs calculated for each sample time and partitioned based on clade membership. Clade B arose between sampling specimens 1 and 2; therefore, there are no data for clade B.

TABLE 2 Selection analysis results

Variable	No. (%)			*dN*/*dS* ratio
	Nonsynonymous	Synonymous	Intergenic	
All SNPs	73 (68.2)	25 (23.4)	9 (8.4)	1.01
Multitime SNPs				
All	29 (74.4)	7 (17.9)	3 (7.7)	1.43
Clade A	15 (71.4)	5 (23.8)	1 (4.8)	1.04
Clade B	14 (77.8)	2 (11.1)	2 (11.1)	2.42

The *dN*/*dS* results supporting positive selection are further bolstered by the significantly negative result for both a Tajima's *D* test (48) and Fu and Li's test (49). Tajima's $D =$ to -2.08 ($P = 0.002$), while Fu and Li's $D^* = -5.58$ ($P = 0.001$) and $D =$ to -5.60 ($P < 0.001$, using the *P. aeruginosa* LESB58 strain as an outgroup). No significant differences were observed between the two clades in these analyses. These results indicate that there has been a recent population expansion after a selective sweep or population bottleneck. Given that there was no change in the absolute size of the *Pseudomonas* population in this patient, the best explanation for this expansion is the replacement of one clone by another.

Parallel pathoadaptation. A striking pattern in the data is the number of loci that were independently mutated multiple times. The occurrence of multiple independent mutations within the same locus can be a very strong indication of adaptive parallel evolution and strong selection on the locus (50–52). Nineteen loci were mutated multiple times (Table 3). Thirty-three of the 107 SNPs (30.8%) occur in these loci, with 15 (38.5%) being multispecimen SNPs, while 20 of the 58 indels (34.5%) are found in loci with multiple independent mutations, 11 of which are multispecimen indels (39.3%).

We compared the mutational class spectrum (relative frequency of nonsynonymous, synonymous, and intergenic changes) in our entire data set to that seen in loci with multiple mutations to determine if the parallel evolution is due to selection or simply a higher local mutation rate. We would expect an elevated mutation rate to maintain the same ratio of nonsynonymous to synonymous changes, while adaptive parallel evolution should predominantly be driven by nonsynonymous changes. We observed a mutational class spectrum of 68.2% nonsynonymous, 23.4% synonymous, and 8.4%, intergenic mutations in our full data set and a spectrum of 91.7%, 0.0%, and 8.3%, respectively, for the loci with multiple mutations. This highly significant skew toward more nonsynonymous mutations (chi-square test, $P = 1.5 \times 10^{-7}$) strongly supports selection-driven parallel evolution.

Many of the loci with multiple mutations are known to play important roles in *P. aeruginosa* virulence in the setting of CF. Among them is the gene that encodes a murein peptide ligase (Mpl), which has been shown to increase expression of AmpC and resistance to the β-lactam antibiotics piperacillin and ceftazidime (53). This locus carries five independent indels and one SNP. Given a genome size of 6,082 genes and making some simplifying assumptions concerning uniformity of gene size, the probability of any gene being mutated six times is 1.2×10^{-19}. Similarly, genes involved in alginate biosynthesis, such as the MucA and AlgU cluster and the locus that encodes AlgG, were each mutated five times ($P = 1.17 \times 10^{-14}$ and $P = 7.3 \times 10^{-16}$, respectively). Many other loci carrying multiple independent mutations also influence antimicrobial resistance, such as the operon encoding multidrug resistance-related loci MexA and MexB, which was mutated four times ($P = 1.08 \times 10^{-07}$), the transcriptional regulator AmpR and its neighboring β-lactamase precursor, AmpC, which were mutated four times ($P = 3.6 \times 10^{-11}$), and the gene encoding penicillin-binding protein 3 (PBP3), which was mutated

TABLE 3 Multiply mutated loci

Encoded protein	Locus	No. of SNPs/indels	Probability [a]
Murein tripeptide ligase (Mpl)	PA4020	1/5	1.20×10^{-19}
Anti-sigma factor (MucA)/sigma factor (AlgU)[b]	PA0763/PA0762	2/3	1.17×10^{-14}
Alginate-C5-mannuronan epimerase (AlgG)	PA3545	5/0	7.31×10^{-16}
Conserved hypothetical protein	PA4701	2/2	3.56×10^{-11}
Transcriptional regulator (AmpR)/β-lactamase precursor (AmpC)	PA4109/PA4110	4/0	3.56×10^{-11}
Penicillin-binding protein 3 (PBP3)	PA4418	3/0	2.70×10^{-08}
Multidrug efflux membrane fusion protein (MexA)/multidrug efflux transporter (MexB)	PA0425/PA0426	0/3	1.08×10^{-07}
Transcriptional regulator (MexT)	PA2492	2/0	1.64×10^{-04}
Outer membrane lipoprotein precursor (OprI)[c]	PA2853	2/0	1.64×10^{-04}
Hypothetical protein	PA3093	2/0	1.64×10^{-04}
Hypothetical protein[d]	NCGM23404	2/0	1.64×10^{-04}
Aminopeptidase P (PepP)/ubiquinone biosynthesis protein (UbiH)[c]	PA5224/PA5223	0/2	3.29×10^{-04}
LD-Carboxypeptidase (LdcA)	PA5198	1/1	1.64×10^{-04}
Conserved hypothetical protein	PA5133	1/1	1.64×10^{-04}
Conserved hypothetical protein/hypothetical protein	PA4962/PA4961	0/2	3.29×10^{-04}
Transcriptional regulator (LasR)	PA1430	1/1	1.64×10^{-04}
Multidrug resistance operon repressor (MexR)	PA0424	1/1	1.64×10^{-04}
Probable transcriptional regulator	PA0535	1/1	1.64×10^{-04}
D-Amino acid dehydrogenase, small subunit (DadA)	PA5304	0/2	1.64×10^{-04}

[a] Calculated based on the probability of resampling with replacement any locus *n* times, given a genome size of *N*. $P = (1/N)\wedge(n-1)$. We used $(n-1)$ since we are calculating the probability for any locus, rather than a specific locus.

[b] Mutations that occurred in two adjacent loci and which may be part of a common regulon according to the *Pseudomonas* Genome Database (97). The probability calculation was adjusted by dividing the total number of loci in the genome in half.

[c] One mutation occurred in the intergenic region flanking this locus.

[d] This locus is not found in PAO1. The homolog with highest similarity is in *P. aeruginosa* NCGM2.S1 (100).

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three times ($P = 2.7 \times 10^{-8}$). Six other loci were mutated twice ($P = 1.6 \times 10^{-4}$ for any one locus to receive two independent mutations), including the gene encoding the transcriptional regulator MexT.

Identification of potential adaptive variants. To detect possible adaptive variants, we determined the ontology of multispecimen segregating variants. Many loci with segregating variation in our sample are implicated in antibiotic resistance and adaptation to the host (see Table S2 in the supplemental material). The six fixed differences distinguishing the two clades include SNPs in genes encoding the two-component regulator NarX, a phosphomannose isomerase, an arginine/ornithine succinyltransferase, and the fimbrial subunit CupC1. All but the last one are nonsynonymous substitutions. One of the fixed indels leads to a frameshift mutation in the locus that encodes AlgF, which controls the addition of acetyl groups to alginate. The second fixed indel is located in the intergenic region upstream from the gene encoding DNA polymerase I (*polA*). Beyond these fixed mutations, there are SNPs in the gene encoding a probable nonribosomal peptide synthetase and alginate-C5-mannuronan epimerase AlgG that are also fixed between the two clades, with the exception of a single clade A specimen 1 isolate that carries the clade B variant at both sites. Additionally, there are SNPs in genes encoding a β-lactamase precursor and the transcriptional regulator MexT that are found only in clade B isolates and that first appear in specimen 2, which is the first specimen containing clade B isolates. These SNPs are found in 29.0% and 17.8% of clade B strains, respectively. Finally, an indel located in the gene that encodes a murein tripeptide ligase (Mpl) segregates exclusively in clade B, where it is found in 76.3% of the isolates. All of the isolates lacking this mutation in clade B also have a second indel in the same locus 1,240 bases upstream.

Given the relatively large number of variants that distinguish clades A and B, it is impossible to conclusively identify the beneficial mutation that initially gave rise to clade B. Nevertheless, it may be possible to identify SNPs that helped maintain one or both populations. As discussed above, three independent nonsynonymous mutations arose during the course of this study in the *pbpB* locus, encoding PBP3. Two derived alleles are found exclusively in clade B isolates (one at very low frequency), and one derived allele is found exclusively in clade A isolates (Fig. 5; see Fig. S5 and S6 in the supplemental material). The ancestral PBP3 allele is found in both clades.

We noted that aztreonam and piperacillin-tazobactam were first administered intravenously during the time that roughly coincides with the emergence of clade B (Fig. 1). Since aztreonam has high affinity for PBP3 (54), we examined the *in vitro* fitness of the clade A-derived *pbpB* allele, the most common clade B-derived *pbpB* allele, and the ancestral *pbpB* allele using standard MIC assays against four antipseudomonal β-lactam antibiotics (Fig. 6) (55). The ancestral allele is present in both clades but carried in different genetic backgrounds; nevertheless, the resistance it confers does not differ significantly among strains, regardless of their clade of origin ($P = 0.15$ to 0.44). The derived PBP3 alleles from both clades A and B increased strain resistance relative to the ancestral allele in the presence of both aztreonam and cefsulodin. In contrast, when tested on ceftazidime and piperacillin, while the clade A allele increased resistance, the clade B allele was no more resistant than the ancestral allele. These results indicate that the *in vitro* fitness contribution of each derived PBP3 allele (as measured by antibiotic resistance) is dependent on the specific antibiotic environment in which it is found.

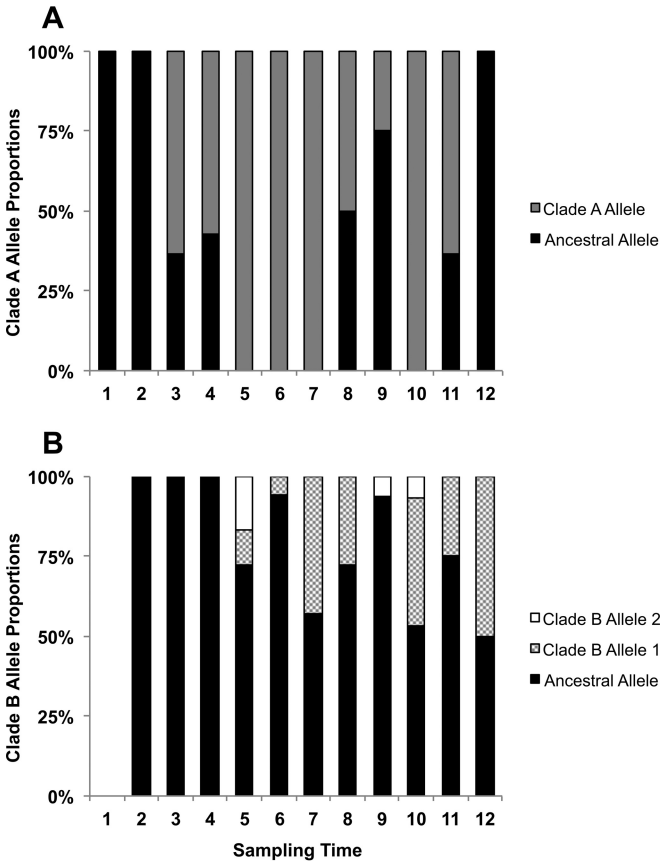

FIG 5 Distribution of *pbpB* alleles in clade A (A) and clade (B) over the 12 sampling time points. Clades A and B share an ancestral major allele. A minor allele is observed specifically in clade A, and two others segregate only in clade B.

DISCUSSION

We have characterized the fine-scale evolutionary dynamics of *P. aeruginosa* during human CF lung infection and have observed a temporal shift in intraspecific population structure consistent with recurrent emergence and expansion of new clonal lineages. Since there is no change in the absolute size of the bacterial population over time, and *Pseudomonas* makes up the bulk of each of these populations (as shown by quantitative PCR [qPCR] analysis and microbiome sequencing, respectively), it is likely that the expansion of any new clonal lineage comes at the cost of preexisting clonal linages. Further, since there is strong evidence for positive selection in the population, we believe the best explanation for these data are recurrent selective sweeps, also known as periodic selection.

Although this patient has been infected with *P. aeruginosa* for at least 12 years, the entire population of *P. aeruginosa* coalesced to a common ancestor less than 3 years before the last specimen sampled for this study, indicating that this sweep went to fixation. It is possible that our sputum-based sampling did not capture the entirety of the *P. aeruginosa* population living within this patient and that there are additional distinct subpopulations of *P. aeruginosa* located in other anatomical compartments. However, if this

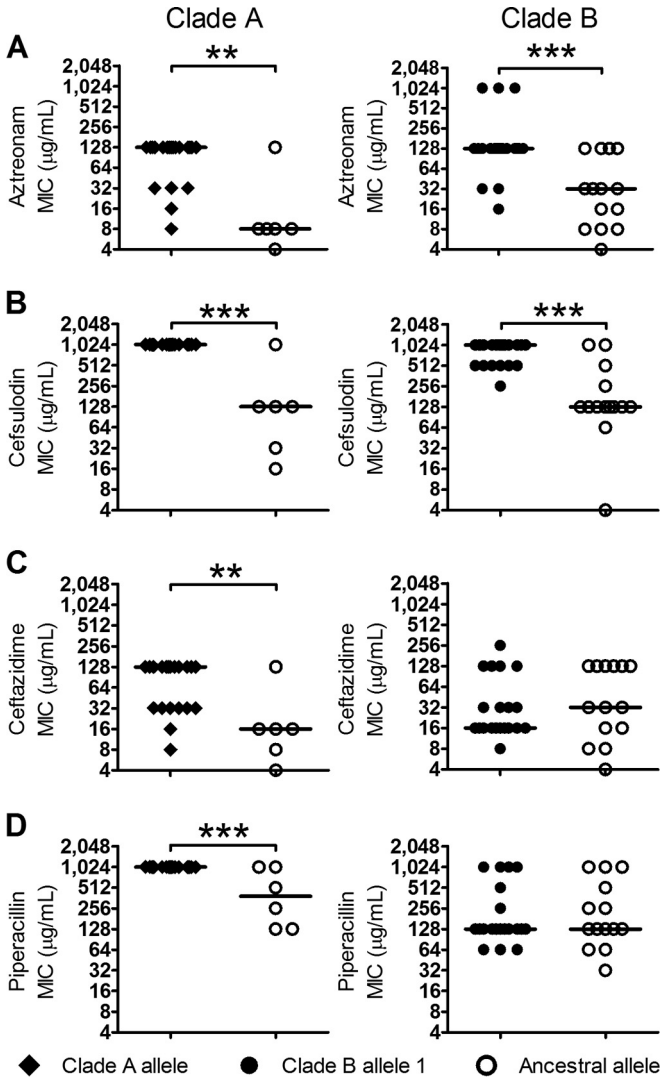

FIG 6 Relationship between *pbpB* alleles and MICs to selected antipseudomonal antibiotics. *Pseudomonas aeruginosa* isolates were grouped by *pbpB* genotype based on the presence of major and minor alleles, as well as clade membership in clade A (ancestral) and clade B (sweep) populations. Antibiotic MICs were assayed for aztreonam (A), cefsulodin (B), ceftazidime (C), and piperacillin (D). The presence of the minor alleles was associated with significantly increased resistance to aztreonam and cefsulodin in both clades but to ceftazidime and piperacillin only for the clade A minor allele. Statistical significance was determined by Mann-Whitney *U* test and is indicated with asterisks. (**, $P \leq 0.01$; ***, $P \leq 0.001$).

were the case, then the migration rate between these subpopulations would have to be so low that we did not detect a single migrant over the year of sampling. Any rare migration from a divergent subpopulation would have been seen in the genealogical structure and time to most recent common ancestry of the overall population. On balance, we believe that such a low rate of migration between anatomical compartments is fairly unlikely and that we have therefore likely sampled the overall diversity of *P. aeruginosa* in this patient.

A second clonal lineage arose ~2 years after the first, giving rise to clade B. We cannot conclusively identify the "origin mutation" that gave rise to this lineage since there are a number of mutations that are fixed between the two clades. Consequently, no single variant is uniquely associated with the origin of clade B. This is perhaps not surprising since clade B originated in the nearly 6-month period between the collection of the first and second specimens. Any mutations that occurred in the emergent clade B common ancestor would be held in linkage disequilibrium with the causative mutation due to the very low rate of recombination.

Incomplete selective sweeps, such as those we observe in clade B, have been documented in other systems and generated substantial experimental and theoretical interest (56–69). A number of hypotheses can be proposed to explain why clade B has not swept to fixation. For example, we may have simply caught the sweep at an intermediate stage on its way to fixation, or the short-term persistence of the two clades could be due to stochastic processes. It is also possible that the ancestor of clade B colonized a distinct anatomical space and has since stayed isolated from its ancestral lineage. However, perhaps the most interesting explanation is clonal interference (70) driven by either competing beneficial mutations, negative epistasis between beneficial and deleterious mutations, or changing selection pressures driven by environmental heterogeneity. An example of the latter can be seen in our PBP3 data. While the clade A PBP3-derived allele confers a fitness advantage relative to the ancestral allele under all antibiotics tested, the clade B-derived allele is more fit than the ancestral allele only under half of the tested antibiotics. This is an example of latent potential for selection (71), in which the selective advantage of an allele is dependent on the environment. Even if the clade B allele is beneficial under certain conditions (such as during aztreonam treatment), it may be disadvantageous in other environments (such as during ceftazidime treatment), thereby, promoting the maintenance of the ancestral population and potentially explaining an underlying mechanism for clonal interference.

The analysis of natural selection within population data is complicated by short-term segregation of genetic polymorphisms, which can confound the more appropriate measure of fixed differences between populations (72). We have attempted to compensate for this issue by comparing *dN*/*dS* signals that emerge in the full data set versus the data set containing only the multispecimen SNPs. While the full data set provides a *dN*/*dS* value that is consistent with the expectation of a purely random mutational process (both synonymous and nonsynonymous mutations occurring at a rate consistent with the number of potential sites), the SNPs that are recovered from multiple specimens (multiple time points) over the course of the study show a signal consistent with strong positive selection in clade B, as would be expected in the case of a selective sweep. Again, it is not possible to use this analysis to identify the causative variant since the sweep population has had very little time for recombination to disentangle the selected substitution from the genomic background.

While *dN*/*dS* values can be difficult to interpret, a particularly notable indicator of selection is the number of loci that have accumulated multiple mutations over the short time since the most recent common ancestor arose. Our population of 233 strains has seven Mpl alleles, six AlgG and MucA/AlgU alleles, five AmpR/AmpC alleles, four PBP3 and MexA/MexB alleles, and three MexT and MucA alleles segregating (Table 3). The probability of even two mutations occurring at the same locus (giving rise to three alleles) is 1.64×10^{-4}, or 0.027 when Bonferroni corrected for 165 independent mutations (107 SNPs and 58 indels). This pattern of multiple independent mutations is a powerful signal of parallel

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pathoadaptation (73) and mirrors the findings of Marvig and colleagues (28), who found extensive evidence for parallel evolution of *P. aeruginosa* in the CF lung. A number of multiply mutated loci overlapped between studies, including those that encode Mpl, MucA, AlgU, MexA/MexB, and LasR. A recent study by Williams et al. (29) also identified the *mpl* locus as carrying homoplasious (parallel) mutations in multiple strains, possibly due to homologous recombination. These patterns of parallel pathoadaptation are particularly striking given that nearly all of the affected loci are involved in either antimicrobial resistance or biofilm production.

The use of deep population sequencing in a single CF patient over a short period reveals community dynamics that are not evident by 16S rRNA gene microbiome sequencing or long-term longitudinal studies. Analyses of CF microbiota using culture-independent techniques (which are typically limited to genus-level taxonomic identification) have demonstrated that *Pseudomonas* commonly dominates microbial communities, is highly resilient, is associated with poor lung function and disease aggressiveness, and is a determinant of community dynamics (5, 6, 74–77). However, these analyses have been limited in their ability to predict temporal variation in patient status, such as the origins of exacerbations, and have not been able to explain the apparent stability of *Pseudomonas* through treatment. Our analysis shows that, although apparently stable at the genus level, *Pseudomonas* (*P. aeruginosa* specifically) can be a highly dynamic pathogen, rapidly adapting to environmental stress, which thus enables it to persist and resist the intended effects of antibiotic therapy.

The rapid evolution of *P. aeruginosa* revealed by deep sequencing in this study may help explain why significant changes in the antimicrobial treatment regimen did not result in any significant change in bacterial community composition. The apparent microbiologic stability observed belies a highly dynamic *P. aeruginosa* population that carries extensive genetic variation in a range of virulence- and resistance-associated loci, which are under strong positive selection. These population dynamics, which are undetectable using lower-resolution methods (e.g., microbiome- or culture-based methods), allow the pathogen to rapidly respond to therapeutic changes and could explain why some changes in treatment do not result in clinical improvement.

The PBP3 data reveal that changes in the selective environment (e.g., different antibiotics) can influence the fitness of strains in unpredictable ways. This observation may even be extended to the microbiome level, where changes in the relative abundance of *Pseudomonas* do not correlate with the anticipated response to antibiotics administered based on their antipseudomonal properties (such as the drop in *Pseudomonas* relative abundance following metronidazole administration at time point 9). Ultimately, we are left with a number of critical questions about the relationships between disease state, treatment approaches, and pathogen evolution. To what extent do irregular and variable antimicrobial treatments drive pathogen population dynamics? Are highly dynamic pathogen populations associated with specific clinical prognoses? Are there population structures or pathogen lineages that would favor better clinical outcomes? Is it possible to select for these lineages, and how could this be accomplished?

This study is limited to a single bacterial species in one infected individual over a defined time period, and thus its results may not generalize to other CF patients, disease stages, or bacterial species. Selective stress in human infection is highly heterogeneous and includes a broad array of differences in host response, microbial milieu, and treatment administration that would be expected to produce various temporal and spatial selective pressures, resulting in multiple patterns of intraspecies evolution. Future efforts to characterize the prevalence and dynamics of different patterns of selection across pathogens, individuals, and disease states are needed to understand their significance in health, disease, and treatment response.

MATERIALS AND METHODS

Specimen collection and microbiological analysis. All protocols for the collection and use of patient sputum were approved by the Research Ethics Boards at the University Health Network (Toronto) and St. Michael's Hospital (Toronto). Sputum samples were voluntarily produced by expectoration over a 350-day period by a single female study patient, CF67. Sputa were processed and *P. aeruginosa* cultured as previously described (40).

Following 72 h of incubation, all cultures were visually inspected and sampled for overall diversity in colony morphology. Twenty colonies resembling *P. aeruginosa* that were oxidase positive and exhibited growth on cetrimide and MacConkey agars (Becton, Dickinson, Hunt Valley, MD) were identified and cryopreserved for further analysis as previously described (38).

To study the bulk *P. aeruginosa* population, patient sputum was also spread plated directly onto *P. aeruginosa*-selective cetrimide agar and incubated at 37°C for 72 h. The resulting lawn of *P. aeruginosa* colonies was scraped from each replicate plate using a sterile inoculating loop and pooled in a single tube of sterile H_2O for population sequencing.

DNA isolation. Genomic DNA was extracted for whole-genome sequencing from 233 individual *P. aeruginosa* isolates and the pooled populations using the DNeasy blood and tissue kit (Qiagen, Ontario, Canada) as per the manufacturer's protocol for Gram-negative bacteria.

Antimicrobial susceptibility testing of *pbpB* variants. MICs for the β-lactam antibiotics aztreonam (Alfa-Aesar, Ward Hill, MA), ceftazidime (Sigma-Aldrich, Ontario, Canada), cefsulodin (A.G. Scientific, Inc., San Diego, CA), and piperacillin (Sigma-Aldrich, Ontario, Canada) were determined for 60 *pbpB* variants (20 per genotype) by agar dilution, as described by the Clinical and Laboratory Standards Institute guidelines (78). Significant differences in fitness defined by variations in MIC associated with clade A or clade B isolates carrying the *pbpB* ancestral major allele, ancestral minor allele, or sweep minor allele were assessed in GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) using a Mann-Whitney *U* test with a 95% confidence interval.

Microbiome analysis and 16S quantitation. Microbiome analysis was performed by analysis of the V5 to V7 hypervariable regions of the 16S rRNA gene using the SI-Seq protocol (38). Sequencing was performed at the University of Toronto, Centre for the Analysis of Genome Evolution and Function (CAGEF). An in-house Java script was used to prepare the sequence for downstream analysis using MACQIIME (79). Chimeric reads were removed using reference-based chimera detection with USEARCH (80). Operational taxonomic units (OTU) were picked against an SI-Seq structured reference set using a similarity of 0.87 (corresponding to the genus level by the SI-Seq method), with premature termination turned off. Taxonomy was assigned using an RDP structured data set using UCLUST with --max_accepts set to 0 and similarity set to 0.97 (empirically determined to produce the highest consistency identification at the genus level using *P. aeruginosa* controls) (80, 81). Unassigned OTU were identified with BLASTN (82). OTU representing less than 0.005% of overall sequence were removed. Quantitative PCR of the 16S rRNA gene was performed as previously described (83).

Sequencing and quality control. Whole-genome sequencing of sampled *P. aeruginosa* isolates was performed on the GAIIx, HiSeq, and MiSeq Illumina platforms. The distribution of the number of bases sequenced per isolate ranged from 167 to 1,426 million bases, and the median was 402 million bases (see Fig. S1A in the supplemental material). This sequencing depth yielded a median read depth per position of 54× (range

25× to 183× [see Fig. S1B]). Sequencing reads were quality trimmed employing Quake and its native k-mer counter (84), and adapters were removed in the CLC Genomics Workbench v. 6.5.1 (Aarhus, Denmark).

***De novo* and reference mapping assembly.** Isolate CF67-71, which was sequenced with 250-bp-long paired-end reads, was assembled *de novo* using the CLC Genomics Workbench (Aarhus, Denmark), using its default assembly settings. Contigs with a scaffolding depth of lower than 10× and/or with a size smaller than 1 kb were removed from this analysis. Contigs that passed this filter were annotated at the RAST server using the native gene caller and Classic RAST as the annotation scheme (85). CF67-71 contigs were used as the reference for mapping assembly of each remaining isolate. We performed six different reference-mapping assemblies using BWA v. 0.7.4 (86), LAST v. 284 (87), and novoalign v. 2/8/03 (Novocraft Technologies), using sequencing reads with and without quality trimming (Fig. 2).

SNP and indel calling. The preliminary list of variants was extracted from the reference-mapping step using SAMtools and BCFtools v. 0.1.19 (88). We then employed a two-step SNP calling process similar to that of Lieberman et al. (89). In the first step, we generated a set of 108 polymorphic positions by identifying the overlapping set of variants called by all six reference-mapping approaches using a custom Java pipeline (available at https://github.com/DSGlab/SNPCallingPipeline). This step used the following criteria: (i) variant Phred quality score of ≥30 and (ii) variants must be found at least 150 bp away from the edge of the reference contig and/or an indel. In the second step, we revised each polymorphic position from the previous step in all of the isolates and included calls that had a Phred score of at least 25. Finally, we tested the sequencing support for either the SNP or the reference base. This multihypothesis correction required that at least 80% of the reads supported the SNP or the reference. If the data did not support either base, then the position was called as an ambiguous base ("N").

We used Dindel v. 1.01 (90) to realign the sequencing reads (mapped and unmapped) and test whether candidate indels produced by BWA and SAMtools were supported by those reads. Putative indel positions were selected as having an indel call in at least one nonreference isolate with the following criteria: (i) a variant Phred quality score of ≥35, (ii) at least 2 forward and 2 reverse reads, and (iii) sequencing coverage of ≥10. Each of these putative indel positions was then reviewed in each of the 233 isolates. An indel was called if the variant had a Phred quality score of ≥25 and the allele frequency was ≥80%. Indel calls where the allele frequency was ≤20% were labeled as ambiguous. All other indel calls were discarded.

Population and single-genome-sequencing evaluation. To confirm that our sampling design adequately sampled the underlying *P. aeruginosa* population, we compared the major allele frequency for each segregating site from individual isolates with that obtained from bulk population sequencing of *P. aeruginosa* isolates collected from 1 of the 12 specimens (see Fig. S3 in the supplemental material).

Sequencing reads from each isolate from specimen 9 were rarified to 1/20th of the total number of reads in the population sequencing experiment such that the overall number of reads would be the same. Reads from both experiments were mapped to the contigs of isolate CF67-71 as described above. Major and minor allele frequencies were calculated using the quality thresholds described by Lieberman et al. (89).

Phylogenetic, population genetic, and coalescent analyses. An alignment of the 107 SNPs was employed to reconstruct the phylogeny of the 233 isolates by both maximum likelihood and neighbor-joining methods using maximum composite likelihood distances and gamma correction ($\alpha = 1$) in MEGA6 (91). The two trees gave nearly identical results. The phylogeny was also investigated with a network-based phylogenetic analysis. SplitsTree4 (92, 93) with the Jukes Cantor distance matrix was used to create a neighbor-net phylogenetic tree.

The time to the most recent common ancestor (tMRCA) among our 233 isolates and between our isolates and the *P. aeruginosa* strain LESB58 was calculated by Bayesian Markov chain Monte Carlo (MCMC) analysis using BEAUTi and BEAST v. 1.75 (43, 44). The parameters deduced from prior information were days between isolation dates and nucleotide substitution model and frequencies. The nucleotide substitution model that best fit our data according to FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html), which is a web application of ModelTest (94), was the general time-reversible (GTR) model (Log likelihood [LnL] = −927.007482, and Akaike information criterion [AIC] = 1,870.014964). The nucleotide substitution frequencies AC = 0.0661, AG = 0.3306, AT = 0.0275, CG = 0.1858, GT = 0.0657, and CT = 0.3241 were estimated employing the Maximum Likelihood Estimate of Substitution Matrix tool in MEGA6 (91). Preliminary MCMC analyses consisting of 10 million generations were employed to infer the appropriate molecular clock and demographic models (95). We inferred the log-normal relaxed uncorrelated molecular clock for the constant size coalescent analysis. Given the estimated priors, the final analysis was run in duplicate through 1 billion MCMC generations sampled every 1,000 MCMC generations, and the burn-in period was set at 100 million MCMC generations. The inferred molecular clock was consistent with the number of mutations observed in our isolates through time (see Fig. S7 in the supplemental material).

Population genetic tests were performed with DnaSP v. 5.10.01 (96). *P* values were determined via coalescent simulations as implemented in DnaSP.

Interstrain whole-genome alignment and phylogeny. The full genomic sequences of the *P. aeruginosa* strains LESB58, PAO1, DK2, PA7, NCGM2, UCBPP, B136, and M18 were obtained from the *Pseudomonas* Genome Database (97). These sequences and the contig sequences of the *de novo*-assembled isolate CF67-71 were aligned using Mugsy v. 1.2.3 (98). The output alignment file in MAF format was converted to the more broadly used FASTA format in the Galaxy platform (99). Finally, the neighbor-joining phylogeny (see Fig. S4 in the supplemental material) was inferred using the CLC Genomics Workbench v. 6.5.1 (Aarhus, Denmark) with a bootstrap value of 500.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00981-15/-/DCSupplemental.

Figure S1, TIF file, 2 MB.
Figure S2, TIF file, 2.6 MB.
Figure S3, TIF file, 1.5 MB.
Figure S4, TIF file, 1.2 MB.
Figure S5, TIF file, 1.4 MB.
Figure S6, TIF file, 1.7 MB.
Figure S7, TIF file, 1.2 MB.
Table S1, DOCX file, 0.01 MB.
Table S2, DOCX file, 0.02 MB.

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FORM1 (v0.2 Oct 2020)

CoE Research Service Research Grants Outline Application

Application to:

Project code: *Internal use only*

1. Title: Whole genome sequencing of potable water derived *Cupriavidus* species

2. Principal applicant, Institution and email address:

Professor Alistair Leanord,

Scottish Microbiology Reference Laboratory Glasgow,

Alistair.leanord[redacted]

3. Co-applicants(s) and Institution(s):

Derek Brown, Scottish Microbiology Reference Laboratory Glasgow

Aleks Marek, Scottish Microbiology Reference Laboratory Glasgow

Cosmika Goswami, University of Glasgow

Stephen Fox, University of Glasgow

Teresa Inkster, Queen Elizabeth University Hospital, Glasgow

4. Duration of project and total funding requested from CoE Research Service:

Duration: 3 months

Total Funding: £[redacted]

5. Lay summary: Concerns about the part that the hospital environment plays in infections has highlighted the importance of water as a source of bacteria that have the potential to infect patients. Drinking water is not sterile and can contain a number of different kinds of bacteria. We do not know what range of bacteria are normally in hospital water One way of tracking these bacteria is to use sequencing of genes to allow us to look at the relationships between bacteria to better understand how they interact. We plan to focus on one of these bacteria , *Cupriavidus*, collect them from a range of hospital waters and see firstly, what the population looks like and secondly, to see if there are genetic differences between those that infect patients and those that do not. Very few reports on the genetic characterisation of this bacteriua exist in the literature.

6. What is the uncertainty that is highlighted in available literature and which gives rise to the research question(s) and what is the importance of the research proposed?

Research questions:

Main question: What is the genetic variability of *Cupriavidus* species isolated from potable water and environmental sources?

Secondary question: Is there evidence of transmission events between the environment and patients within samples collected?

Secondary question: What genes, if any, are associated with environmental and/or clinical isolates?

Secondary question; Current national guidance asks laboratories to select a single colony from an agar plate for typing of positive water samples. This approach may underestimate exogenous acquisition of waterborne pathogens. Outbreaks may be polyclonal, particulary if mature and extensive biofilm is present in pipework/outlets. Not detecting a polyclonal outbreak is a lost opportunity for future preventative measures.

This will have direct translation as an existing sequencing pipeline with a background genomic population that can be used, in near real time, for the understanding of transmission dynamics, both as part of an outbreak and as an understanding of the ecology of *Cupriavidus* within water systems.

7. What is the intended impact of the research? Who will use the research findings, for what purposes, and how will they be disseminated to maximise impact?

This work will describe the environmental transmission dynamics of *Cupriavidus*, an organism which is common in water and capable of causing significant infections in susceptible patients.

This work will:

- Describe the natural genomic population of *Cupriavidus* that will allow for the placement of outbreak strains into an ecological context
- Develop the sequencing pipeline that can be used for outbreak investigation
- Develop understanding of which virulence genes allow for human infection
- Inform guidance on the typing of isolates during water related outbreaks with a view to detection of polyclonal outbreaks
- Be part of any Board program that gives assurance around water safety

The outputs will be used by Infection Control specialists, Estates and Facilities management, Board Clinical Governance and Board Senior management to investigate, reassure and monitor water safety within the Healthcare build environment.

Dissemination will be via a report to NHS Scotland Assure, submitting Boards and via publication.

Raw sequence data, with minimal metadata, will be made available to the wider scientific community by submission to the European Nucleotide Archive.

8. Research plan

1. Isolates from clinical infections and from environmental sources, already collected over a 3 year period (2018-2020) from GGC will be sequenced using an already developed bioinformatic pipeline. 152 isolates will be available for immediate sequencing over a 2 month period. SNP analysis will be used to describe both the genetic heterogeneity and possible transmission events.
2. Further analysis of identified clinical and environmental isolates genes looking for genetic associations in these organisms will be done using already developed analytical methodology as used for *E.coli* and *S.aureus* as part of the SHAIPI genomics work.

3. A separate concurrent study is looking to see how common it is to find Cupriavidus in healthcare water systems across Scotland and England. As the water is being tested in the Glasgow laboratory it will provide the opportunity to perform whole genome sequencing on several isolates from the same sample to further aid establishing biodiversity in water systems. This would add £[redacted] to the costs which would permit forty extra isolates to be genome sequenced – the number of colonies to be tested from specimen will be determined by the number of positive water samples.

9. Subject Matter Experts

Tim Wafer: Water Solution Group.
Ginny Moore ginny.moore[redacted].
Susanne Lee susannelee[redacted].

10. References

Kweon OJ, Lim YK, Kim HR, Kim TH, Ha Sm, et al. (2020) Isolation of a novel species in the genus *Cupriavidus* from a patient with sepsis using whole genome sequencing. PLOS ONE 15(5): e0232850.
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11. Funding requested from CoE Research Service

Clinical Researchers x2 for 1 month = £[redacted]

Sequencing costs 192 isolates x £[redacted] = £[redacted]

12. Declaration

Principal Applicant: To my knowledge the project outline described here represents the ideas, concepts and writings of myself and co-investigators and is not a modification of projects submitted by others elsewhere.

Signature of Principal Applicant	Name	Date
[redacted]	Alistair Leanord	22/12/2020

Sponsor(s): I agree to be sponsor/co-sponsor/joint sponsor (delete as appropriate) for this project under the requirements of the UK Policy Framework for Health and Social Care Research.

Signature *for and on behalf of the Sponsor Organisation(s)*	Name/Organisation	Date
[redacted]	Rob Gardiner NHS GGC	30/12/2020
		Click here to enter a date.

Please return completed outline application to Geraldine.OBrien[redacted], michael.weinbren[redacted] & Sarah.Gibson[redacted]

Outlook

FW: WGS RESEARCH PROJECT

From Teresa Inkster [redacted]
Date Wed 09/10/2024 15:35
To teresa inkster [redacted]

1 attachments (359 KB)
Cupriavidus CoE WGS proposal 221220 Final v3 Al edits.docx;

From: Leanord, Alistair [redacted]
Sent: Monday, January 18, 2021 9:15 AM
To: Michael Weinbren [redacted]; Inkster, Teresa [redacted]; Sarah Gibson [redacted]; Geraldine O'Brien [redacted]; Chris McVey [redacted]
Subject: RE: WGS RESEARCH PROJECT

Thanks Mike

Changed the final amount requested to reflect the increased sequencing ask.

All else fine.

Al

From: Michael Weinbren
Sent: 17 January 2021 18:05
To: Inkster, Teresa [redacted]; Sarah Gibson [redacted]; Geraldine O'Brien [redacted]; Chris McVey [redacted]; Leanord, Alistair [redacted]
Subject: WGS RESEARCH PROJECT

Hi,

Have made some changes- this will allow the testing of multiple picks from water samples being collected from the other Cupriavidus project.

Results of which will give us further information on biodiversity.

Alistair ? Teresa could you let me know if this is Ok?

Best wishes,

Mike

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Patterns of colonization by *Pseudomonas aeruginosa* in intubated patients: a 3-year prospective study of 1,607 isolates using pulsed-field gel electrophoresis with implications for prevention of ventilator-associated pneumonia

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Abstract *Objective:* To identify routes and patterns of colonization with *Pseudomonas aeruginosa* in intubated patients to design strategies of prevention for respiratory infection. *Design and setting:* Prospective and observational study in the 16-bed intensive care unit of a teaching hospital. *Patients and participants:* Ninety-eight intubated patients were investigated over a 3-year period. Those ventilated less than 72 h were excluded. *Measurements and results:* Samples from the tap water from each patient's room, stomach, oropharynx, subglottic secretions, trachea, and rectum were collected when the patient was intubated, and then three times per week. Pulsed-field gel electrophoresis was performed to type the strains. We identified 1,607 isolates pertaining to 35 different pulsotypes. Overall 54.2% of patients presented colonization, and tracheal colonization was present in 30.5%. Ten patients had colonization at intubation, and four of these developed ventilator-associated pneumonia (VAP) after a mean of 4±2 days. ICU-acquired colonization occurred in 31 patients, and 4 of these developed VAP after a median of 10±5 days. *P. aeruginosa* was isolated from the room's tap water in 62.4% of samples. More than 90% of tap water samples had pulsotypes 1 and 2, which were frequently isolated in the stomach (59%) but were only rarely associated with VAP. *Conclusions:* Although colonization/infection with *P. aeruginosa* in intubated patients tends to be endogenous, exogenous sources should not be ruled out. A combination of early identification (and eradication) of airways colonization by *P. aeruginosa* plus infection control measures targeted to reduce cross-contamination should be the basis to prevent pulmonary infection.

Keywords *Pseudomonas aeruginosa* · Colonization · Pneumonia · Mechanical ventilation · Intensive care

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Introduction

Pseudomonas aeruginosa (PA) is highly endemic in intensive care units (ICUs), where it causes urinary tract infections, surgical wound infections, bacteremia, and pneumonia [1, 2]. According to the report of the National Nosocomial Infections Surveillance System [3], PA is the second most frequent pathogen in ICUs. Although colonization by PA frequently precedes overt infection, the origin of the organism and the precise mode of transmission are often unclear [4, 5]. The origin may be exogenous (the environment, other patients, or respiratory equipment) or endogenous (the oropharynx, stomach, or gut). Establishing the source of strains causing colonization is crucial for the development of effective preventive measures.

The main goal of this study was to identify the routes of colonization by PA in intubated patients and the sources of the strains causing colonization or respiratory infection over a long period and without an epidemic outbreak. The findings may have important implications for the design of evidence-based preventive measures for ventilator-associated pneumonia (VAP). A secondary goal was to determine whether patients were colonized or infected with a single or with multiple genotypes of PA, an issue that may have implications for diagnosis.

Material and methods

This prospective study was conducted over a 35-month period (1 April 1996 to 28 February 1999) in a medical-surgical ICU of a teaching hospital. The Human Subjects Review Committee of the Hospital approved the study protocol (96-027).

Study population

The study originally enrolled 98 patients, 26 of whom were later excluded because the duration of MV was less than 72 h; thus 72 patients were finally evaluated (Table 1). All patients intubated with Hi-Lo Evac (Mallinckrodt Laboratories, Athlone, Ireland) were eligible; patients with tracheotomies and different tubes were not included. A maximum of three patients were evaluated simultaneously. The nurse-patient ratio was 1:2. The Acute Physiology and Chronic Health Evaluation II score [6] was used to assess the severity of illness at admission. The infection control measures used in the ICU during the study period consisted of hand hygiene with clorhexidine, use of gloves, and cleaning once daily with tap water of the sink and environment surfaces using glutaraldehid solutions. Eight patients died during the study.

Table 1 Characteristics of the 72 patients included in the colonization study

Age (years)	65±17
Sex: M/F	51/21
APACHE II at admission	20±8
Cause of ICU admission	
Respiratory failure	40.8%
Cardiovascular failure	22.5%
Neurological diseases	15.5%
Renal failure	8.5%
Metabolic disturbances	5.6%
Gastrointestinal diseases	4.2%
Hematological disturbances	2.8%
Days in the study (range)	7.6±5.5 (3–21)
Days of ICU stay (range)	18.5±17.1 (3–91)

Surveillance cultures

Samples from gastric aspirate, oropharyngeal swab, subglottic secretions, internal surface of endotracheal tube, tracheal secretions, and rectal swab were taken in each patient after intubation and then three times per week. Tap water from the individual ICU room was also cultured at admission and every 72 h. Follow-up was considered complete at 21 days of mechanical ventilation (MV) or when the patient was extubated, a tracheotomy was carried out, the patient died, or an episode of VAP by PA was diagnosed. Surveillance cultures from environmental surfaces of the ICU and from the hands of health care workers (HCW) were taken on five separate days during the study period, without prior warning.

Definitions

Colonization was defined as the isolation of PA from specimens taken from any body site studied in the absence of infection. Colonization at intubation was defined as that occurring within 24 h of entry into the study. When colonization occurred more than 48 h after entry in the study, it was defined as ICU acquired. The trachea was considered as the initial site of colonization when no prior colonization was documented at other sites. The routes of PA colonization leading to the trachea were determined by analyzing the chronological comparison of the pulsotypes (PTs) obtained from the different samples.

Primary or endogenous colonization was defined as colonization by a strain of PA that had not been previously isolated from another studied patient, the hands of HCW, or another site analyzed in the ICU environment. Exogenous colonization was defined as colonization by a strain of PA with a PT previously isolated from another studied patient, hands of HCW, or another environmental surface in the ICU. Diagnosis of VAP has been reported elsewhere and confirmed by a positive protected specimen brush culture containing at least 10^3 colony-forming units (CFU)/ml, a positive bronchoalveolar lavage culture with at least 10^4 CFU/ml or by quantitative endotracheal aspirate with 10^6 CFU/ml or more [7, 8].

Microbiological and bacterial typing

Hands of HCW were studied by the rinse sampling method [9]. All surfaces studied and taps were studied by the wipe-rinse sampling method or the swab-rinse sampling method [9]. Samples were plated in cetrimide agar plates and in nonselective broth. Pharyngeal swab, gastric aspirate, subglottic secretions, tracheal aspirate, endotracheal tube, and rectal swab of each patient were sampled on cetrimide agar plates and chocolate agar plates, and the cultures were reported semiquantitatively. Whenever possible at least four colonies representative of the different morphological types of PA present on the culture plate were picked for subsequent characterization beyond species level.

Pulsed-field gel electrophoresis (PFGE) was performed using a previously described method [10]. Electrophoresis was performed with a Chef DRIII System apparatus (Bio-Rad, Richmond, Calif., USA) under conditions appropriate for the enzymes. Analysis of PFGE profiles was made with the software Bio Image Whole Band Analyzer (Genomic Solutions, Ann Arbor, Mich., USA).

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Each strain was first compared with all others to calculate similarity using the Dice correlation coefficient (S_D). S_D was defined as twice the number of shared bands divided by the total number of bands. Isolates were defined as belonging to the same clonal lineage if their patterns exhibited less than six band differences (two genetic events) [11]. The most common profile among the isolates of each lineage was designated as the parental profile. Numbers designated strains corresponding to each major macrorestriction type, and a letter suffix indicated each of their subclonal variants (subtypes). The patterns were ordered chronologically according to time of collection. All isolates were first digested with *Xba*I. Moreover, a representative strain of the different PTs and subtypes were subjected to further digestion with a second enzyme (*Dra*I). The final types and subtypes are the result of the combined two-enzyme analysis.

Statistical analysis

Descriptive analysis was performed. Continuous variables were expressed as means (±SD). Associations of categorical variables were assessed with the χ^2 test or with Fisher's test.

Results

In 39 (54.2%) PA colonization could be proven during the study period (Fig. 1). Ten patients (13.9%) were already colonized at intubation. Two of these acquired different PA strains during the study period. Thus 31 patients (43%) were colonized during the period of MV and were classified as ICU acquired.

Genotyping

When available, four colonies were subcultured and independently typed, and a total of 1,612 isolates were obtained; 5 of these could not be studied by PFGE. Thirty-five different PFGE patterns or pulsotypes (PTs) were found. Among 279 cultures performed from the five environmental surveillance cultures 41 (14.7%) were positive for PA, and 15 different PTs were identified: the most frequent were PT1 (31.7%), PT7 (17.1%), and PT8 (14.6%). The cultures from the inanimate surfaces of the ICU were positive for PA in 34.3% of samples and the hands of HCW in 6% of cases. On the other hand, 93 of 149 (62.4%) cultures from the tap water of patients' rooms were positive for PA. Two PTs were identified in 26 cultures. Eleven different PTs were identified among PA cultures isolated from tap water, PT2 (74.2%) and PT1 (32.3%) being the most frequently isolated.

In the 39 colonized patients 288 positive cultures for PA were obtained. In this case, 22 different PTs were obtained, PT2 (51.3%) and PT1 (41%) being the most frequently isolated. The distribution of PTs differed significantly in the stomach than in other sites (Fig. 2).

Of the cultures analyzed 13% were polyclonals, 24.8% of environmental and tap water cultures, but only 7.3% of those from the various patient sites ($p<0.05$). The stomach (19.1%) and rectum (14.3%) had the highest incidence of polyclonals. Only one of the lower respiratory tract cultures was polyclonal.

Pseudomonas aeruginosa colonization

Ten of the 39 patients with PA (25.6%) were already colonized at intubation. Eight had prior hospitalization, and three were intubated after the day of admission in the ICU. Two had multiple PTs. Seven of the 13 strains of PA isolated in this subset (53.8%) had primary colonization (single PFGE pattern). The remaining six strains (46.2%) were considered exogenous colonization (three from tap water). Detailed information on distribution is shown in Table 2. Only four strains were initially isolated from the digestive tract (stomach or rectum).

Most of the 31 patients who suffered ICU-acquired PA colonization during ventilation presented simultaneous colonization at multiple sites. Distribution of colonization and days of colonization is shown in Table 3. Among the 31 patients with ICU-acquired colonization 47 strains of

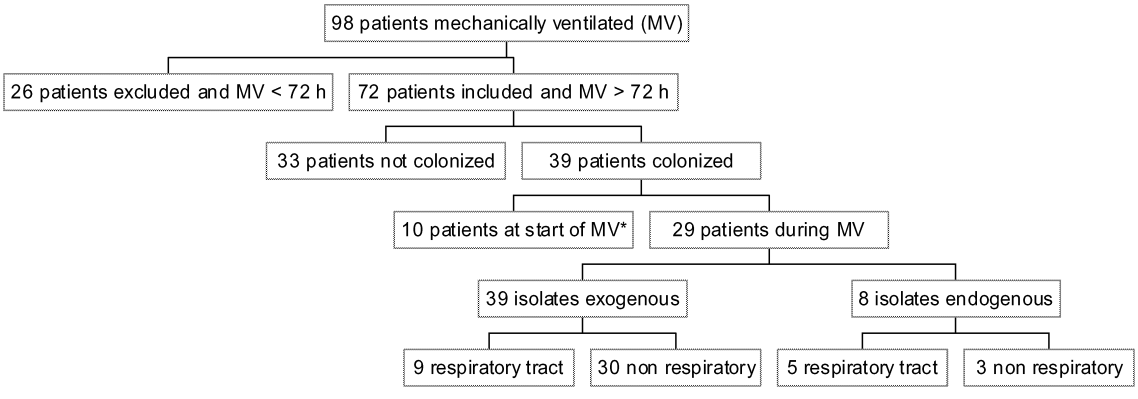

Fig. 1 Patients colonized by *P. aeruginosa* during the period of the study (*Two patients colonized by different strains during ICU stay)

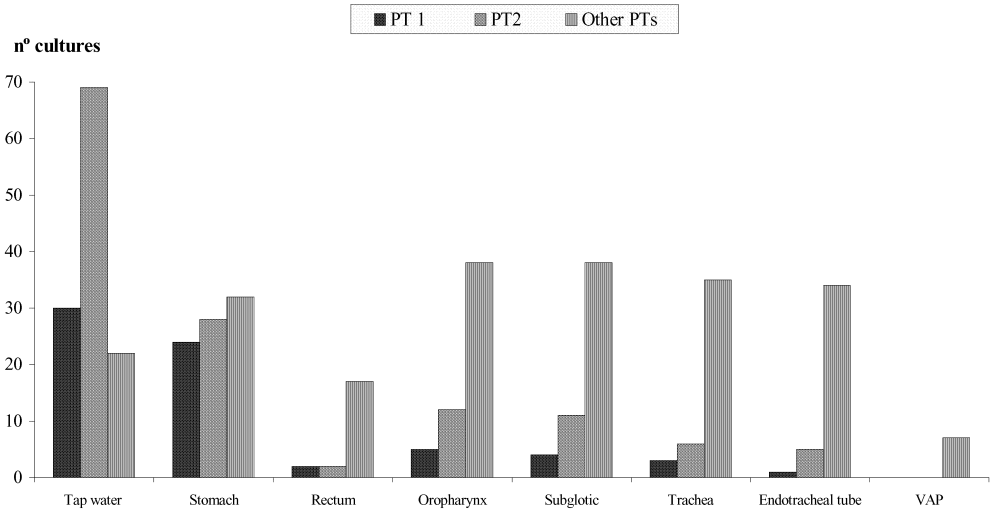

Fig. 2 Distribution of pulsotypes 1 and 2 and the remaining pulsotypes according to localization

$p < 0.01$ for pulsotypes 1 and 2 when comparing stomach with other sites from colonized patients

Table 2 Pattern of colonization among 10 patients colonized at onset of mechanical ventilation; first day of isolation in each site (*S* stomach, *Ophx* oropharynx, *SG* subglottic, *ET* endotracheal tube, *T* trachea, *R* rectum, *VAP* ventilator-associated pneumonia, *E* exogenous, *P* primary, *PT* pulsotype)

Patient no.	Intubation day	PT	Tap water	Localization (first day detected colonization)						VAP	Source
				S	Ophx	SG	ET	T	R		
8	1	1	1	–	–	–	–	–	–	–	P
		5	1	–	–	–	–	–	–	–	
		6	–	1	–	1	1	1	–	3	
31	1	1	1	1	–	–	–	–	–	–	E
53	1	1	1	–	–	–	–	–	–	–	P
		2	1	–	–	–	–	–	–	–	
		16	–	1	1	1	1	1	–	5	
55[a]	1	18	–	–	–	–	–	–	1	–	P
57	3	22	1	1	1	3	3	3	3	–	E
		19	–	–	–	–	–	–	1	–	P
		21	–	–	–	–	–	–	1	–	P
75	6	22	–	1	1	1	3	1	–	–	E
88	4	2	1		1	6	–	9	–	–	E
		34	–	3	3	1	1	1	–	–	P
90	1	1	1	–	–	–	–	–	–	–	P
		35	–	1	1	1	1	1	–	–	
92	1	1	1	–	–	–	–	–	–	–	E
		2	1	–	–	–	–	–	–	–	
		7	–	3	3	3	1	3	3	6	
94[a]	1	1	1	–	–	–	–	–	–	–	E
		6	–	3	1	1	3	3	–	5	

[a] Patients also colonized with different pulsotypes during the period of mechanical ventilation

PA were isolated. Eighteen patients were colonized with a single PT. Of the 47 strains of PA 39 (83%) were classified as exogenous. The stomach was the site with the highest incidence of exogenous colonization (94% of strains). PT1 and PT2 isolated from the tap water and environment were the most frequent PTs colonizing patients with ICU-acquired colonization. Excluding these PTs, the majority of the remaining PTs were considered endogenous. Detailed information on body sites colonized and PTs of PA in this group of patients is shown in Fig. 3 and Table 3.

Most of patients included in the study (94.4%) received antibiotic treatment prior to PA colonization. Sixteen patients (22.2%) were treated with third-generation cephalosporins, and 35 of 72 patients (48.6%) received antibiotics affecting PA. Only the prior treatment with

Table 3 Sequences, first day of colonization and sources of strains of *P. aeruginosa* in 31 patients with colonization acquired during mechanical ventilation; first day of isolation in each site (*S* stomach, *Ophx* oropharynx, *SG* subglottic, *ET* endotracheal tube, *T* trachea, *R* rectum, *VAP* ventilator-associated pneumonia, *E* exogenous, *P* primary, *PT* pulsotype)

Patient	Intubation day	PT	Tap water	S	Ophx	SG	ET	T	R	VAP	Source
2	2	1	1	3	3	–	3	3	–	3	E
		2	1	3	3	–	–	–	–	–	E
4	1	1	1	6	–	–	–	–	–	–	E
		2	1	6	–	–	–	–	–	7	E
		3	–	–	–	–	–	–	–	–	P
5	1	1	–	3	–	–	–	–	–	–	E
		2	3	–	3	3	–	–	3	–	E
		4	–	–	–	–	–	–	–	–	P
6	1	1	6	15	–	–	–	–	–	–	E
		2	6	12	–	–	–	–	–	–	E
7	1	2	3	3	–	–	–	–	–	–	E
11	1	1	–	–	3	3	–	3	–	–	E
		2	1	–	–	–	–	–	–	–	
13	4	1	–	3	3	3	–	–	3	–	E
		2	1	–	–	–	–	–	–	–	
14	1	2	1	3	–	–	–	–	–	–	E
16	1	2	1	–	6	–	–	–	–	–	E
18	1	1	1	–	–	–	–	–	–	–	P
		10	–	–	6	6	–	–	9	–	
21	1	2	1	–	–	–	–	–	–	–	P
		11	–	–	–	9	9	9	–	–	
22	1	2	1	3	3	3	–	–	–	–	E
26	3	12	–	–	–	–	9	9	–	–	P
32	1	1	–	3	–	–	–	–	–	–	E
36	1	2	3	6	6	–	–	–	–	–	E
43	1	1	–	9	–	–	–	–	–	–	E
		14	1	3	9	9	9	9	9	–	E
		15	–	–	–	–	–	–	–	–	
55[a]	1	2	1	15	–	–	–	–	–	–	E
		17	1	15	18	18	21	21	18	–	E
66	1	2	3	3	–	–	–	–	–	–	E
68	2	1	1	3	–	–	–	–	–	–	E
		2	1	9	–	–	–	–	15	–	E
		23	1	–	–	–	–	–	–	–	
69	2	1	1	–	–	–	–	–	–	–	–
		2	3	6	6	9	12	6	–	–	E
71	1	1	1	3	–	3	–	–	–	–	E
72	1	2	1	3	–	–	12	–	–	–	E
		24	3	–	–	–	–	–	–	–	
		25	9	3	6	3	6	3	–	–	P
73	1	1	–	3	–	–	–	–	–	–	E
		2	1	6	–	–	–	–	6	–	E
		26	–	–	–	–	–	–	–	–	P
76	1	1	1	12	–	–	–	–	–	–	E
		2	1	9	–	–	–	–	–	–	E
77	1	1	–	6	3	3	–	6	–	–	E
		2	1	–	6	3	–	–	–	–	E
		27	1	–	–	–	–	–	–	–	
78	1	28	1	3	6	9	9	9	6	–	E
82	1	2	1	12	–	–	–	–	–	–	E
83	3	1	–	–	3	–	–	–	–	–	E
		2	1	–	–	–	–	–	–	–	
		27	9	–	–	–	–	–	–	–	
		29	–	3	3	6	6	6	–	17	P
86	3	2	1	–	–	–	–	–	–	–	E
		7	–	–	3	3	6	6	–	8	
		17	–	6	–	–	–	–	–	–	E
89	1	2	1	3	9	3	6	3	–	–	E
		24	1	–	–	–	–	–	–	–	
94[a]	1	1	1	3	–	–	–	–	3	–	E

[a] Patients also colonized with different pulsotypes at beginning of mechanical ventilation

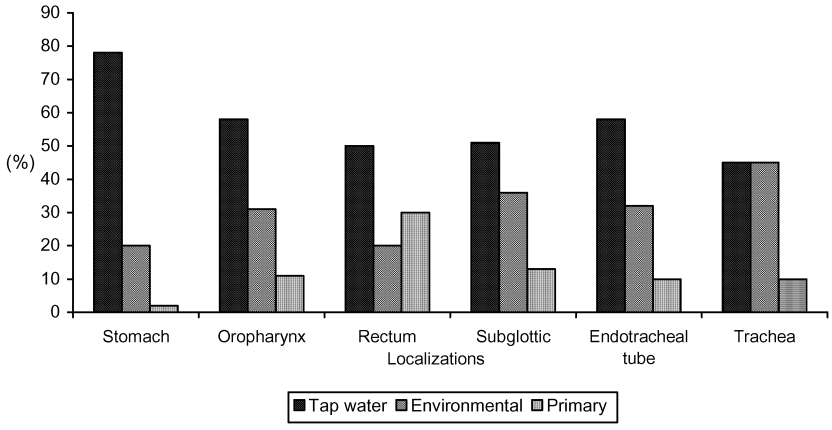

Fig. 3 Origin of *P. aeruginosa* strains according to localization among 31 patients with ICU-acquired colonization

Trachea vs. stomach between strains originated from tap water ($p< 0.01$)

third-generation cephalosporins was associated with a risk of colonization with PA (p=0.03).

Respiratory colonization

In our study 22 of 72 patients (30.5%) had colonization of the lower respiratory tract, 5 (6.9%) at intubation. Among the five patients colonized at the onset of MV four were primary. In contrast, 14 of 17 PTs isolated from the 17 patients with ICU-acquired tracheal colonization were of exogenous origin. Eight of these 17 PTs simultaneously colonized airways and gut; in three cases the first site colonized was the stomach. Only one patient had two different PTs.

Ventilator-associated pneumonia by *Pseudomonas aeruginosa*

Eight patients (11.1%) presented VAP caused by PA. Seven were confirmed by protected specimen brush and one by quantitative tracheal aspirate. The mean period of MV prior to the VAP onset was 7±4.7 days (range 3–17). Only two patients presented VAP within the first 4 days of MV. Among the five patients with upper respiratory tract colonized at onset of MV, four had VAP with 4±2 days (range 3–7) of intubation. The remaining four episodes of VAP were diagnosed in patients with ICU-acquired colonization, and the mean period of MV prior to the diagnosis of VAP was 10±5 days (range 6–17). The respiratory tract in seven of eight patients with VAP had been colonized previously by the same strain causing the pneumonia. The initial site of colonization for these strains was the upper airways in seven of eight patients (87.5%), but in four cases simultaneous digestive tract colonization was found. PFGE typing identified neither the stomach nor the rectum as the only initial site of colonization in any case. The mean period of previous tracheal colonization before the diagnosis of pneumonia was 4.3±3.4 days (range 2–11). In four cases (50%) the origin of strains causing VAP was considered exogenous (one tap water). In the remaining four patients with VAP the strains causing infection were considered primary.

Discussion

This study is unique in evaluating more than 1,600 isolates over a long period or time. More than one-half of intubated patients were colonized with PA, and this colonization was not limited to the respiratory tract. An important finding was that the source of strains causing ICU-acquired colonization was predominantly environmental, in most cases the tap water from the patient's room. However, this colonization was mainly in the gastrointestinal tract and did not affect the clinical course of the patients, because our results showed that the strains causing respiratory infections were endogenous in one-half of VAP episodes, emphasizing the limitations of infection control measures to prevent VAP by PA.

The incidence rate of colonization by PA in a range the ICU ranges between 4.5% and 37% [5, 12, 13, 14]. Our incidence was higher, although comparison of incidence rates with those other ICUs is difficult, mainly because of differences in patient populations and in the samples analyzed. Indeed, in our study among only the patients with prolonged MV (>72 h) the incidence rate of colonization by PA was 54.2%. Differences in patients who were already colonized at intubation, colonization pressure, and number of samples may explain these differences [15].

The stomach showed the highest incidence (84%) of ICU-acquired colonization, and this location was the earliest site of colonization in most of the patients. Previous studies [5, 16] have suggested that the origin of digestive colonization was endogenous, but they did not

simultaneously analyze patients' samples and environmental and tap water cultures, nor did they subculture and independently type four colonies. In our study more than 60% of the tap water in the ICU was contaminated by PA, mainly PT1 and PT2, the PTs that predominated among strains isolated from the stomach. These results suggest that the origin of gastric colonization by PA is predominantly exogenous, and that PA was transmitted directly through the water infused in the stomach by gastric tube or indirectly via handling by the HCW after handwashing. Tap water and sinks have been recognized as the origin of outbreaks of respiratory and urinary infections in pediatric and adult ICUs due to PA [17, 18, 19].

Our findings agree with a study carried out in a surgical ICU [17]. A follow-up study in the same surgical ICU [20] confirmed that all taps in the patients' rooms were contaminated with PA, and in 32% of cases a tap water isolate from the room was shown to be of the same genotype as the patient's isolate. Our findings suggest that in our ICU the tap water was the source of acquisition because in most cases the detection of PA took place prior to the detection of a strain in the patient, and because the tap water was colonized mainly by the same PTs (1 and 2) whereas the pattern of cultures of other environmental surfaces of the ICU and patient samples was more heterogeneous.

Our results indicate that the clinical importance of gastrointestinal colonization by PA in mechanically ventilated patients is low. Indeed, although most of our ventilated patients presented a gastric ICU-acquired colonization, we found that tracheal ICU-acquired colonization was present in only 23.6% of patients and was due predominantly to strains with PTs other than those found in tap water and in the stomach. Moreover, when we analyzed only patients with VAP caused by PA, no strain was found previously in the stomach. These results agree with a study on the pathogenesis of VAP [21] and demonstrate that a gastric origin is unlikely in strains of PA causing VAP.

This study is also unique in subculturing at least four colonies that were representative of the different morphological types of PA present on each culture plate. It allowed the origin of the strains to be identified and reduced the risk of underestimating exogenous colonization. Unlike environmental strains, isolates from airways were mainly monoclonal. The implications for diagnosis and therapy in clinical practice are evident.

One particularly interesting finding was that strains present in tap water or in the environment were associated with colonization, but only infrequently with infection. Indeed, our results show that more than 70% of strains causing gastric or respiratory colonization come from tap water or environmental surfaces of the ICU, whereas among strains causing pulmonary infection, 50% were of endogenous or primary origin, and only one of the remaining strains come from tap water. Further studies analyzing exoproducts or different virulence proteins secreted by different strains of PA may help to explain these findings. In fact, recent studies [22, 23] have reported that isolates of PA able to secret type III proteins are of increased virulence, and pulmonary infections caused by these strains are associated with higher mortality or recurrence. Further studies should investigate whether intubated patients colonized with type III secreting isolates are at a higher risk of developing pneumonia than patients colonized by nonsecretory strains of PA.

Our findings suggest that efforts to prevent colonization by PA should be directed toward decontamination of tap water and infection control measures that reduce cross-contamination from exogenous sources. However, recent reports [17, 20] have noted how difficult it is to reduce colonization in tap water. Cross-contamination from exogenous origin was involved in one-half of our episodes of pneumonia, and our findings support the use of alcoholic solutions for hand antisepsis [24]. In fact, our findings changed the infection control strategies in our ICU: only mineral water to administer drugs through the gastric tube, and alcohol solution was used before contact with a new patient.

Important limitations of our study were that not all intubated patients were consecutively included, and that samples for culture were obtained each 72 h. Cross-contamination from other colonized patients could thus not be detected, and in the cases with simultaneous detection of PA at different sites the true sequence of colonization remains uncertain. Other studies applying similar methods for sample collection (twice weekly) have had the same problem of interpretation [25]. Another limitation is the that only a reduced number of surveillance cultures of the environmental surfaces and hands of HCW were carried out. Although the incidence rate of hands colonized by PA was similar to that in a study [26] carried out in a neonatal ICU with endemic PA infection, increasing the number of cultures from environment and HCW should allow us to determine more exactly the importance of cross-colonization. Finally, our findings should not be generalized to ICUs with different colonization pressures and case-mixes.

In summary, although the origin of PA colonization in intubated patients is often exogenous, in patients who developed pulmonary infection the origin can be either exogenous or endogenous. Our results confirm that the ICU environment is a major source of micro-organisms colonizing critically ill patients and in particular emphasize the importance of exogenous colonization/infection of PA. A combination of early identification (and eradication) of airways colonization by PA plus infection control measures targeted to reduce cross-contamination should be the basis to prevent pulmonary infection.

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METHOD

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Bacterial genomic epidemiology with mixed samples

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Abstract

Genomic epidemiology is a tool for tracing transmission of pathogens based on whole-genome sequencing. We introduce the mGEMS pipeline for genomic epidemiology with plate sweeps representing mixed samples of a target pathogen, opening the possibility to sequence all colonies on selective plates with a single DNA extraction and sequencing step. The pipeline includes the novel mGEMS read binner for probabilistic assignments of sequencing reads, and the scalable pseudoaligner Themisto. We demonstrate the effectiveness of our approach using closely related samples in a nosocomial setting, obtaining results that are comparable to those based on single-colony picks. Our results lend firm support to more widespread consideration of genomic epidemiology with mixed infection samples.

DATA SUMMARY

Supplementary figures, tables, and a file describing the implementation details of Themisto have been submitted to the Microbial Genomics figshare account: https://doi.org/106084/m9figshare15177591v1 [1]. Source code and precompiled binaries (generic Linux and macOS) for both mGEMS and Themisto are freely available in GitHub at https://github.com/PROBIC/mGEMS (MIT license) and at https://github.com/algbio/themisto (GPLv2.0 license). A tutorial describing how to reproduce the synthetic mixed samples, bin the mixed reads, and infer the phylogenies is available in the mGEMS GitHub repository. Accession numbers and other information about the synthetic mixed samples is available in Table S1 (available in the online version of this article). The reference data used is available from Zenodo (*E. coli* doi: 10.5281/zenodo.3724111, *E. faecalis* doi: 10.5281/zenodo.3724101, *S. aureus* doi: 10.5281/zenodo.3724135), and the accession numbers are available in Table S2. Accession numbers for the data used in the *in vitro* mixture experiments is available in Table S3.

INTRODUCTION

Public health epidemiology for bacterial infections has been transformed by the use of high-throughput sequencing data to analyse and identify the source of an outbreak and to trace circulating pathogenic strains based on routine surveillance [2–6]. Standard genome-based epidemiological linking of cases requires accurate genome sequences for the pathogens derived from high coverage sequencing data for pure-colony isolates. The isolates are obtained by an enrichment and separation step in the form of a plate culture and subsequent colony picks based e.g. on morphology and colour. Typical workflow of genomic epidemiology may thus necessitate multiple colony picks per sample with the corresponding DNA library preparation and sequencing steps done individually for each of them. DNA isolation, library prep and sequencing require a significant amount of laboratory effort and time per colony, and lead to increased costs since the price of library preparation is becoming comparable to the cost of sequencing itself [7]. This can act

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Abbreviations: BGM, Bayesian Gaussian mixture; BIGSdb, bacterial isolate genome sequence database; bp, base pair; ENA, European Nucleotide Archive; GTR, generalized time reversible; MLST, multilocus sequence type; MRSA, methicillin-resistant Staphylococcus aureus; NCBI, National Center for Biotechnology Information; RAM, random-access memory; ST, sequence type.
†These authors contributed equally to this work
Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary table and seven supplementary figures are available with the online version of this article.
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as a barrier to more widespread genomic pathogen surveillance even in well-resourced public health laboratories, and prevent application of genomic epidemiology altogether in poorer settings.

Whole-genome shotgun metagenomics has been proposed as a solution for getting rid of the culturing step entirely when storing the isolates for record keeping or later phenotyping is not necessary. In this approach, sequencing is performed directly on the DNA extracted from the original sample and the resulting reads computationally binned or assembled. While tools capable of pangenome-based analyses [8], metagenome assembly [9–12], or taxonomic binning [13–15] from metagenomic short-read sequencing data have been developed, these methods typically require that the samples do not contain many closely related organisms. In particular, the strain-variation within a species is assumed to be large enough not to be confused with sequencing errors or variation in the assembly graph [16]. When more complex strain-level diversity is present, benchmarking these tools shows reduced performance in both taxonomic binning and metagenomic assembly [17–20]. In practice, natural strain-level variation is harboured ubiquitously in epidemiologically relevant samples [21–30] and it is reflected by the transmission events occurring between individuals and their environment [31]. Although some sample types may be dominated by one or two strains [32], direct sequencing of clinical samples may result in an overabundance of host DNA [29, 33–35], or lack detection power for strains with low abundance in environments with high species diversity [19, 33, 36]. These challenges are overcome in genomic epidemiology by enriching the target species through the use of plate cultures. Since established protocols and growth media are available for most bacteria of clinical relevance [37], enrichment provides an effective means to deplete the host DNA and increase the sequencing depth for target organisms when working with well-characterized species.

In this article, we introduce the mGEMS pipeline for performing genomic epidemiology with mixed cultures from samples that may harbour multiple closely related bacterial lineages. mGEMS requires only a single culturing and library preparation step per sample, which can significantly reduce the cost of performing genomic epidemiology in the standard public health setting and make the whole process more streamlined. We demonstrate the effectiveness of our approach in SNP calling and phylogenetic analyses by using *in vitro* mixed samples of *Escherichia coli* and *Enterococcus faecalis* strains, as well as DNA reads synthetically mixed from closely related samples obtained from previous genomic epidemiology studies [30, 38, 39] tracking *E. faecalis*, *E. coli* and *Staphylococcus aureus* in public health settings. Likewise, the *E. coli* and *E. faecalis* strains used in the *in vitro* samples were hospital isolates and selected as representatives of clinically highly relevant sequence types. Our results illustrate that accurate transmission and case-linking analyses are possible at reduced cost levels by enabling sample de-mixing and subsequent variant calling.

Impact Statement

The reduced cost of high-throughput sequencing has enabled genomic epidemiology to be adopted widely for public health applications worldwide. In this paper we propose the mGEMS pipeline that promises to make genomic epidemiology even more affordable by replacing separate analysis of colony picks with a single analysis per plate sweep, which significantly reduces the costs of the DNA isolation, library preparation and sequencing steps, facilitating scaling up genomic epidemiology studies. Our mGEMS pipeline provides a tool to perform typical genomic epidemiological analyses directly from plate sweeps of all colonies on a single plate with a single DNA extraction, library preparation and sequencing step. We expect mGEMS to facilitate more widespread application of genomic epidemiology in public health laboratories and enable the development of entirely novel types of analyses and pipelines.

Key parts of our pipeline presented in this paper are the mGEMS binner for short-read sequencing data, and the scalable pseudoaligner Themisto, which provides an exact version of the kallisto pseudoalignment algorithm [40] and significantly reduced RAM usage for large reference databases of single-clone sequenced bacterial pathogens. Together with recent advances in both probabilistic modelling of mixed bacterial samples [41] and genome assembly techniques [42], these methods form the mGEMS pipeline. A central step in mGEMS is an application of the recent mSWEEP method [41], which estimates the relative abundance of reference bacterial lineages in mixed samples using pseudoalignment and Bayesian mixture modelling. While Themisto enables upscaling of mSWEEP to significantly larger reference databases, the mGEMS binner is a novel sequencing read binning approach. Our binner is based on leveraging probabilistic sequencing read classifications to reference lineages from mSWEEP, and notably allowing a single read to be assigned to multiple bins. Using mGEMS to bin the reads in the original mixed samples produces sets of reads closely resembling standard isolate sequencing data and additionally acts as a denoising step for removing possible contaminant DNA. These advances enable efficient application of the existing tools for genomic epidemiology to the analysis of mixed culture samples, paving the way to a more widespread consideration of genomic epidemiology for public health applications.

METHODS

mGEMS workflow

Our pipeline for performing genomic epidemiology with short-read sequencing data from mixed samples, mGEMS, requires as input the sequencing reads and a reference database representing the clonal variation in the organisms

likely contained in these reads. The reference database must additionally be grouped accordingly into clonal groups representing lineages within the species. We used either the multilocus sequence types (*E. faecalis* experiments) or sublineages within the sequence types (*E. coli* and *S. aureus* experiments) as the clonal grouping. With these pre-processing steps performed, the first step in the mGEMS pipeline is to pseudoalign [40] the sequencing reads against the reference database using our scalable implementation of (exact) pseudoalignment with the Themisto software (in this article we used v0.1.1 with the optional setting to also align the reverse complement of the reads enabled). The pseudoalignments and the clonal grouping are then supplied as input to the mSWEEP software (v1.3.2; doi: 10.5281/zenodo.3631062 [41], with default settings) which estimates the relative sequence abundances of the clonal groups in the mixed sample. Consequently, mSWEEP produces a probabilistic assignment of the sequencing reads to the different reference clonal groups. This tentative assignment is subsequently processed by the mGEMS binner (v0.1.1, default settings), which assigns the sequencing reads to bins that correspond to a single reference clonal group — with a possibility for a sequencing read to belong to multiple bins. As the last step, the bins are (optionally) assembled with the Shovill (v0.9.0, with default settings [42]) assembly pipeline. mGEMS and Themisto are freely available on GitHub (https://github.com/PROBIC/mGEMS and https://github.com/algbio/themisto).

Reference data for mSWEEP and mGEMS

We used assemblies of three different sets of sequencing data as the reference for the three different experiments presented. The three different reference datasets represent a local (*S. aureus* experiment, [30]), a national (*E. coli*, [43]) and a global collection (*E. faecalis* downloaded from the NCBI) of strains from these species. Accession numbers and multilocus sequence types for the reference data are available in Supplementary Table 2 accompanied with rudimentary assembly statistics from both the isolate sequencing data and the assemblies from the mGEMS pipeline. In each experiment, we only aligned against the reference sequences from the relevant species.

In the *E. coli* experiments, our collection of 218 *E. coli* ST131 isolates originated from the British Society for Antimicrobial Chemotherapy's bacteraemia resistance surveillance programme and were originally isolated from 11 hospitals across England [43]. These isolates were assigned to six ST131 sublineages (A, B0, B, C0, C1, or C2) as described in a previous study [43]. As the reference sequence for calling the SNPs in building the phylogeny, we used the ST131 strain NCTC13441 (European Nucleotide Archive [ENA] sequence set UFZF01000000).

For the *E. coli* experiment where the six sublineages were further split, the split was generated by clustering the sequences with PopPUNK (v2.3.0, [44]) with the BGM model using four components and then performing the subsequent refinement step. The resulting PopPUNK clustering was combined with the sublineage numbering by concatenating the two together. This clustering is included in Supplementary Table 2.

The global collection of *E. faecalis* reference data was obtained by downloading all available *E. faecalis* assemblies (1484 as of 2 February 2020) from the NCBI, which were assigned to STs with the BIGSdb [45, 46] mlst software at pubmlst.org and assigned to the assemblies with the *mlst* software (v2.18.1) [47]. Sequence type could not be determined for 177 assemblies. These were discarded, leaving a total of 1307 assemblies assigned to 203 distinct sequence types. We used the ST6 strain V583 [48] as the reference for SNP calling (NCBI RefSeq sequences NC_004668.1-NC_004671.1).

The *S. aureus* reference data were obtained from the same study as the experiment data [30]. We used Shovill (v0.9.0 [42], with default settings) to assemble the isolate sequencing reads from the first sampling of the staff members at the veterinary hospital, and assigned the assembled sequences to the ST22 sublineages according to the information provided in original study [30]. The reference sequence used in calling the SNPs was the ST22 strain HO 5096 0412 ([49], ENA sequence HE681097.1)

If the reference sequence in any of the experiments consisted of multiple contigs, we concatenated the contigs together by adding a 100-base gap between them. The final reference file that was used as input for Themisto indexing was produced by concatenating all reference sequences processed in this way together.

In vitro mixture experiment setup

We first generated reference genomes for the three *E. coli* and three *E. faecalis* strains used in the *in vitro* benchmarking of mGEMS. In order to obtain as accurate reference genomes as possible, we combined short-read Illumina sequencing data (Table S3) with long-read Oxford Nanopore sequencing data.

In the first mixture experiment, single colonies of each strain were grown up overnight in liquid medium (LB broth [Sigma-Aldrich] for *E. coli* and brain heart infusion broth [Fluka Analytical] for *E. faecalis*), and DNA extracted for short-read sequencing. The DNA concentration was quantified using the Qubit system (Invitrogen) and purified DNA, diluted to 30 ng μl^{-1}, from the three strains of *E. coli* and the three strains of *E. faecalis* were used to prepare three different mixtures with varying ratios (1:1:1, 1.4:1.4:0.2, and 2.2:0.4:0.4; Table 1). These mixtures were then prepared for Illumina sequencing, and analysed as described earlier in the manuscript. All sequencing data generated for these mixture experiments is available under BioProject PRJNA720284.

For the second experimental setup, the six reference strains were again grown in single-clone cultures overnight as described above, and 1 : 1 : 1 mixtures of the liquid cultures from the three strains per species were made, centrifuged, and used as sample for DNA extraction for short-read sequencing. Unlike in the first experimental setup, in this setup the concentrations of the different strains were not measured

Table 1. Mixture concentration ratios in the six *in vitro* experiment samples. The column labels contain the sample identifiers and lineages for the strains that were mixed in each of the six experiments, represented by the rows of the table. The values are the proportions of each strain in the sample out of the total concentration of 30 µl.

	NORM7910041 ***E. coli* ST131-C2-4**	**NORM7911464** ***E. coli* ST131-C2-6**	**NORM7908673** ***E. coli* ST131-A-14**
Exp. 1 *E. coli* (1 : 1 : 1)	0.33	0.33	0.33
Exp. 2 *E. coli* (1.4 : 1.4 : 0.2)	0.47	0.47	0.07
Exp. 3 *E. coli* (2.2 : 0.4 : 0.4)	0.73	0.13	0.13
	51 271 926 ***E. faecalis* ST6**	**51 271 052** ***E. faecalis* ST40**	**51 271 223** ***E. faecalis* ST28**
Exp. 4 *E. faecalis* (1 : 1 : 1)	0.33	0.33	0.33
Exp. 5 *E. faecalis* (1.4 : 1.4 : 0.2)	0.47	0.47	0.07
Exp. 6 *E. faecalis* (2.2 : 0.4 : 0.4)	0.73	0.13	0.13

with Qubit and thus are not available beyond the initial 1 : 1 : 1 mixture of the liquid cultures.

DNA extraction

DNA was extracted using the MagAttract HMW kit (Qiagen) for Oxford Nanopore sequencing, and the DNeasy Blood and Tissue kit (Qiagen) for short-read sequencing.

Sequencing

DNA libraries for long-read sequencing were prepared using the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore) in combination with the native barcoding kits EXP-NBD104 and EXP-NBD114 (both Oxford Nanopore) according to the manufacturer's instruction. DNA was sequenced using an Oxford Nanopore GridION on a R9.4.1 flow cell with an input of 337.5 ng. For short-read sequencing, DNA libraries were prepared using the Nextera XT DNA library kit (Illumina) and sequenced on an Illumina NextSeq550 using a mid-output flow cell, 300 cycles and 2×150 bp paired-end set-up.

Reference genome assembly for the *in vitro* mixed experiments

We performed hybrid assembly from the long and short reads by first assembling only the long-reads and then using the short-reads for error correction. The initial long-read only assembly was created from the raw long-read sequencing data with Flye (v2.8.2, [50]) and polished with quality controlled long reads (QC'd with filtlong v0.2.0; https://github.com/rrwick/Filtlong) using medaka (v1.2.1, https://github.com/nanoporetech/medaka/). The short reads were used for error correction by first quality controlling them with fastp (v0.21.0, [51]) and then using pilon (v1.23, [52]) to perform the error correction on the long-read assembly. This procedure resulted in closed chromosome and plasmid sequences for all six reference strains. The short reads, long reads, and the produced genomes have been submitted and made available in standard repositories (Table S3).

Synthetic mixture generation

We produced our three synthetic mixture sets by synthetically mixing together the isolate sequencing data from distinct lineages in each of the three studies [30, 38, 39]. In the *E. coli* experiments, we produced ten mixed samples with one strain from each of the three main ST131 lineages (A, B, or C) in each sample. In the *E. faecalis* experiments, we mixed together seven strains from seven different sequence types to produce a total of 12 mixed samples. The strains included in each sample were chosen at random without replacement in the *E. coli* and *E. faecalis* experiments. The *S. aureus* mixed samples were produced by randomly mixing together one strain from each of the three sublineages with replacement while ensuring that each strain appears at least once. The sequencing data that were used in the reference dataset were not included in any of the experiments. In all three experiment sets, we used all available sequencing data in the mixed samples, resulting in 8–15 million reads in the experiments. Table S1 contains the accession numbers and lineage assignments of the isolate sequencing data in each sample, as well as the assembly statistics from both isolate sequencing and the synthetic mixed samples processed with mGEMS.

Pseudoalignment

We used Themisto (v0.1.1) with the default settings. Themisto is a *k*-mer-based pseudoalignment tool which encodes sets of *k*-mers as a succinct coloured de Bruijn graph. A read is considered to pseudoalign against a reference sequence if at least one *k*-mer of the read is found in the reference, and each *k*-mer of the read is either found in the reference or

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not found at all in the database of all references. This can be seen as an exact version of the pseudoalignment algorithm implemented by the tool kallisto [40]. Unlike kallisto, Themisto constructs the alignment index utilizing external memory, leading to massively reduced RAM consumption during index construction. Additionally, the index structure in Themisto is implemented using advanced compact data structures [53] to minimize the amount of memory required to store the index. Themisto is also able to exploit redundancy in the colour sets of the nodes to compress the index further.

In all of our experiments, the index was constructed using *31*-mers. Themisto does not distinguish between paired-end reads and single reads, so we decided to consider a paired-end read as pseudoaligned only when both fragments pseudoaligned. We have included this functionality for supporting paired-end reads in both the mSWEEP and mGEMS software implementations.

Abundance estimation and probabilistic read assignment

We used the mSWEEP [41] software (v1.3.2; doi: 10.5281/zenodo.363106210.5281/zenodo.3631062) with default settings. The programme was altered to support pseudoalignments from Themisto, and to output the read-level probabilistic assignments to the reference lineages. We also improved the scalability of mSWEEP by parallelizing the abundance estimation part and reducing memory consumption. These alterations have been included in versions v1.3.2 (Themisto and mGEMS support) and v1.4.0 (parallelization and memory usage improvements) of the software.

Read binning

In order to collect all reads in a mixed sample that likely originate from the same target lineage, we consider a binning strategy that allows associating the same read with multiple reference lineages. We assume that each reference lineage is represented by, at most, only one target sequence in the mixed sample, and that the sets of reference sequences capture the variation in the reference lineages sufficiently to use them as a substitute for the target sequence which may not be included in the reference sequences. In our formal treatment of the task of binning a set of sequencing reads, we define the task in terms of finding K subsets (bins), one for each reference lineage $k = 1, ..., K$, of the full sets of reads $R = \{r_1, ..., r_N\}$ denoted by $G_k \subset R$ that contain reads likely originating from the target sequence belonging to the reference lineage k. The reads assigned to each subset G_k are determined based on read-level probabilities $\gamma_{n,k}$, $\sum_{k=1}^{K} \gamma_{n,k} = 1$, $n = 1, ..., N$ to assign the read r_n into the reference lineage k by defining the subsets G_k such that

Equation 1:

$$G_k = \{r_n : \gamma_{n,k} \geq q_k\}$$

holds for some threshold $q_k \in [0, 1]$ which may vary between the lineages k. The formulation in Equation (1) has the benefit of allowing the read r_n to possibly belong to several subsets G_k, which is an important property for dealing with multiple closely related lineages in the same mixed sample.

In order to find a suitable value for the threshold q_k, and to determine the corresponding assignment rule, we consider two binary events: (1) $I_{n,k}$: the reference lineage k generated the read r_n, and (2) $J_{n,k}$: the true nucleotide sequence represented by the read r_n is part of the target sequence belonging to the reference lineage k. Knowing the probability of the event $J_{n,k}$ would directly enable us to assess the plausibility of assigning the read r_n to the reference lineage k but its value is difficult to estimate directly. However, we can determine and write down the values of the conditional probabilities $P[I_{n,k} = 1 \mid J_{n,k} = 0]$ and $P[I_{n,k} = 1 \mid J_{n,k} = 1]$ as

Equation 2:

$$P[I_{n,k} = 1 \mid J_{n,k} = 0] = 0, \text{ and}$$

$$P[I_{n,k} = 1 \mid J_{n,k} = 1] = \frac{\theta_k}{\sum_{c\,:\,J_{n,c}=1} \theta_c}$$

where θ_k is the proportion of reads from the reference lineage k, and $\sum_{c\,:\,J_{n,c}=1} \theta_c$ is the proportion of reads from any reference lineages $\{c : J_{n,c} = 1\}$ which contain the sequence represented by the read r_n. The conditional probabilities in Equation (2) allow us to write the unconditional probability $P[I_{n,k} = 1]$ as

Equation 3:

$$\begin{aligned} P[I_{n,k} = 1] &= P[I_{n,k} = 1 \mid J_{n,k} = 0]\, P[J_{n,k} = 0] + \\ &P[I_{n,k} = 1 \mid J_{n,k} = 1]\, P[J_{n,k} = 1] \\ \Leftrightarrow\ P[I_{n,k} = 1] &= \frac{\theta_k}{\sum_{c\,:\,J_{n,c}=1} \theta_c} P[J_{n,k} = 1]. \end{aligned}$$

Using the formulation in Equation (3) and the fact that we can approximate

$\frac{\theta_k}{\sum_{c\,:\,J_{n,c}=1} \theta_c} \approx \theta_k$ if we assume that the mixed sample is mostly composed of closely related organisms (the denominator $\sum_{c\,:\,J_{n,c}=1} \theta_c$ approaches 1), we can rewrite Equation (3) as

Equation 4:

$$P[I_{n,k} = 1] \approx \theta_k P[J_{n,k} = 1]$$

Equations (4) and (3) together imply that if the value of the probability $P[I_{n,k} = 1]$ that the read r_n was generated from the lineage k exceeds the relative abundance θ_k of that lineage in whole sample ($P[I_{n,k} = 1] \geq \theta_k$), then the value of the probability $P[J_{n,k} = 1]$ that the nucleotide sequence represented by the read r_n is contained in the target sequence from the reference lineage k must be 'large' ($P[J_{n,k} = 1] \to 1$). This statement about the magnitude of $P[J_{n,k} = 1]$ derives from our assumption that the denominator in Equation (3) is close to 1.

Since we have an estimate of the probabilities $P[I_{n,k} = 1]$ available in the form of the read-level probabilistic assignments $\gamma_{n,k} \approx P[I_{n,k} = 1]$, we can plug these values in Equation (4) and use the result to derive the assignment rule

Equation 5:

$$\text{if } \gamma_{n,k} \geq \theta_k, \text{ assign the read } r_n \text{ to } G_k$$

The assignment rule in Equation (5) gives us a way to assess the validity of the statement contained in the probability $P\left[J_{n,k} = 1\right]$ which we could not estimate directly.

Because of computational accuracy, we cannot obtain meaningful relative abundance estimates θ_k for lineages with a relative abundance less than $\frac{1}{N}$ (less than one read from the lineage k in the sample). Since there are K lineages in total, in the worst-case scenario $K\frac{1}{N}$ units of the relative abundance fall into this meaningless range. Therefore, only a fraction of the total relative abundance of 1 can be considered to be accurately determined when using computed values of θ_k, and this fraction d is determined in the worst-case scenario through the formula

Equation 6:

$$d = 1 - K\frac{1}{N}$$

Equation (6) means that when evaluating the validity of the assignment rule presented in Equation (5) with computed values, we have to replace θ_k with the value $d\theta_k$ which depends on the value of d in Equation (6). Merging the result from (Equations 5; 6) leads us to the final assignment rule of if

Equation 7:

$$\text{if } \gamma_{n,k} > d\theta_k, \text{ assign the read } r_n \text{ to } G_k$$

In practice, reads which pseudoalign to exactly the same reference sequences have identical values $\gamma_{n,k}$. The reads can thus be assigned to equivalence classes defined by their pseudoalignments, which enables a speedup in the implementation of the binning algorithm by considering each equivalence class as a single read. Due to this speedup and the computational simplicity of evaluating the assignment rule in Equation (7), the memory footprint of the mGEMS binner is determined by the number of equivalence classes and reference lineages in the input pseudoalignment and the runtime limited by disc I/O performance.

Genome assembly from mGEMS bins

After binning the sequencing reads in our experiments with the aforementioned assignment rule, we assembled the sequencing reads assigned to the bins using the Shovill (v0.9.0 [42], with default settings) assembly optimizer for the SPAdes assembler [54, 55]. This step concludes what we in this article call the mGEMS pipeline.

SNP calling and phylogeny reconstruction

We used snippy (v4.4.5, [56]) to produce a core SNP multiple-sequence alignment from the assembled contigs. Since the *E. coli* and *S. aureus* strains used were from the same sequence type, the core alignment for these two species contained almost the whole genome. After running snippy, we used snp-sites (v2.5.1, [57]) to remove sites with ambiguous bases or gaps from the alignment (*E. coli* experiments only) and then ran RAxML-NG (v0.8.1, [58]) to infer the maximum-likelihood phylogeny under the GTR+G4 model. Since some of the *S. aureus* strains from the same clade were identical, we changed the default value of the minimum branch length parameter in RAxML-NG to 10^{-10} in the *S. aureus* experiments and printed the branch length with eight decimal precision to identify branches of length zero. In all experiments, we ran RAxML-NG with 100 random and 100 maximum parsimony starting trees, and performed 1000 bootstrapping iterations to infer bootstrap support values for the branches. We used the phytools R package (v0.6–99, [59]) to perform midpoint rooting for the tree, and the ape R package (v5.3, [60]) to create the visualisations.

RESULTS

Read binning and genome assembly from mixed samples with mGEMS

Our mGEMS read binning algorithm, part of the mGEMS pipeline (Fig. 1), requires probabilistic assignments of sequencing reads to reference taxonomic units (lineages or sequences) and an estimate of the relative sequence abundance of these same references in the full set of reads. mGEMS then bins the reads by assigning a read to a bin (corresponding to a target sequence from a given reference lineage) if the read-level assignment probability of the lineage is greater than or equal to the sequence abundance of that particular lineage in the full set of reads. Notably, this algorithm allows a single sequencing read to be assigned to multiple bins which is a crucial feature for considering strain-level variation. As shown in the Methods section, this algorithm assigns reads to reference lineages only if the sequence represented by a read is likely contained in a target sequence that belongs to the reference lineage.

In the pseudoalignment part of the pipeline (Fig. 1), we use our own more efficient and accurate implementation of the pseudoalignment algorithm in kallisto [40], called Themisto, to pseudoalign the sequencing reads against the reference sequences. Themisto is based on using coloured de Bruijn graphs to represent the reference sequences, and utilizes disc storage during indexing to control the amount of RAM required to construct the pseudoalignment index. These choices lead to Themisto aligning a similar number of reads per hour as kallisto, while being 70 times faster to load in an example pseudoalignment index consisting of 3682 *E. coli* sequences (28 min for kallisto and 0.55 min for Themisto; Supplementary Methods). Implementation of the method is described in more detail in Supplementary Methods.

The pseudoalignments from Themisto are used as input to the mSWEEP method [41] to estimate the probabilistic read assignments and whole-sample relative sequence abundances. These values provide the necessary input to the mGEMS binner which assigns the sequencing reads to the bins. Finally, we use the Shovill [42] assembly optimizer for the SPAdes assembler [54, 55] to assemble the bins. On an example synthetic mixed sample (the *E. coli* sample with the most reads), the full mGEMS pipeline took 112 min to run (Themisto 26 min, mSWEEP 4 min, mGEMS

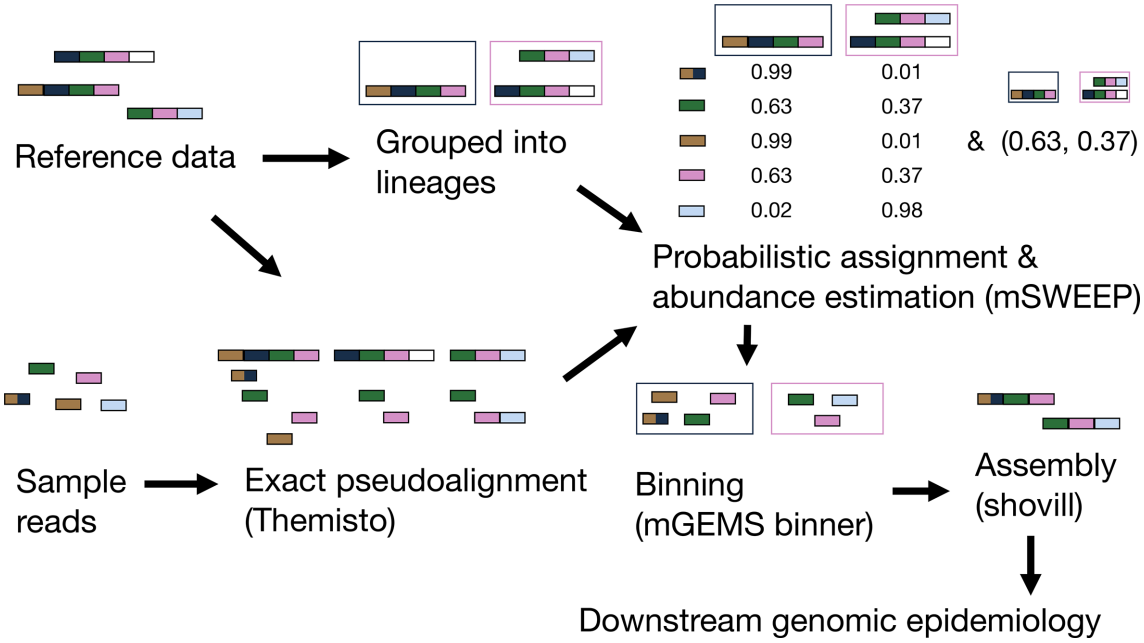

Fig. 1. Flowchart describing a genomic epidemiology workflow with the mGEMS pipeline. The figure shows the various steps of the pipeline. Steps with programme names in brackets constitute the parts of the mGEMS pipeline. Presented values from mSWEEP and mGEMS binner are the actual results of running the pipeline with the described input.

binner 16 min, and Shovill 66 min) using two threads on a laptop computer with two processor cores and 16 gigabytes of memory. C++ implementations of both the mGEMS binner and the Themisto pseudoaligner are freely available on GitHub (https://github.com/PROBIC/mGEMS, MIT license, and https://github.com/algbio/themisto, GPLv2 license).

Overview of the experiments used in benchmarking mGEMS

We assessed the accuracy and effectiveness of mGEMS by considering data from three genomic epidemiological studies [30, 38, 39] and by generating a benchmarking dataset of *in vitro* mixed samples with measured DNA concentrations. The *in vitro* dataset was generated by first growing three strains of *E. coli* and three strains of *E. faecalis* separately, resulting in six overnight cultures in liquid medium. Next, the amount of DNA extracted from the overnight cultures was measured and six mixtures, each consisting of three strains of either *E. coli* or *E. faecalis*, with known concentrations of DNA for each isolate, were created. This resulted in a benchmark dataset where the relative abundances of the different strains in each mixture are known. We also generated two additional mixtures where the *E. coli* or *E. faecalis* strains were mixed in 1 : 1 : 1 proportions from the liquid culture without measuring the amount of DNA, and the DNA extraction was then performed on these already-mixed bacterial samples. To our knowledge, these benchmarking samples constitute the first published dataset where DNA from three strains of the same species has been mixed with known concentrations, providing an important resource for development of methods aimed at untangling strain-level variation.

In the synthetic mixture experiments, we used sequencing reads from previously published genomic epidemiological studies [30, 38, 39] as the basis for creating synthetic mixture data. The synthetic mixtures were processed with the mGEMS pipeline, and the output was compared against the benchmark of having non-mixed data available by running the same epidemiological analyses on both the mGEMS output and the non-mixed data. The synthetic experiments presented are: (1) mixing reads from three clones of *E. coli* sequence type (ST) 131 sublineages obtained from a study of multidrug-resistant *E. coli* ST131 strains circulating in a long-term care facility in the UK [38], (2) mixing reads from seven *E. faecalis* STs identified in a study of the population structure of hospital-acquired vancomycin-resistant *E. faecalis* lineages in the UK and Ireland [39], and (3) mixing reads from three *S. aureus* ST22 sublineages from a study of the transmission network of methicillin-resistant *S. aureus* (MRSA) among staff and patients at an UK veterinary hospital [30]. We also provide three different approaches to constructing the reference datasets for the pseudoalignment step: (1) a national (UK) collection of *E. coli* ST131 isolates associated with bacteremia [43], (2) a global collection of all available *E. faecalis* genome assemblies from the NCBI as of 2 February 2020, and (3) a local collection of *S. aureus* sequencing

data from the staff members at the veterinary hospital at the earliest possible time point in the same study [30]. A detailed description of the generated experiments and the accession numbers of the isolate sequencing and reference data used is presented in the Methods section.

Evaluation of mGEMS and mSWEEP on the *in vitro* benchmark data

We first evaluated the accuracy of mGEMS and mSWEEP on the six *in vitro* experimental samples, where the true relative abundances of the three strains in each sample are known. In the *E. faecalis* samples each of the three strains originated from a different multilocus sequence type (MLST) and the measurements were accordingly performed on the level of the MLST grouping [45]. In the *E. coli* samples, the strains originated from sublineages within ST131 as defined in a previous study [43], with one strain from sublineage ST131-A and two from sublineage ST131-C2. In order to distinguish between the two strains from ST131-C2, we further split the strains based on their accessory genomes using PopPUNK [44], which provided us with a grouping where all three strains were split into three separate groups (A-14, C2-4, and C2-6), enabling us to differentiate between them with mSWEEP and mGEMS.

We assessed the accuracy of mGEMS by comparing the results of SNP calling from a hybrid long +short read assembly obtained from a single-colony derived sample of the strains used in the *in vitro* mixed experiments with calling the SNPs from an assembly obtained by processing the mixed experiment samples with the mGEMS pipeline. The results of the SNP calling are highly similar in both datasets (Fig. 2a, b) with the exception of the *E. coli* ST131-C2-6 strain from the experiment labelled 'Exp 2 *E. coli*'. In this experiment, the sample consisted of equal amounts of DNA from the ST131 C2-4 and C2-6 strains and a small amount of ST131-A-14, causing some confusion between the reads originating from the closely related C2-4 and C2-6 strains which resulted in a difference between the observed and expected SNP counts.

Similarly, the mSWEEP relative abundance estimates for both the *E. coli* and *E. faecalis* samples correspond well with the true values when measured by both absolute and relative error (Fig. 2c, d, respectively). Slightly higher errors were observed in the estimates for the *E. coli* ST131 C2-4 and C2-6 strains when compared to the estimates for the *E. coli* ST131 A-14 strain and all three *E. faecalis* strains. Akin to the results of SNP calling with mGEMS, these differences in the relative abundance estimates are likely a result of using the highly detailed *E. coli* within-ST clustering, which is significantly harder to differentiate than the between-ST clustering used for *E. faecalis*. Regardless, in all cases, there are no false positive or false negative detections of lineages reported in the mSWEEP relative abundance estimates.

SNPs from synthetic mixtures match SNPs called from isolate data

In the first synthetic mixture benchmark, we compared the accuracy of SNP calling with the snippy software (v4.4.5) [56] from the bins obtained by processing the abundance estimation results from the mixed samples with the mGEMS binner with the results of the same analyses from the isolate sequencing data (Fig. 3). In the *E. coli* and *E. faecalis* experiments (Fig. 3a, b, respectively), the SNPs were called from assembled contigs while in the *S. aureus* experiment (Fig. 3c), we called the SNPs directly from the sequencing reads because calling the SNPs from the contigs resulted in poorer performance (Fig. S1). In all experiments, the SNPs called from the mixed samples closely resemble the results of isolate sequencing data in both the samples that are similar and dissimilar to the reference sample. Although in the *E. coli* experiment mGEMS produced slightly more SNPs on average, the results were consistently higher for all samples and did not affect the results of the analyses presented further in this article.

We suspected that the observed differences in the SNP counts may have been caused by issues in the sequence assembly due to mGEMS allowing a read to belong to multiple bins, which results in variable coverage between the regions with and without the clade-specific SNPs. We tested this assumption by replacing the Shovill assembler in the mGEMS pipeline with metagenomic assemblers, which naturally handle variable coverage. Using the metagenomic assemblers marginally improved the results in some of the experiments (Figs 3d and S2) with MEGAHIT [10, 11] in particular outperforming Shovill when the coverage is markedly varied like in the *S. aureus* experiment. However the improvements were not drastic enough to decisively confirm our suspicions about the accuracy of the SNP calling being limited by the choice of assembler. We did observe that when measured by reference-independent assembly statistics (sum of all contig lengths, total number of contigs, sequence length of the shortest contig at 50% genome length N50, and the smallest number of contigs whose sum is at least 50% of the genome length L50), the statistics obtained from the standard configuration of mGEMS with the Shovill assembler resemble those from isolate sequencing data.

We further assessed the accuracy of the called SNPs by fitting a Bayesian linear regression model to the same SNP data with the isolate results as the sole explanatory variable and the results from the bins or the metagenomic assemblers as the response variable (Figs 3 and S2) using the brms R package [61–63]. In both the *E. coli* ST131 sublineage and the *E. faecalis* experiments, the 95% posterior credible interval for the slope from mGEMS with all assembler choices except metaSPAdes contains the correct value of 1.0. The *S. aureus* experiments produce worse 95% credible intervals for the slope compared to the *E. coli* and *E. faecalis* experiments with none of the intervals containing the correct value. However, the regression model is not well

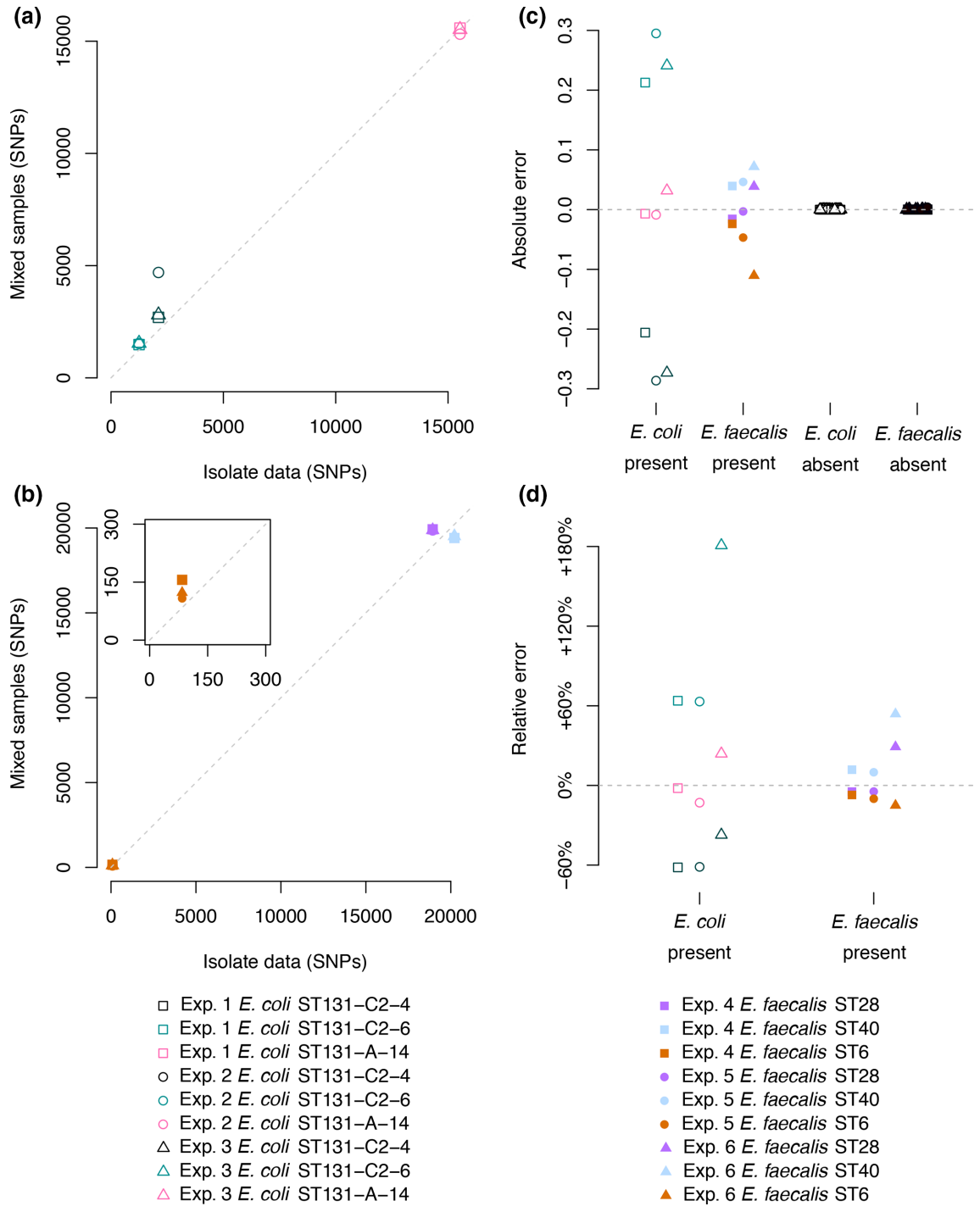

Fig. 2. Evaluating mGEMS and mSWEEP on the *in vitro* benchmark data. Panels (a) *E. coli* and (b) *E. faecalis* compare the results of SNP calling from the isolate sequencing data (horizontal axis) against the results of SNP calling from the mixed samples with the mGEMS pipeline (vertical axis). The subplot in panel (b) contains a zoomed-in view of the points around the origin. Panels (c) and (d) compare the abundance estimates from mSWEEP to the ground truth relative abundances. Panel (c) shows the absolute difference between the estimates from mSWEEP and the true abundance. The values shown are split into *E. coli* and *E. faecalis* lineages truly present in the samples, and lineages truly absent. Panel (d) shows the relative error in the truly present lineages.

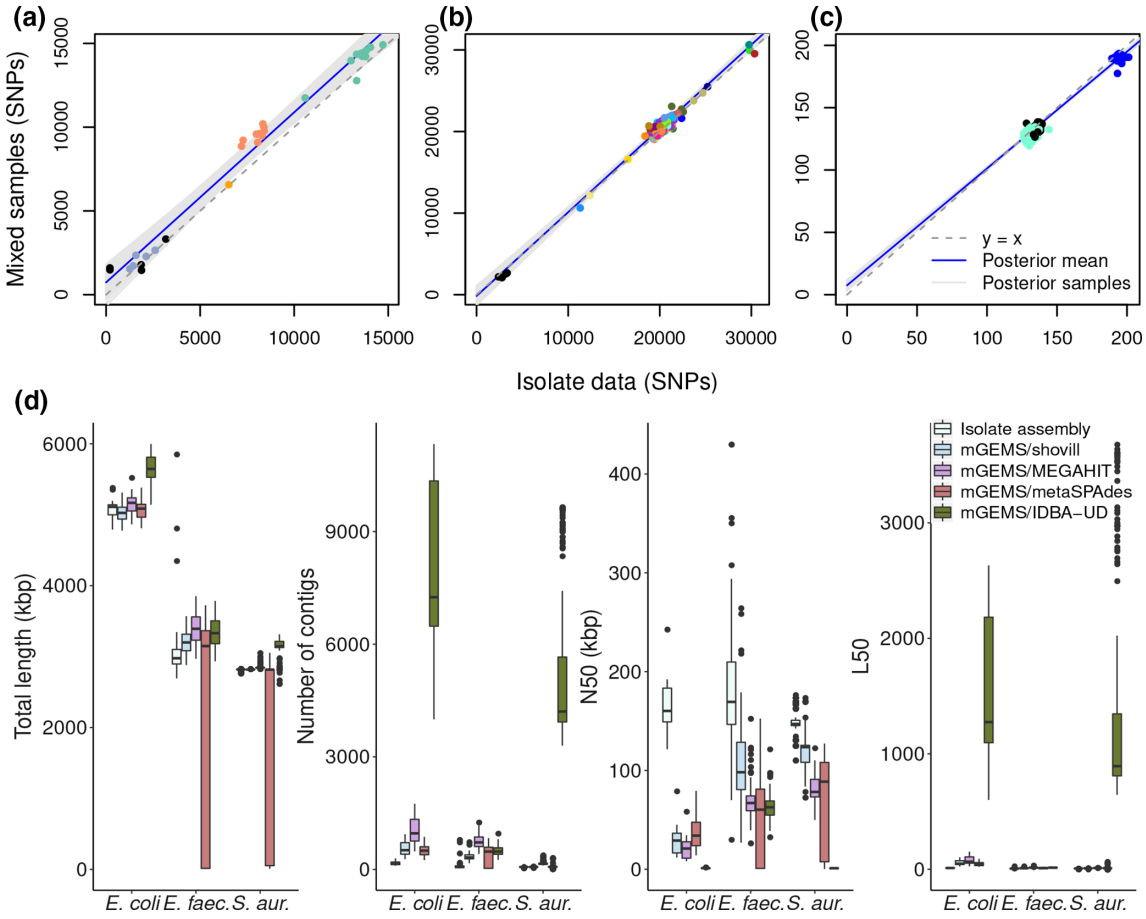

Fig. 3. Comparing mGEMS and synthetic mixtures with isolate sequencing data. Panels a–c compare the results of SNP calling from mixed samples with the mGEMS pipeline against the results from isolate sequencing data. Panel d compares reference-free assembly statistics from mGEMS pipeline with different assemblers against the results from assembling the isolate sequencing data with Shovill. The results in panel a are for the *E. coli* ST131 isolates, panel b the *E. faecalis* isolates, and panel c the *S. aureus* ST22 isolates. In panels a and b, SNPs were called from contigs after assembling the reads. In panel c, the SNPs were called directly from the reads. Points are colored according to the lineage within the species (the full legend is available in Fig. S3). The dashed gray line represents a hypothetical perfect match between the binned and isolate reads. The blue line is the posterior mean while the shaded area contains the 95% posterior credible region calculated from 10000 posterior samples from a Bayesian regression model with the SNPs from the binned reads as the response and the SNPs from the isolate sequencing data as the sole explanatory variable. In panel d, the boxes are colored according to the type of assembly. The presented statistics are the summed lengths of all contigs (total length), the number of contigs, the sequence length of the shortest contig at 50% genome length (N50), and the smallest number of contigs whose sum of lengths is at least 50% of the genome length (L50).

suited to analysing the *S. aureus* samples since the number of SNPs between the strains is minimal (0–10 SNP differences within the lineages) and there are only three lineages, which translates poorly to finding a linear relationship.

Split-*k*-mer comparison between isolate reads and mGEMS bins in synthetic mixtures

We also examined the accuracy of the mGEMS binner without assembling by using the split *k*-mer analysis provided by the SKA software (v1.0, [64]). In a split-*k*-mer analysis, each nucleotide in the read is flanked by two *k*-mers. The nucleotide in the middle position plus the flanking *k*-mers constitute a single split-*k*-mer. If the split-*k*-mers are calculated for all nucleotides in two samples, they can be used to compare the samples on the basis of matching or mismatching split-*k*-mers or to call SNPs by comparing two split-*k*-mers where the flanking *k*-mers match but the nucleotide in between does not.

We first used SKA to call split-*15*-mer-SNPs in the three reference sequences from the binned sequencing reads, and calculated the difference in the count of SNPs called in the reference sequence between the isolate and the binned reads (Fig. S4). Since the results in Fig. 3 for *S. aureus* were obtained without assembly, there is no notable difference when compared to the SKA results. However, the SKA results for *E. coli* and *E. faecalis* contain fewer SNPs called from the binned reads, implying that binning with mGEMS acts as filtering for the sequencing data, since the results from the assemblies display no stark differences.

In our next assessment, we performed pairwise comparisons within the separate sets of (1) all isolate reads, and (2) the binned reads. First we called the split-*15*-mer SNPs pairwise between all samples containing the isolate reads, and pairwise between all samples containing the binned reads. We then calculated the differences in the pairwise SNP counts obtained from the isolate reads and the binned reads. Secondly, we performed the same pairwise analysis but instead of the split-*15*-mer SNP counts we looked at the numbers of split-*15*-mers that either were the same (matching) or different (mismatching) between each pair of samples. The results from these two comparisons (Fig. S5) show more discrepancy than the earlier results considering only SNPs called in the reference genome (Fig. 3), but the pairwise SNP counts are still relatively well preserved in all three species.

Phylogenetic analysis of *E. coli* ST131 sublineages in a long-term care facility with synthetic mixtures

We used a set of 30 multidrug-resistant *E. coli* ST131 strains sequenced from the residents of a long-term care facility in the UK [38] to produce a total of ten synthetic mixed samples. Each sample was the result of mixing isolate sequencing data from three *E. coli* ST131 sublineages (one from each of the main lineages A, B, or C) together. We attempted to preserve the potential sequencing errors and biases by using all available reads from each of the isolate samples.

We applied the mGEMS pipeline to the ten synthetic mixed samples with a national (from the UK) collection of *E. coli* ST131 strains as the reference data [43], and used RAxML-NG (v0.8.1) [58] to infer a phylogenetic tree from both assemblies obtained from the isolate sequencing data (ground truth) and the assemblies from the mGEMS pipeline. Comparisons of these two trees (Fig. 4), show that the overall structure of the trees is highly similar, with the deep branches within the tree well reconstructed and differences in the tree topology appearing only at the very recent short branches.

Population structure of nosocomial *E. faecalis* infections in the UK from synthetic mixtures

Our next experiment was performed on sequencing data from bloodstream-infection-associated *E. faecalis* strains with a high prevalence of vancomycin resistance circulating in hospitals in the UK [39]. In this experiment, we mixed isolate sequencing data from seven distinct *E. faecalis* STs [45], producing a total of 12 synthetic mixed samples with seven lineages present in each. Each synthetic mixed sample included all sequencing reads from the mixed isolate sequencing data similarly to the *E. coli* experiment. We used a global collection of *E. faecalis* strains (all *E. faecalis* genome assemblies submitted to the NCBI as of 2 February 2020) as the reference data for the mGEMS pipeline, and again inferred the phylogenies for assemblies from both the isolate sequencing data and the results of the mGEMS pipeline. The more complex structure of these phylogenies was compared by plotting the two phylogenies against each other in a tanglegram (Fig. 5). Apart from a few structural mismatches in branches with poor bootstrap support values in both phylogenies (indicating uncertainty in the structure to begin with), the tree structure is strikingly well-recovered from the binned reads.

In fact, the tree inferred with the mGEMS pipeline has better bootstrap support values in the lower parts of the tree, suggesting that using mGEMS provides a better supported phylogeny than using the isolate sequencing data alone. We suspect this improvement in the bootstrap support values was caused by contamination in the isolate sequencing data for BSAC ec750, which produces an assembly 5.8 Mb long — nearly twice the length of the reference *E. faecalis* strain V583 (3.2 Mb). Similar changes in the bootstrap support values and additional structural changes occur in the parts of the tree containing the isolates BSAC ec294 and BSAC ec655 which both produce abnormally long assemblies (4.8 and 4.4 Mb, respectively). The assembly lengths for both the isolate and mGEMS-binned sequencing reads are provided in Table S1.

Methicillin-resistant *S. aureus* transmission patterns among staff and patients at a veterinary hospital from synthetic mixtures

In our last experiment, we used a dataset containing three *S. aureus* ST22 sublineages (called clade 1, clade 2, and clade 3) circulating among the staff and patients at a veterinary hospital in the UK [30] and separated by less than 150 SNPs. Because of the minimal differences between the clades, and a lack of isolates from these specific clades in published sources, we decided to use the isolates from the temporally first sample from the staff members as the reference data (representing a local reference collection). We separated the reference isolates from our experiment cases, which consist of all samples sequenced after the reference isolates, and proceeded to mix the remaining isolate sequencing data together. We generated a total of 312 synthetic mixed samples, each containing the sequencing data from three isolate samples from each of the three clades. Because the numbers of samples in each clade were not equal, the data from some of the isolate samples were included in multiple mixed samples. Since we wanted to represent each isolate with only a single instance in the phylogeny, we randomly chose one corresponding bin from mGEMS as the representative for an isolate that was included in multiple mixed samples.

The phylogenies in Figs. 6 and 7 were inferred with RAxML-NG (v0.8.1, [58]) from the results of the mGEMS pipeline. We plotted the subtrees of the overall phylogeny separately for the clade 1 isolates (Fig. 6) and clade 2 and 3 isolates (Fig. 7) without changing the underlying tree structure. Phylogenies inferred from the isolate sequencing data using the same pipeline are available in Figs S6 and S7. In the original study [30], staff member A was inferred as having introduced the MRSA strain from clade 1 into the veterinary hospital. In our phylogeny (Fig. 6), the initial samples from staff member A (timepoints labels 1 and 2) are indeed

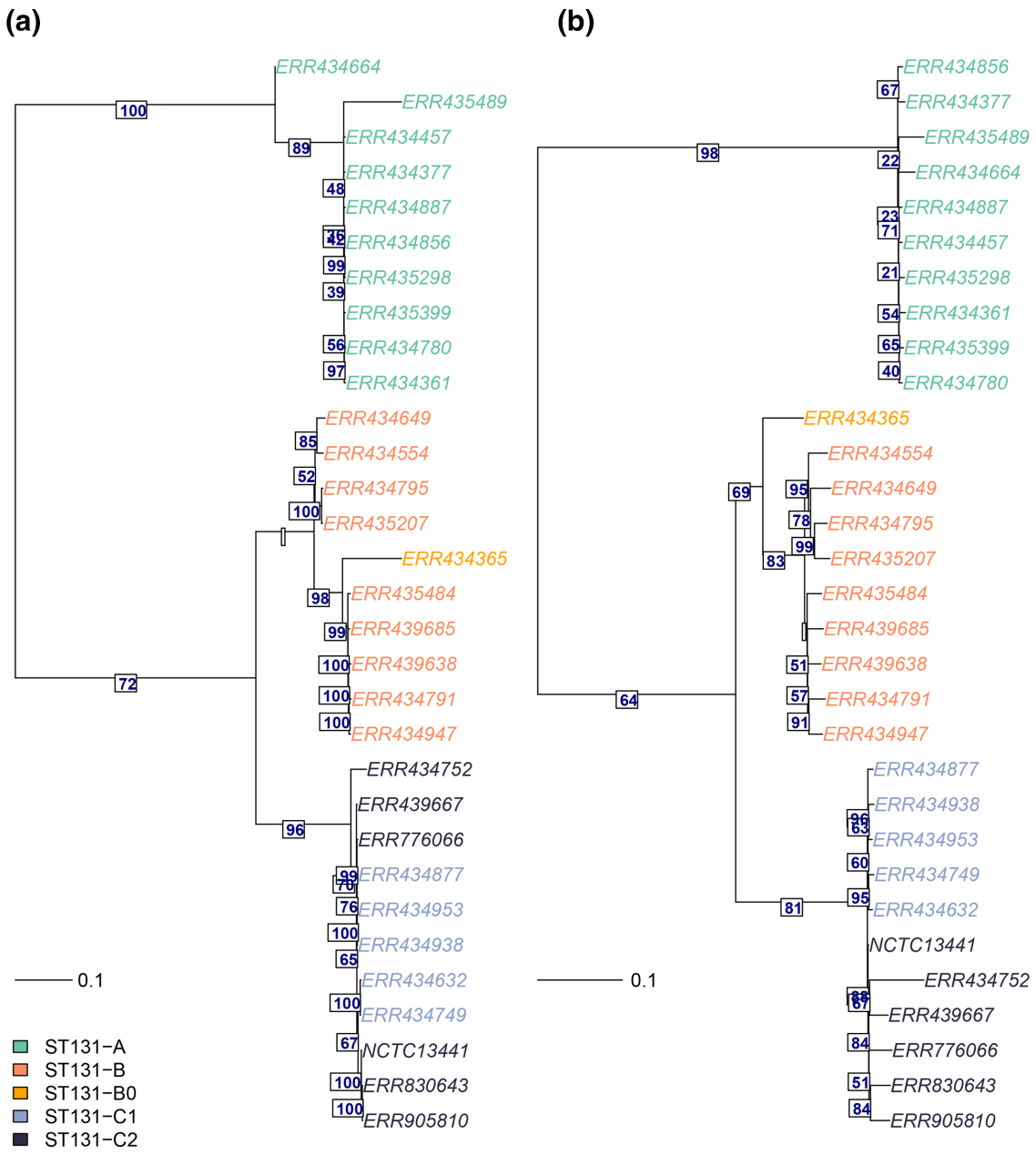

Fig. 4. Midpoint-rooted maximum likelihood trees from core SNP alignment of *E. coli* ST131 strains. The phylogeny in panel a was constructed from isolate sequencing data from 30 *E. coli* ST131 strains, and the phylogeny in panel b with the mGEMS pipeline from ten synthetic plate sweep samples, each mixing three isolate samples from the three main ST131 lineages (a–c; one strain from each per sample). Both phylogenies were inferred with RAxML-NG. Numbers below the edges are the branch support values from RAxML-NG for the next branch. Leaves are coloured according to the *E. coli* ST131 sublineage (a, b, B0, C1, or C2), and branch lengths in the tree scale with the mean number of nucleotide substitutions per site on the respective branch (GTR+G4 model). Leaves are labelled with the ENA accession number and the leaf labelled NCTC13411 corresponds to the reference strain used in calling the core SNPs.

contained at the root of the tree inferred from the mGEMS pipeline, although the placement of the strains further up the tree vary when compared to the results presented in the original study. The original study performed manual quality control of the SNP data by removing transposable elements which was not replicated in our experiment, possibly explaining some of the observed differences between the tree structures. The phylogenies for clades 2 and 3 (Fig. 7) follow the results of the original study more closely with most subclades found in both the isolate and the mixed sample phylogenies. Importantly, in all three clades no assembly from the mGEMS pipeline was assigned to the wrong clade in the phylogeny despite the minimal distances between the clades.

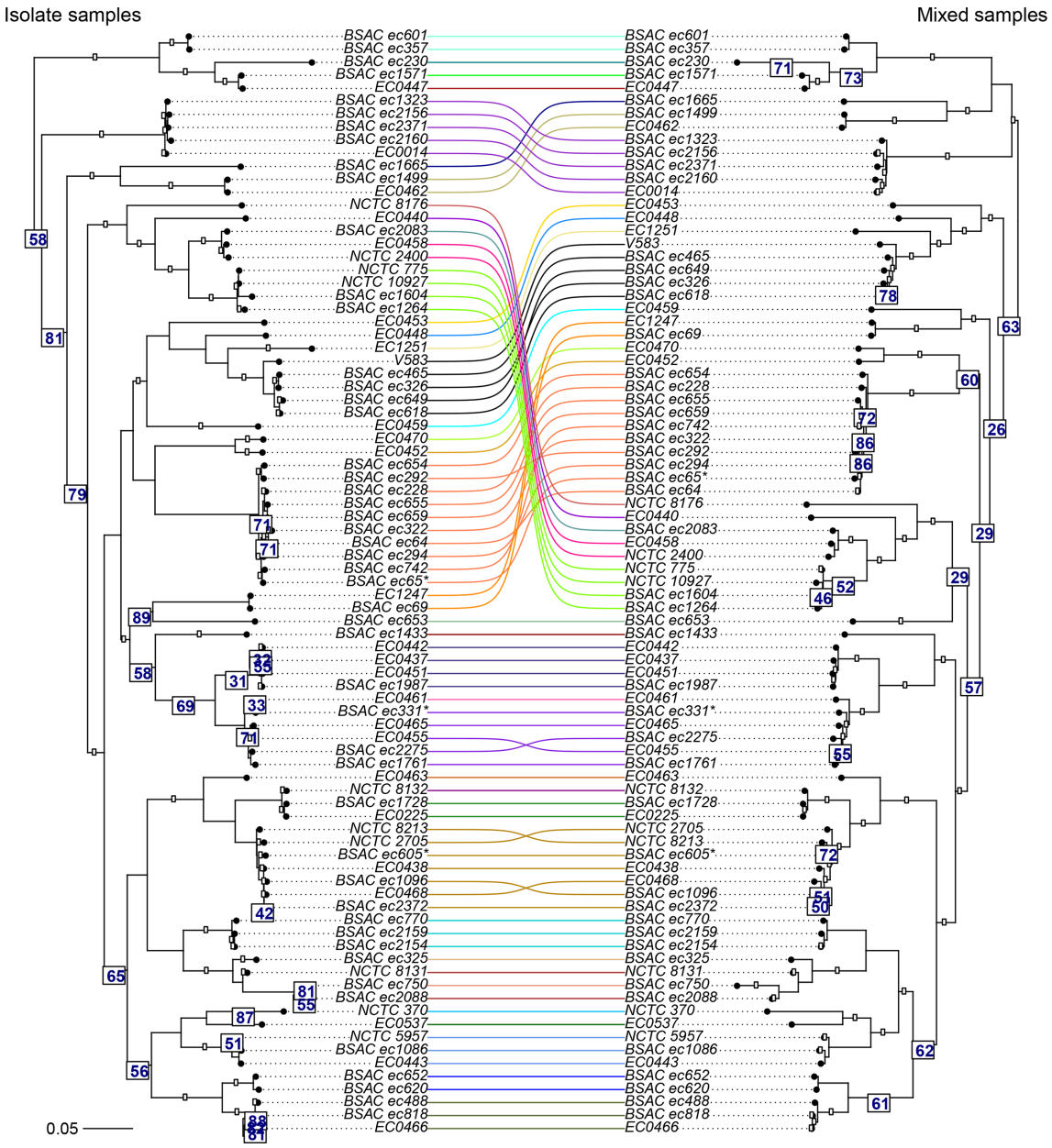

Fig. 5. Tanglegram of two midpoint-rooted maximum likelihood trees from core SNP alignment of *E. faecalis* strains. The phylogeny labelled Isolate samples (left side of the tree) was inferred with RAXML-NG from assembling the isolate sequencing data from 84 *E. faecalis* strains. The phylogeny labelled Mixed samples (right side of the tree) was inferred from 12 synthetic mixed samples, each containing sequencing data from seven different *E. faecalis* STs randomly chosen from the isolate sequencing data. Numbers below the edges indicate bootstrap support values from RAxML-NG for the next branch towards the leaves of the tree. Only support values less than 90 are shown. Branches are coloured according to the *E. faecalis* STs, and branch lengths in the tree scale with the mean number of nucleotide substitutions per site on the respective branch (GTR+G4 model). Leaves are labelled with the strain name from NCBI and the leaf labelled V583 corresponds to the reference strain for calling the core SNPs.

DISCUSSION

Adopting a plate-sweep approach, where DNA from the individual bacteria growing on the same plate is prepared and sequenced as a single library, shows clear promise in reducing the amount of manual and costly laboratory work that has been identified as an emerging bottleneck for epidemiological analyses at many public health laboratories [7]. We see two main applications for this. On the one hand, diagnostics where usually a single clone is picked can now capture the whole diversity at much lower additional costs. Whilst single-clone culturing might be required depending on the application (e.g. if more detailed speciation is required, or if further

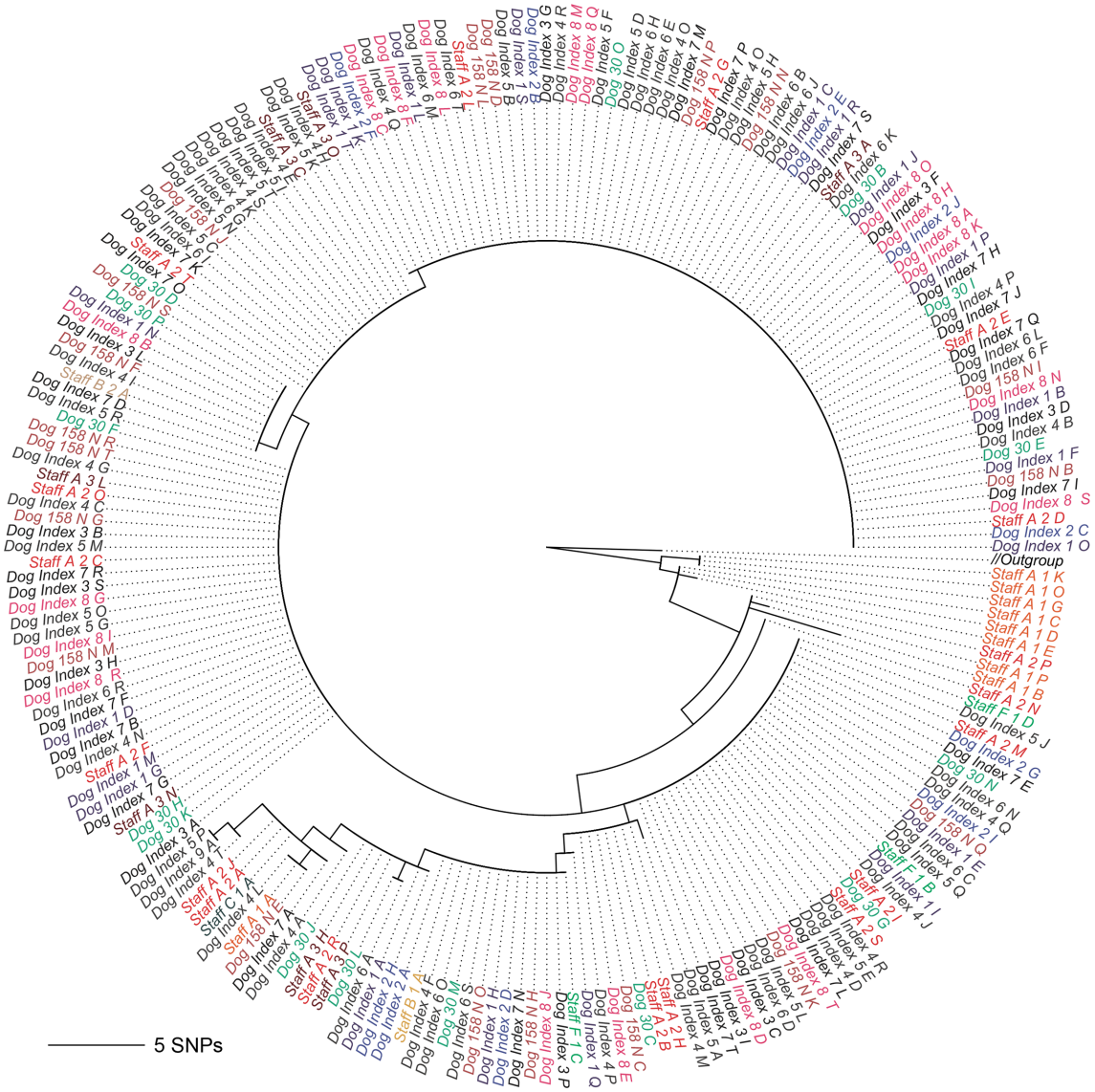

Fig. 6. Midpoint-rooted maximum likelihood tree from core SNP alignment of *S. aureus* ST22 showing strains from a single lineage within the sequence type. The phylogeny was inferred from a combined set of assemblies from 60 isolate sequencing samples (leaves labelled Staff A-G 1 A-T, corresponding to the temporally first samples from each staff member) and 312 assemblies obtained from the mGEMS pipeline applied to synthetic mixed samples of sequencing data from each of the three different *S. aureus* ST22 clades (1, 2, and 3). Only strains from clade 1 are displayed in the tree, with the branch labelled Outgroup leading to the collapsed clades 2 and 3. The mixed samples were produced from the isolate sequencing data collected from the patients, or from the staff members after the first sampling time. Branch labels are coloured according to the plate the isolate sequencing data was picked from. Branch lengths in the phylogeny scale with the mean number of SNPs obtained by multiplying the mean nucleotide substitutions per site on the respective branch (GTR+G4 model) with the total number of alignment sites. Leaves are labelled with the format: staff or patient, a letter indicating the donor, plate number (ascending in time), and a letter indicating the colony pick id.

phenotyping experiments are planned), a major cost- and time-factor - the DNA isolation, library prep and sequencing - are covered with a single experiment for the whole diversity, saving time and costs. On the other hand, this method might greatly support work on samples where the high diversity has so far proved as a major challenge, like longitudinal transmission or carriage studies especially in complex samples such as human faeces, where it is appreciated that only a fraction of the diversity can be covered with single-colony sequencing approaches.

In this article, we have introduced the mGEMS pipeline, which includes novel pseudoalignment and read binning methods, for genomic epidemiological analyses of plate sweeps. Our pipeline provides means to accurately recover the genomes, or corresponding sequencing reads, from mixed samples with

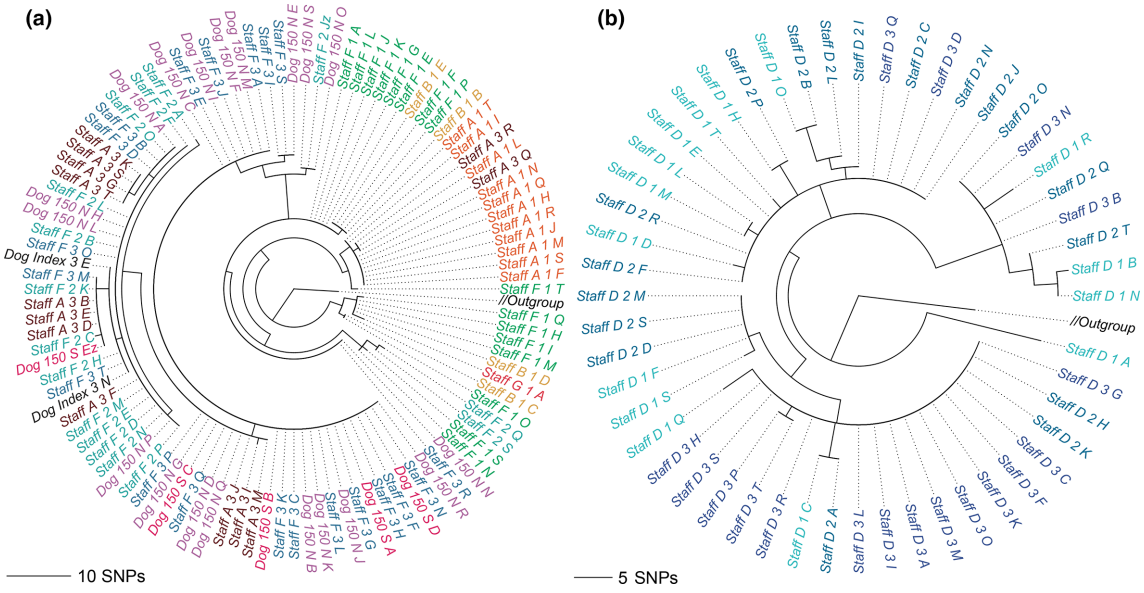

Fig. 7. Midpoint-rooted maximum likelihood trees from core SNP alignment of *S. aureus* ST22 showing clade 2 and clade 3 strains. The underlying phylogeny is the same as in Fig. 6. The phylogeny in panel a contains the clade 2 strains, and panel b the clade 3 strains. Branches leading to clade 1 and clade 3 (panel a), or clade 1 and clade 2 (panel b), labelled Outgroup in both panels, were collapsed. Branch labels are coloured according to the plate the isolate sequencing data was originally picked from with darker shades indicating later sampling times. Branch lengths in the phylogeny scale with the mean number of SNPs obtained by multiplying the mean nucleotide substitutions per site on the respective branch (GTR+G4 model) with the total number of alignment sites. Leaves are labelled with the format: staff or patient, a letter indicating the donor, plate number (ascending in time), and a letter indicating the colony pick id.

extremely closely related strains separated by less than a few dozen SNPs. In these settings, where the differences between the strains are at or under the sequence type level, isolate sequencing is traditionally required to draw epidemiological conclusions.

Using both samples based on synthetically mixed reads, as well as experimentally generated benchmark samples mixing bacterial DNA and strains, we have shown that with mGEMS we can robustly infer the same conclusions from plate sweeps that can be inferred from single-isolate sequencing data. Additionally, since mGEMS relies on modelling counts of pseudoalignments against grouped reference sequences, the inclusion of the alignment step causes the pipeline to also acts as quality control for sequencing reads from samples that inadvertently contain multiple lineages or contamination, which can disrupt downstream analyses like SNP calling [65]. In analysing sequencing data from closely related mixed samples, our pipeline reaches accuracy levels likely constrained by technical variation in the sequencing data and limitations in assembling sequencing data with variable coverage. Although existing tools like StrainPhlAn [32, 66] are capable of determining and analysing the dominant strains in complex mixed samples, to our knowledge mGEMS is the first tool capable of reliable recovery of the full strain variety when samples may contain multiple strains from the same species.

mGEMS demonstrates the power of plate sweep sequencing in genomic epidemiology and enables a change in the currently dominant framework that confers multiple benefits over both whole-genome shotgun metagenomics and isolate sequencing. Studies of the population structures of opportunistic pathogens have revealed extensive strain-level within-host variation [21, 23, 27, 67, 68] with adverse implications for transmission analyses relying solely on isolate sequencing [31, 69] and colony pick based longitudinal studies reporting the absence or re-emergence of strains in a host [30, 38, 70] or antimicrobial profiles [28, 71]. While whole-genome shotgun metagenomics solves these issues to some extent [35, 72], the culture-free nature suffers from issues with both bacterial and host DNA contamination particularly affecting the sensitivity for detecting strains in low abundance [29, 33, 34, 73, 74]. Using mGEMS in conjunction with plate sweep sequencing data avoids these issues altogether, paving way for more representative studies of pathogen population structure and providing higher-resolution data for more complex models of transmission dynamics incorporating within-host variation and evolution [75–79].

mGEMS is designed for high-throughput short read sequencing data and requires a representative reference collection of the lineages present in the processed samples. When the reference data or the lineages are not sufficiently well-defined — like in the *E. coli in vitro* benchmark, where the unstable *E. coli* accessory genome was required

to separate the two C2 strains into distinct lineages — the binning can be unreliable, leading to less accurate results. Mobile elements and plasmids that are not tied to a specific lineage may also pose a problem for mGEMS but we expect that their handling could be improved by including them as separate entities in the reference collection.

Since our method relies on available single-clone genomic reference data and plate cultures of the bacteria to sequence them at a sufficient depth for assembly, it obviously cannot be applied to the study of uncharacterized or unculturable species. However, culture media do exist for most human pathogens of public health relevance [37] or can be developed for some of the until recently unculturable bacteria [78–80]. Moreover, the availability of single-clone bacterial genome sequences is still increasing at a high rate, such that for many species or lineages plenty of sufficiently representative reference sequences would be available [81, 82]. In these cases, the drastic reduction in the costs of library preparation, and the better capture of the underlying genomic variation between closely related bacteria in a set of mixed samples provided by mGEMS is extremely valuable. We hope that by enabling significant streamlining of the process of producing data for public health genomic epidemiology, our approach inspires both applications and further method development within this exciting research area.

CONCLUSIONS

We have developed the mGEMS pipeline for performing genomic epidemiological analyses from mixed samples containing multiple closely related bacterial strains. The two crucial novel enabling aspects introduced in this paper are the mGEMS read binner and the Themisto pseudoaligner. The mGEMS binner is a binning method based on turning probabilistic assignment of sequencing reads to reference lineages, while the Themisto pseudoaligner is a high-throughput exact pseudoaligner for short-read sequencing data that features external memory construction for compressed coloured de Bruijn graphs for scalability, providing significant runtime savings over conventional pseudoalignment. mGEMS addresses several major issues related to the cost, applicability, and sensitivity of the current approach in genomic epidemiology and enables entirely new types of analyses using mixed samples without sacrificing accuracy.

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Author contributions
T.M., T.K., J.C. and A.H., conceived the study, developed the full mGEMS pipeline, and designed the synthetic mixture benchmarking experiments. E.H., K.H., Ø.S., T.M., J.C. and A.H., designed the *in vitro* mixture study. K.H., Ø.S. and E.H., performed the experiments for the *in vitro* study. T.M. and A.H., developed the mGEMS binning algorithm. J.A. and V.M., developed the Themisto pseudoaligner. T.M., implemented the mGEMS binner. J.A., implemented the Themisto pseudoaligner. T.M., ran the experiments and created the visualisations. T.M., T.K., J.C. and A.H., interpreted the results and wrote the main article. JA wrote the supplementary file describing Themisto. K.H., Ø.S. and E.H., wrote the sections describing the *in vitro* mixture sample generation. All authors participated in reviewing and editing the article and discussed the results.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study used only data from published sources, which have sought the appropriate ethical permissions.

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Genomic epidemiology and longitudinal sampling of ward wastewater environments and patients reveals complexity of the transmission dynamics of *bla*$_{KPC}$-carbapenemase-producing Enterobacterales in a hospital setting

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Background: Healthcare-associated wastewater and asymptomatic patient reservoirs colonized by carbapenemase-producing Enterobacterales (CPE) contribute to nosocomial CPE dissemination, but the characteristics and dynamics of this remain unclear.

Methods: We systematically sampled wastewater sites ($n=4488$ samples; 349 sites) and patients ($n=1247$) across six wards over 6–12 months to understand bla$_{KPC}$-associated CPE (KPC-E) diversity within these reservoirs and transmission in a healthcare setting. Up to five KPC-E-positive isolates per sample were sequenced (Illumina). Recombination-adjusted phylogenies were used to define genetically related strains; assembly and mapping-based approaches were used to characterize antimicrobial resistance genes, insertion sequences (ISs) and Tn*4401* types/target site sequences. The accessory genome was evaluated in some of the largest clusters, and those crossing reservoirs.

Results: Wastewater site KPC-E-positivity was substantial [101/349 sites (28.9%); 228/5601 (4.1%) patients cultured]. Thirteen KPC-E species and 109 strains were identified using genomics, and 24% of wastewater and 26% of patient KPC-E-positive samples harboured one or more strains. Most diversity was explained by the individual niche, suggesting localized factors are important in selection and spread. Tn*4401* + flanking target site sequence diversity was greater in wastewater sites ($P<0.001$), which might favour Tn*4401*-associated transposition/evolution. Shower/bath- and sluice/mop-associated sites were more likely to be KPC-E-positive (adjusted OR = 2.69; 95% CI: 1.44–5.01; $P=0.0019$; and adjusted OR = 2.60; 95% CI: 1.04–6.52; $P=0.0410$, respectively). Different strains had different bla$_{KPC}$ dissemination dynamics.

Conclusions: We identified substantial and diverse KPC-E colonization of wastewater sites and patients in this hospital setting. Reservoir and niche-specific factors (e.g. microbial interactions, selection pressures), and different strains and mobile genetic elements likely affect transmission dynamics. This should be considered in surveillance and control strategies.

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Introduction

Carbapenemase-producing Enterobacterales (CPE) are a global health threat,[1] and treatment of CPE infections remains difficult. Major global carbapenemases include the MBLs (bla_{NDM}, bla_{VIM}, bla_{IMP}), some oxacillinases ($bla_{OXA-48/48\text{-}like}$ variants) and the *Klebsiella pneumoniae* carbapenemase (bla_{KPC}).[2] Intra- and inter-species horizontal transfer of these genes is facilitated by mobile genetic elements such as transposons and plasmids, resulting in rapid dissemination of carbapenem resistance.[3,4]

CPE have been isolated from human and animal gastrointestinal tracts, and from sewage, rivers and sink drains.[5–7] Recent studies have highlighted that hospital wastewater sites may act as a CPE reservoir,[5] but the diversity within this reservoir, genetic overlap with patient isolates, and likely directionality and rates of transmission remain unclear. Studies characterizing patient-to-patient transmission as the sole explanatory factor in dissemination have been unable to robustly explain most transmission events, suggesting that patient, staff and/or environmental reservoirs remain insufficiently considered.[8] Few studies have evaluated within-niche diversity by sampling multiple isolates per individual and/or site.[9–11]

The Manchester University NHS Foundation Trust (MFT; UK) has experienced bla_{KPC}-positive Enterobacterales (KPC-E) cases since 2009. Following a $bla_{KPC\text{-}2}$-ST216-*Escherichia coli* outbreak on two cardiac wards and subsequent ward closure and plumbing infrastructure replacement, early environmental sampling after the ward reopened suggested rapid recolonization of wastewater sites with KPC-E, likely from transfers of colonized patients to the ward and ongoing evolution of new KPC-E strains in the environment.[12] We therefore undertook a prospective study systematically sampling all wastewater sites on six wards for 6–12 months alongside patient rectal screening/clinical sampling, and used an anonymized electronic database to characterize patient admission, ward movement, sampling profiles and culture results. Multiple colonies from KPC-E-positive samples were sequenced to define within-niche diversity and consider modes of evolution and transmission.

Patients and methods

Study setting

MFT is a large centre in northwest England, UK, managing >10 000 patients per year. In response to the regional emergence of KPC-E,[13] the Trust implemented an extensive Infection Prevention and Control (IPC) programme, consistent with UK guidelines.[14] Despite this, in April 2015 there was a large bla_{KPC}-*E. coli* outbreak in the cardiac unit,[12] leading to its closure (September 2015 to January 2016) and complete refurbishment, including plumbing replacement. Subsequently, systematic wastewater site sampling was undertaken (see below).

To evaluate patient-level microbiological/admissions data, MFT electronic bacteriology records were linked, based on NHS numbers, to patient administration data, and anonymized. For this study, we analysed anonymized patient data from 1 January 2016 to 31 December 2016 inclusive, restricted to patients with exposure to any of six wards within the three study units (acute medicine, cardiology, geratology) during that time.

Patients and wastewater sites were considered as two distinct KPC-E reservoirs, with individual patients and specific environmental sites considered as niches.

Environmental sampling and laboratory processing

All sink/drain/wastewater sites on the units/wards were sampled: the cardiac unit [wards 3 (W3)] and 4 (W4)]; the geratology unit [wards 45 (W45) and 46 (W46)]; and the acute medicine unit (wards AM1 and AM2) (Table S1, available as Supplementary data at *JAC-AMR* Online, shows sampling site designations/wastewater site types). All wastewater sites on both W3 and W4 were sampled fortnightly on rotation from 8 January 2016 to 28 December 2016. W45, W46, AM1 and AM2 wastewater sites were similarly sampled fortnightly, but from 18 July 2016 to 31 December 2016.

Environmental sampling was carried out by aspirating ~20 mL of wastewater from sink P-traps, shower drains or toilets and performing enrichment-based culture (see Supplementary methods). Multiplex, real-time, quantitative PCR (for bla_{NDM}, bla_{KPC}, $bla_{OXA\text{-}48}$)[12] was performed on broths following incubation. A 10 μL sample of any bla_{KPC}-positive-broth was streaked onto CPE-selective agar plates (Chromid CARBA) and reincubated (aerobically, 37°C).

Patient sampling and laboratory processing

Routinely collected patient clinical samples were processed using standard operating procedures in line with UK Standards.[15] In addition, a rectal CPE screening programme was in place from 2014 in line with national guidance (see Supplementary methods). Whereas all rectal swabs were directly screened for carbapenem resistance genes using the Cepheid Xpert Carba-R assay or an in-house multiplex PCR (identifying bla_{KPC}, bla_{NDM} and $bla_{OXA\text{-}48}$), clinical isolates were typically only profiled routinely in terms of carbapenem susceptibility. Species identification of isolates was performed by MALDI-TOF (Bruker Biotyper); antimicrobial susceptibility testing was performed as per EUCAST guidelines.[16] To evaluate within-niche diversity in the environment and patients, up to five different CPE colonies were individually subcultured and stored for sequencing.

DNA extraction and isolate sequencing

DNA was extracted from frozen subculture stocks with the QuickGene kit, as per the manufacturer's instructions, with an additional mechanical lysis step post-chemical lysis (6 m/s at 40 s ×2 FastPrep; MPBio). Isolates were sequenced on the Illumina HiSeq 4000 generating 150 bp paired-end reads.

Sequence data processing and analysis

Sequencing reads were processed;[17] species identification was performed with Kraken2 (v2.0.8-beta; default settings),[18] and if ≥50 sequenced isolates were of a single species, reads were mapped to species-specific references and variation called (see Supplementary methods). Recombination-adjusted phylogenies by species were created from core chromosomal single nucleotide variants (SNVs; padded to the reference genome length) using IQtree[19] (v1.5.3; flags: -m GTR+G -blmin 0.00000001 -t PARS) and then ClonalFrameML[20] (v1.0; default parameters). A threshold of ≤400 SNVs was used to define strain clusters (similar to Sheppard *et al.*;[21] distribution of within-cluster SNV distances in Figure S1a–e for commonest species). For detailed sub-strain analysis for *K. pneumoniae* strain 9, sub-strains were defined on the basis of accessory genome clusters characterized using Panaroo (v1.2.9; default parameters including-clean-mode strict)[22] and clustered using the R heatmap package.

Sequences were assembled using SPAdes (v3.6; default parameters).[23] Multilocus sequence types were derived using BLASTn and species-specific databases (https://pubmlst.org). AMR genes were identified using ARIBA[24] (v2.11.1) and the CARD database[25] (version 2.0.0) and ISs using ISFinder (https://isfinder.biotoul.fr). bla_{KPC} genes, Tn*4401* transposons and the 5 bp target site sequences (TSSs) on either side of the Tn*4401* transposon reflecting transposition signatures were characterized using TETyper[26] (version 1.1), a Tn*4401*b reference sequence, a 5 bp flank length and the default structural/SNV profiles. Arbitrary profile

numbers were assigned based on the presence of unique profiles of AMR genes, plasmid replicons and ISs (Supplementary datasets 1 and 2).

For the analysis of *K. pneumoniae* strain 9 (see Results), putative transmission networks were inferred first using SCOTTI[27] (2022–12-21) and then heuristically, linking isolates identified as clusters on the basis of accessory component profiles and geographic/temporal overlap (Supplementary methods).

Statistics and data visualization

Descriptive statistics were calculated in R v3.6.2. For permutational analyses of variance, we used the 'adonis' function (vegan R package, v2.5-7)[28] on a matrix of pairwise Gower distances based on a composite genetic profile of species-strain type, plasmid replicons, AMR genes, ISs and Tn*4401* + flanking TSSs, which was calculated using the 'daisy' function (R cluster package). To evaluate the impact of unit location or environmental site type on the odds of CPE positivity, we used logistic regression with robust standard errors clustered by environmental site sampled (R rms package, v6.2-0).[29] For data visualization we used the ggplot2 R packages[30] and BioRender (www.biorender.com).

Data availability

Sequence data have been deposited in NCBI (BioProject accessions PRJNA768622, PRJNA514245; Supplementary dataset 2). We are not able to share the complete database of patient tests, results and admissions episodes; dates for patient admission/sampling are given as month/year only. Of note, labels represented in the figures and

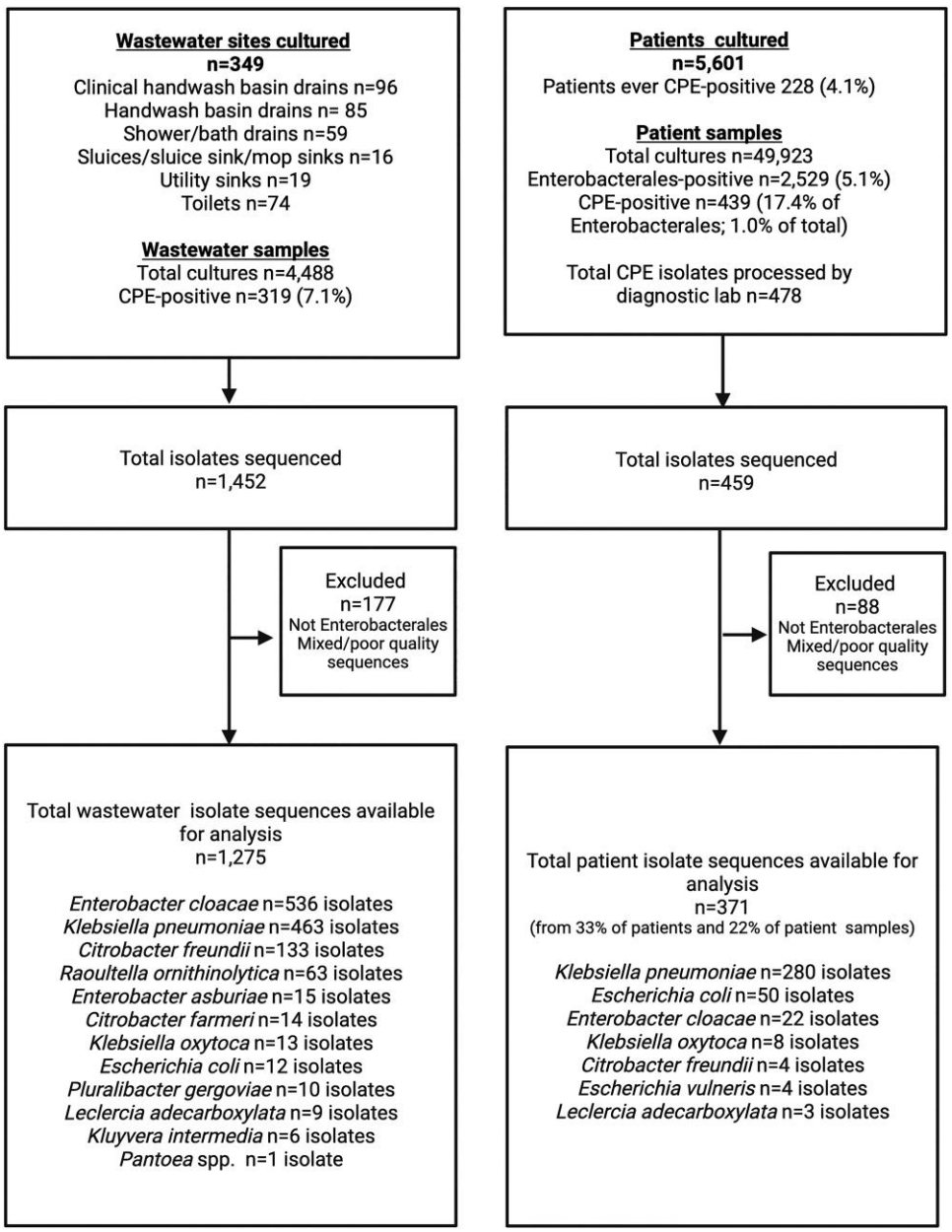

Figure 1. Sampling and sequencing flowchart for the study (created using www.biorender.com).

supplementary data are anonymized study-specific labels that are not patient-identifiable.

Ethical considerations

This study was part of a Trust board-approved and UK Health Security Agency long-term outbreak response, and ethical approval was not required under NHS governance arrangements; all analyses were undertaken using anonymized data. This was reviewed by the Joint Research Office of the Oxford University Hospitals NHS Foundation Trust and the University of Oxford, who deemed that neither sponsorship nor research ethics review was required.

Results

Environmental sampling revealed high prevalence and clustering of wastewater KPC-E colonization

To characterize how densely and persistently ward wastewater sites were colonized with KPC-E over time, 349 sites across the six study wards were sampled a total of 4488 times over 6–12 months (Figure 1). We found that 101/349 (28.9%) sites and 319/4488 (7.1%) sampling events were KPC-E-positive, with no difference by unit (Figure 2, $P=0.908$), or over time. Adjusting for environmental site type and unit location, shower/bath drain sites and sluices/sluice sinks/mop sinks were significantly more likely to be positive for KPC-E than others [adjusted OR (AOR) (95% CI): 2.69 (1.44–5.01), $P=0.0019$; and AOR 2.60 (1.04–6.52), $P=0.041$, respectively], and toilet water sampling less likely to be positive [AOR (95% CI): 0.28 (0.10–0.78), $P=0.015$]; there was no evidence of a unit-dependent difference (Table 1). We found 7/349 (2.0%) sites to be persistently KPC-E positive (≥10 consecutive KPC-E-positive sampling events); interestingly five of these were medicines/treatment room handwash basin drains (of eight medicines/treatment room sinks in total) (Table S1, Figure S2).

Approximately 1 in 25 patients were colonized or infected by KPC-E, with most cases reflecting rectal colonization

To determine the prevalence of infection in and asymptomatic colonization of patients with CPE, we analysed 49 923 culture results from 5601 patients. Of 2529 Enterobacterales-positive

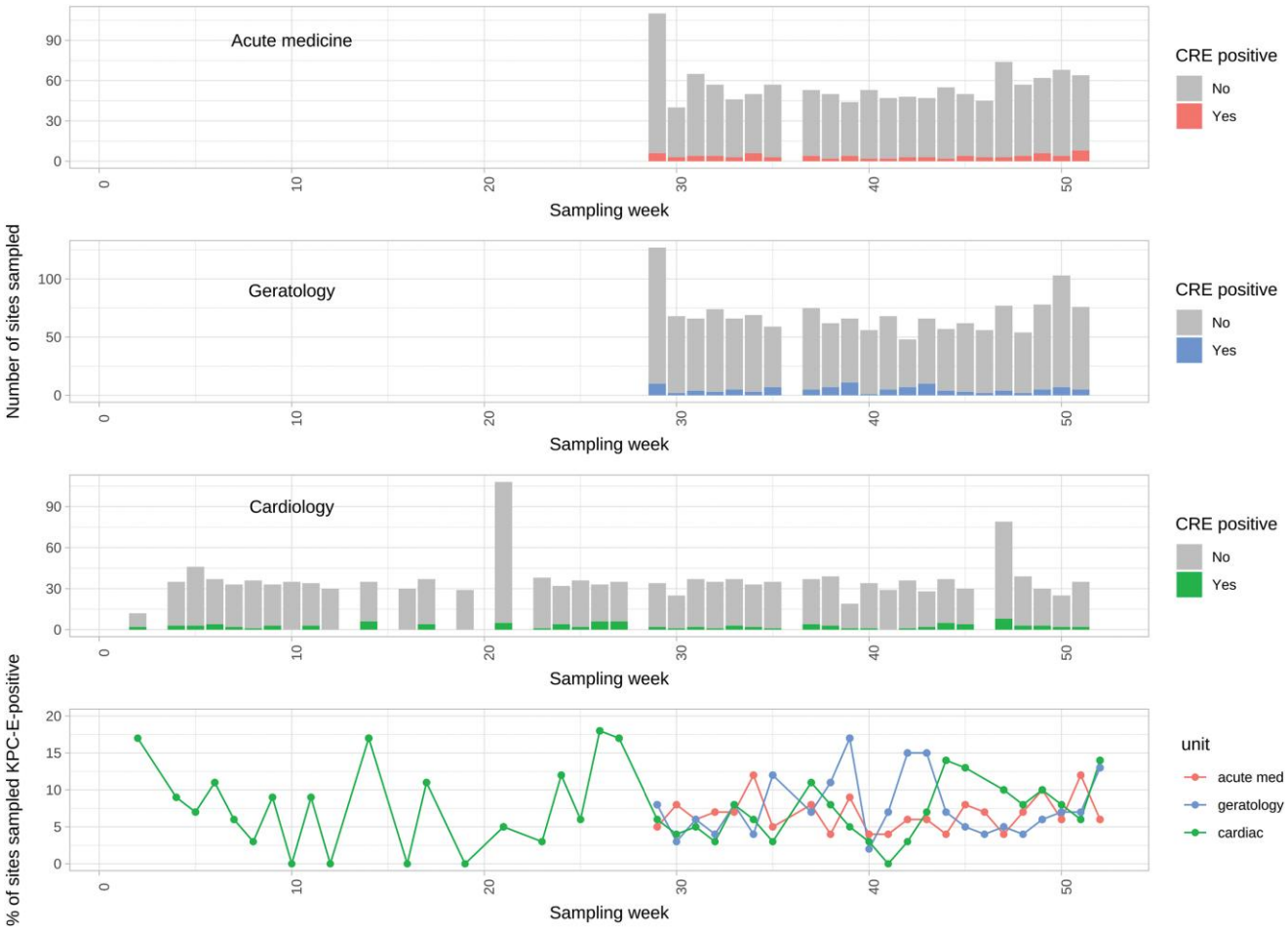

Figure 2. bla$_{KPC}$-Enterobacterales (KPC-E)-positive environmental sites by Week in 2016 and hospital unit. The number of environmental site samples performed and KPC-E-positive samples by week of sampling, stratified by unit (top three panels), and the proportion of KPC-E-positive sites by week of sampling, again stratified by unit (bottom panel). Plumbing replacement on the cardiac unit (wards 3 and 4) was carried out at Week 0.

Table 1. Association of environmental site type and unit location with bla_{KPC}-Enterobacterales (KPC-E) positivity in environmental samples[a]

Environmental site	Number of sites	Number of sampling events KPC-E-positive (% of total sampling events)	Number of sampling events KPC-E-negative	OR (95% CI)	*P*
Environmental site type					
Clinical handwash basin drain	96	79 (5.9%)	1270	Reference	
Handwash basin drain	85	52 (4.6%)	1077	0.99 (0.51–1.93)	0.9735
Shower/bath drain	59	100 (19.2%)	421	2.69 (1.44–5.01)	**0.0019**
Sluice/sluice sink/mop sink	16	47 (17.9%)	215	2.60 (1.04–6.52)	**0.0410**
Toilet	74	5 (0.5%)	931	0.28 (0.10–0.78)	**0.0152**
Utility sink	19	36 (12.4%)	255	1.32 (0.47–3.68)	0.5965
Environmental site location					
Acute medicine unit	129	86 (6.6%)	1223	Reference	
Cardiac unit	76	111 (7.0%)	1453	1.07 (0.57–2.00)	0.8412
Geratology unit	144	122 (7.6%)	1493	0.88 (0.52–1.48)	0.6243

[a]Outputs of a multivariable logistic regression model with robust standard errors clustered by environmental site type and unit location; no other variables were considered in the model. *P* values <0.05 were considered significant and are highlighted in bold. No interactions with an interaction Wald $P < 0.05$ were observed.

samples (2622 Enterobacterales isolates, 1247 patients), 439/2529 (17.4%) samples cultured at least one carbapenem non-susceptible Enterobacterales ($n = 478$ isolates in total) (Figures 1 and S3). For 344/439 (78.4%) of these isolates for which carbapenemase PCR data were also available, all contained at least one known carbapenemase gene, of which 303/344 (88.0%) were bla_{KPC} (Table S2). CPEs were cultured from 228 patients (4.1% of all patients cultured, 18.2% of all patients with an Enterobacterales-positive culture) during the study period (median = 1; IQR: 1–2; range: 1–17 CPE isolates/patient).

Most CPE-positive isolates came from rectal screens [$n = 387/478$ (81.0%)]; others came from non-screening clinical specimens [91/478 (19.0%)] (Table S3). Most CPE-positive patients [178/228 (78%)] were CPE-positive only on rectal screen, but 23/228 (10%) were positive only on clinical culture, and 27 (12%) on both. By routine laboratory methods, >20 species and eight genera were represented amongst the CPEs, with *K. pneumoniae*, *E. coli* and *Enterobacter cloacae* complex predominating [434/478 (90.8%)]; notably, the proportions of carbapenem-resistant isolates within these three species varied [$n = 284/593$ (48%) versus 106/1404 (8%) versus 44/110 (40%), respectively; Fisher exact test, $P < 0.001$, Figure S3].

The proportion of patient CPE-positive cultures was lower than amongst environmental samples, typically ∼3%–4% (Figure 3, bottom panel). Occasional peaks in prevalence were observed, consistent with outbreaks (e.g. Week 31, acute medicine unit). CPE prevalence amongst patient cultures on the refurbished cardiology unit was 0% (97.5% CI: 0–0.02) for the first 10 weeks post-reopening, although two utility room sink drains were positive for KPC-E a day after W3 reopened to patients (11 January 2016). After Week 20 the unit was clearly recolonized (Figure 2), with positive environmental CPE-cultures observed in 26/27 (96%) weeks of sampling and positive patient CPE-cultures observed in 16/32 (50%) weeks of sampling.

CPE culture-positivity was therefore approximately seven times higher in wastewater sites (28.9%) sampled than in patients sampled throughout the study period, and just over three-quarters of patient CPE culture-positives reflected carriage (i.e. positive on rectal screens), highlighting these as major potential reservoirs for transmission. Plumbing replacement on the cardiac unit had only a very transient effect on environmental and patient CPE culture-positivity.

Structuring of KPC-E diversity by patient and environmental niche, with most diversity explained by the individual niche

Given that culture-based microbiology cannot characterize genetic diversity within species, and this diversity is relevant to understanding carbapenem gene dissemination and transmission, we undertook genome sequencing of isolates. We successfully sequenced 1646 CPE isolates (1275 environmental, 371 patient isolates; Figure 1 and Supplementary dataset 2); all contained bla_{KPC-2}, and 13 KPC-E species were identified across patients and environmental reservoirs. Amongst five species with ≥50 sequenced isolates for which phylogenetic analysis and clustering was performed, 109 unique strains were represented. Of the 109 strains, 61 (56%) were found only in the environment, 27 (24%) only in patients, and 21 (19%) in both patients and the environment (Figure 4); the median strain cluster size was five isolates (range: 1–122), and the median number of unique niches (i.e. unique patients or wastewater sites) affected by a strain was two (range: 1–23).

To identify the diversity of mobile genetic elements that could support horizontal gene transfer (HGT), we evaluated plasmid replicon and IS profiles in isolates. We also defined Tn*4401* TSS diversity because Tn*4401* mobilizes bla_{KPC} and TSS diversity can be a marker of transposition frequency.

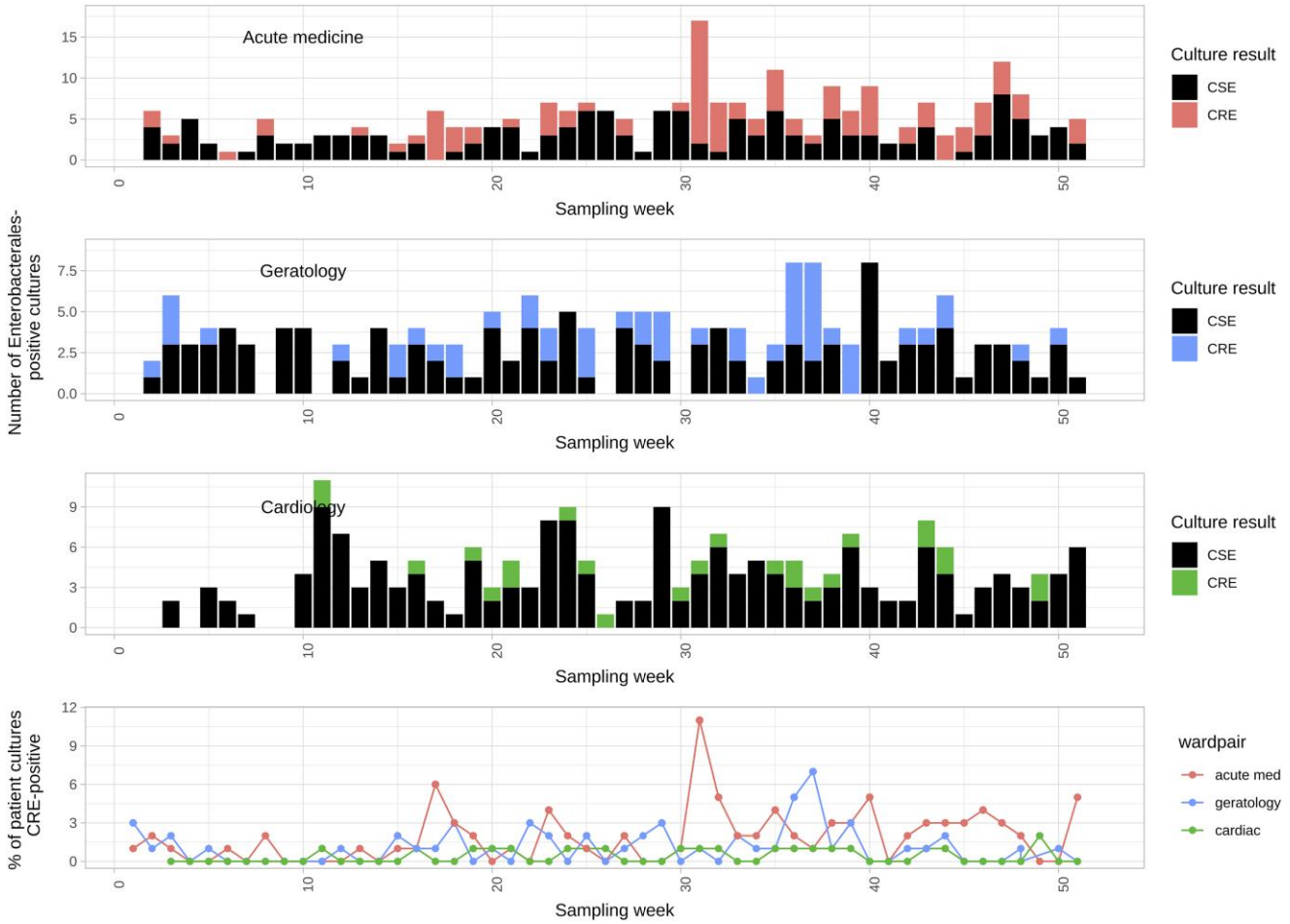

Figure 3. Carbapenem-susceptible Enterobacterales (CSE) and carbapenem-resistant-Enterobacterales (CRE) patient cultures by week in 2016 and hospital unit. Top three panels show counts of Enterobacterales culture-positive samples stratified by unit and carbapenem susceptibility; bottom panel shows the proportion of all cultures taken that were CRE, again stratified by unit.

Amongst sequenced isolates, 100 plasmid replicon profiles, 754 IS profiles, 939 AMR gene profiles and 70 Tn*4401*/TSS types were identified (Supplementary dataset 2). More Tn*4401*/TSS types were observed in the environment versus patients [66/70 (94%) of types versus 12/70 (17%), respectively; Fisher exact test, $P<0.001$], and all the isolates with more than one set of TSSs were seen in environmental sites, consistent with these representing a more favourable reservoir for Tn*4401*-associated transposition/evolution and dissemination.

For colonized niches within patient or environmental reservoirs, using a metric of genomic diversity based on strain, plasmid replicon profile, AMR gene profile, IS profile, and Tn*4401*+5 bp TSS profiles, most variance was explained by the specific individual niche (i.e. patient or environmental site) sampled ($R^2=50.5\%$; $P<0.001$), versus small but significant contributions made by reservoir type (i.e. patient versus environmental reservoir; $R^2=5.6\%$; $P<0.001$) and unit location ($R^2=2.8\%$; $P<0.001$).

For the 15 cases where exactly the same strain, AMR gene profile, IS profile, plasmid profile and Tn*4401*/TSS type were seen in patient and environmental niches (Figure S4), and considering only event-pairs where chromosomal SNP distances were within ≤5 SNPs and there was a change in sampling site, the temporal relationship of these supported patient-to-environment transmission in 13/29 (45%) events, environment-to-patient transmission in 2/29 (7%) events, environment-to-environment transmission in 1/29 (3%) events, and patient-to-patient transmission in 13/29 (45%) events (test of equality of proportions, $P<0.001$).

Environmental and patient niches harboured diverse KPC-E within single samples and over time

Environmental sites showed evidence of major strain-level diversity: from the 101/349 sites from which bla_{KPC-2}-Enterobacterales isolates were successfully sequenced at any timepoint, 78/319 (24.4%) KPC-E-positive samples had more than one bla_{KPC-2}-Enterobacterales strain, namely 52/319 (16.3%) with two strains, 16/319 (5.0%) with three, 9/319 (2.8%) with four and 1/319 (0.3%) with five strains (Figures 5 and S5). Over the study period, colonized environmental sites had a median of three (range: 1–17, IQR: 2–5) bla_{KPC-2}-Enterobacterales strains; 49/101 sites (49%) were apparently colonized by a single bla_{KPC-2}-Enterobacterales strain only,

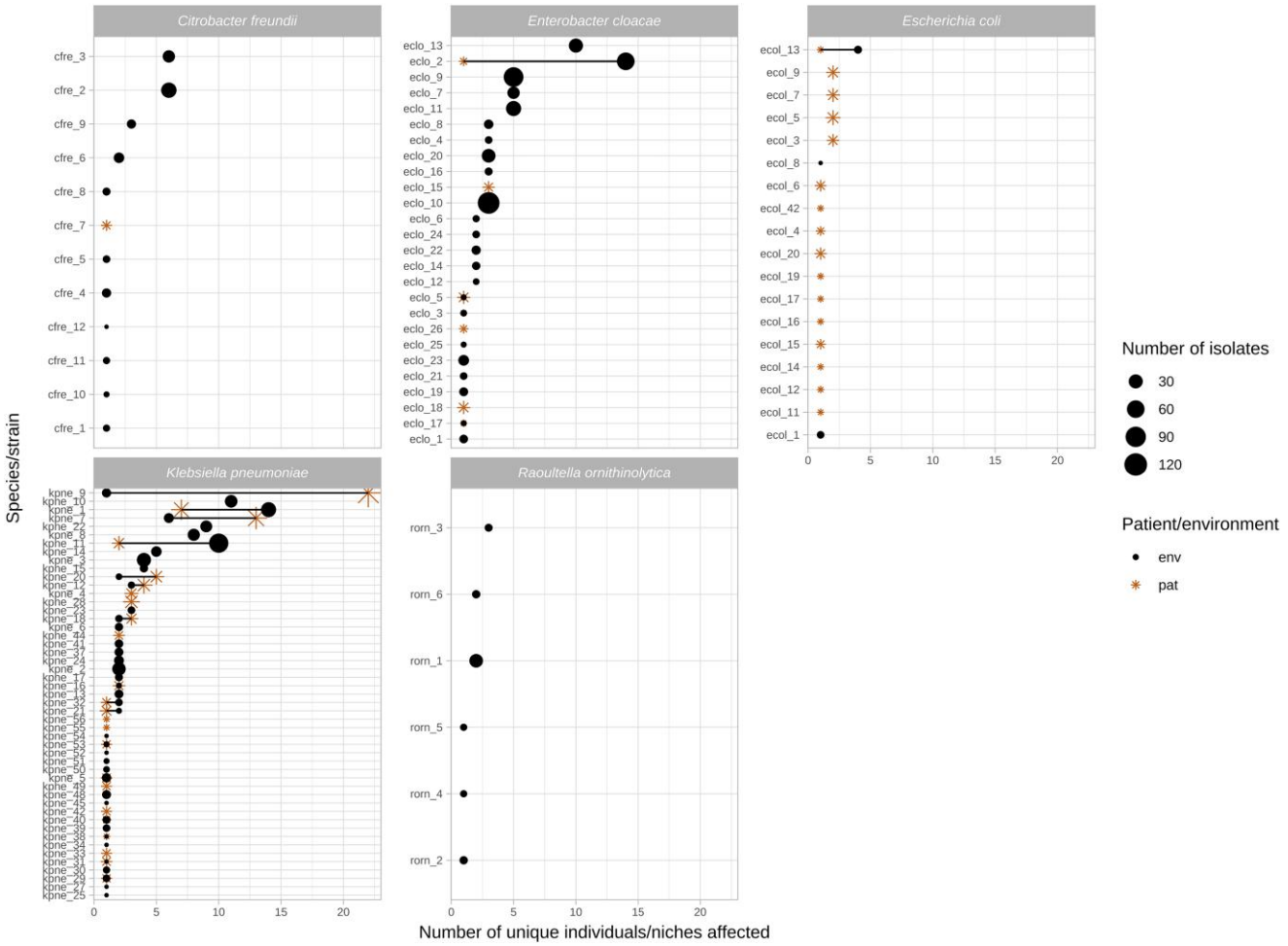

Figure 4. Number of unique patient/environmental niches colonized by common *bla*$_{KPC-2}$ Enterobacterales species/strains and the total number of isolates for each cluster. Includes species/strains if >50 isolates of a species were identified (see Methods). Stars denote patient niches, circles wastewater niches; larger shape size denotes a larger number of isolates sequenced. Strains observed in both niches are either overlapping shapes (same number of niches), or joined by a line (different numbers of niches).

of which 38 were at single sampling-points, and 11 sites were positive with the same strain at two or more timepoints.

For patient samples, based on routine laboratory microbiology, evidence of mixed-species KPC-E colonization/infection was found in 35/439 (8%) samples from 26/228 (11%) patients; these were mostly *E. coli*/*K. pneumoniae* mixtures ($n=17$ samples; maximum two species identified). Based on a genomic evaluation of 457 colonies from 97 samples from 76 patients, 25/97 samples (26%) had more than one strain per sample, namely 17/97 samples (18%) with two PKC-E strains, 6/97 (6%) with three strains and 2/97 (2%) with four strains (Figures 5 and S5)—i.e. considerably more diversity identified than based on routine laboratory species identification data (26% versus 8% samples; Fisher exact test, $P<0.001$).

For a subset of 14 patients and 53 wastewater sites where within-sample diversity was evaluated by sequencing and two or more longitudinal samples were positive, longitudinal switches in KPC-E species-strain composition were common, occurring in 7/14 (50%) of patients and 41/53 (77%) wastewater sites (Figures 5 and S5).

Genomic analysis revealed distinct population biology for different bla$_{KPC-2}$-Enterobacterales strains

To investigate different modes of bla$_{KPC-2}$ dissemination, we characterized three sequenced KPC-E strain clusters, including the largest environmental-only cluster (*E. cloacae* strain 10; $n=122$ isolates), a large cluster involving patients and the environment (*K. pneumoniae* strain 9; $n=106$ isolates) and a cluster in which signatures of Tn*4401* transposition appeared highly frequent (*K. pneumoniae* strain 11; $n=96$ isolates).

Scenario 1: dynamic changes in AMR gene, IS, plasmid replicon and Tn4401 profiles in E. cloacae strain 10 in sink drains

E. cloacae strain 10 (eclo10, ST32) was a site-restricted but persistent colonizer of three co-located sink drains on a geratology ward [in a drug room clinical handwash basin drain (F45), utility sink drain (F46), and a separate treatment room clinical handwash basin drain (F47)] over ~5 months (18 July 2016 to 20 December 2016). At the core chromosomal level, strains appeared largely structured by site, with 7 SNVs separating the

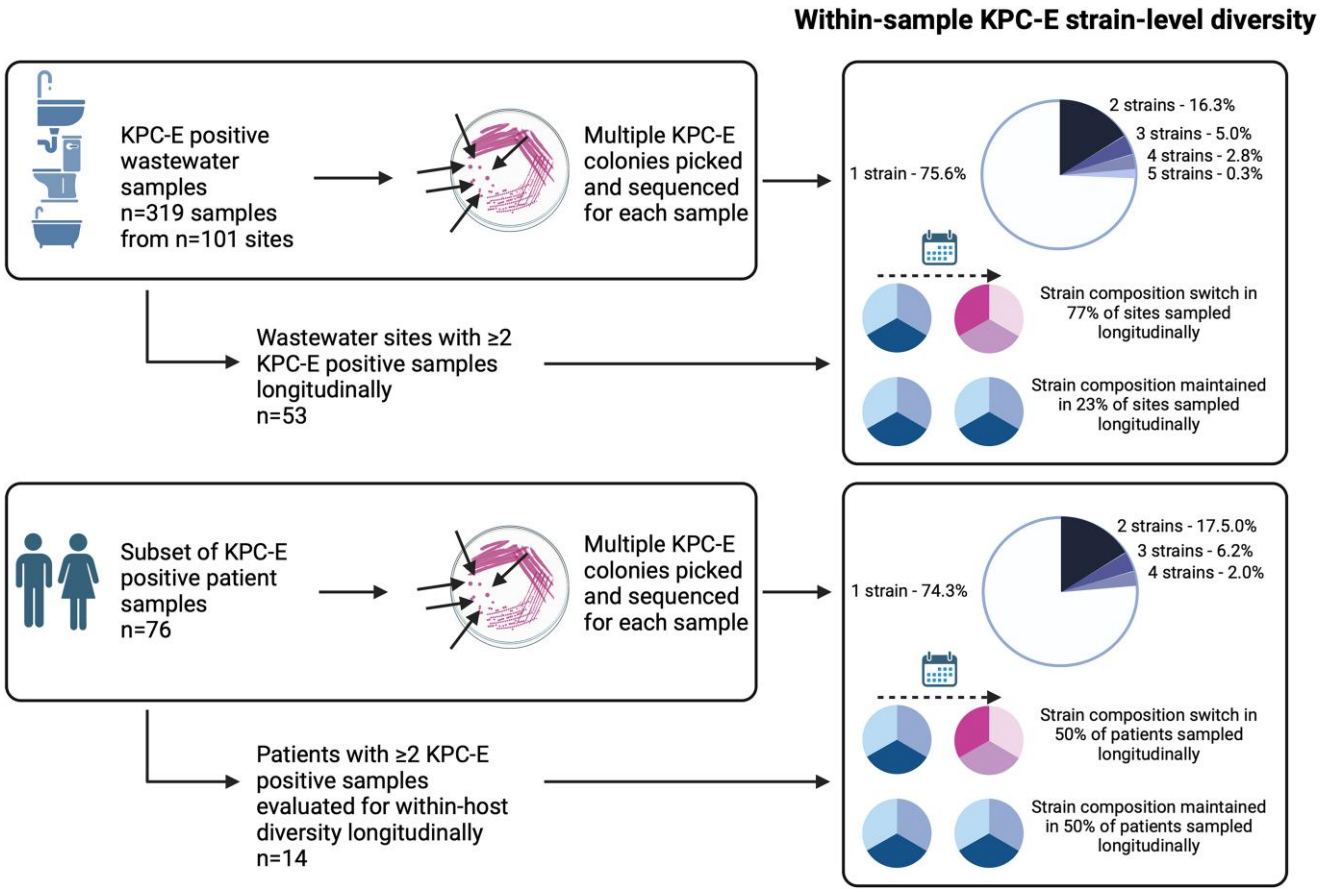

Figure 5. Schematic of within-reservoir and KPC-E sample diversity using genomics (generated in www.biorender.com).

majority of isolates in F45 from those in F47, and 14 SNVs those in F46 from F45. Three isolates in F47 and one isolate in F46 clustered with the F45-associated isolates; sampling dates suggest that these represented transmission events from F45 (Figure 6a). There was evidence of rapid churn in AMR gene and IS content (111 and 36 different profiles respectively; Figure 6bi and 6bii) in the relatively stable strain background, and almost all isolates in F47 had acquired an additional set of IncHI2/HI2A replicons on a background of FIB/FII, consistent with plasmid gain/loss (Figure 6c). Although the Tn*4401* type and flanking sequences were largely identical [Tn*4401*a-ATTGA-ATTGA in 105/122 (86%) isolates across sinks], there was evidence of unique additional Tn*4401* deletions occurring in multiple isolates at several distinct timepoints in the three sink sites (Figure 6).

Scenario 2: rapid clonal patient–patient transmission of K. pneumoniae strain 9

K. pneumoniae strain 9 (kpne9, ST252) was highly related (Figure S6; maximum six SNVs across strain, 68 isolates with zero SNVs between them), and spread among 21 patients across three wards on two units (acute medicine AM1 and AM2, and geratology W46), with a dense outbreak in August 2016, most consistent with direct/indirect patient–patient transmission (Figure 7). bla_{KPC-2} was consistently nested in Tn*4401*-ATTGA-ATTGA, except for one isolate, which acquired a Tn*4401*-associated mutation (isolate: 2216698_14, C4620T), and another in which the right TSS could not be identified (isolate: 2035791_11). Among the 106 isolates there was evidence of four transient plasmid replicon acquisitions on a background of the stable presence of a set of IncFIB/IncFII replicons (Figure S6). Transmission probabilities inferred by SCOTTI were weak (see Supplementary methods and Figure S7), as seen in other studies,[8] but using accessory cluster profiling to further discriminate amongst strains suggested that there were several discrete transmission clusters (Figure S8), with some individuals involved in several of these (i.e. harbouring multiple distinct sublineages simultaneously, Figure 7). A single shower drain site was involved (A13), which was likely a seeded bystander from affected patients, but may have contributed to transmission of sub-strain 3g (Figure 7).

Scenario 3: cross-unit dissemination with evidence of substantial Tn4401-associated bla_{KPC} transposition in K. pneumoniae strain 11

Given that Tn*4401* transposition is associated with bla_{KPC} dissemination,[21] we also evaluated whether the number of different Tn*4401*-TSS types was different across strains, noting that the observed frequency of different Tn*4401*-TSS combinations was higher in *K. pneumoniae* strain 11 (kpne11, ST11) than in other strains (Figure S9). On further analysis, kpne11

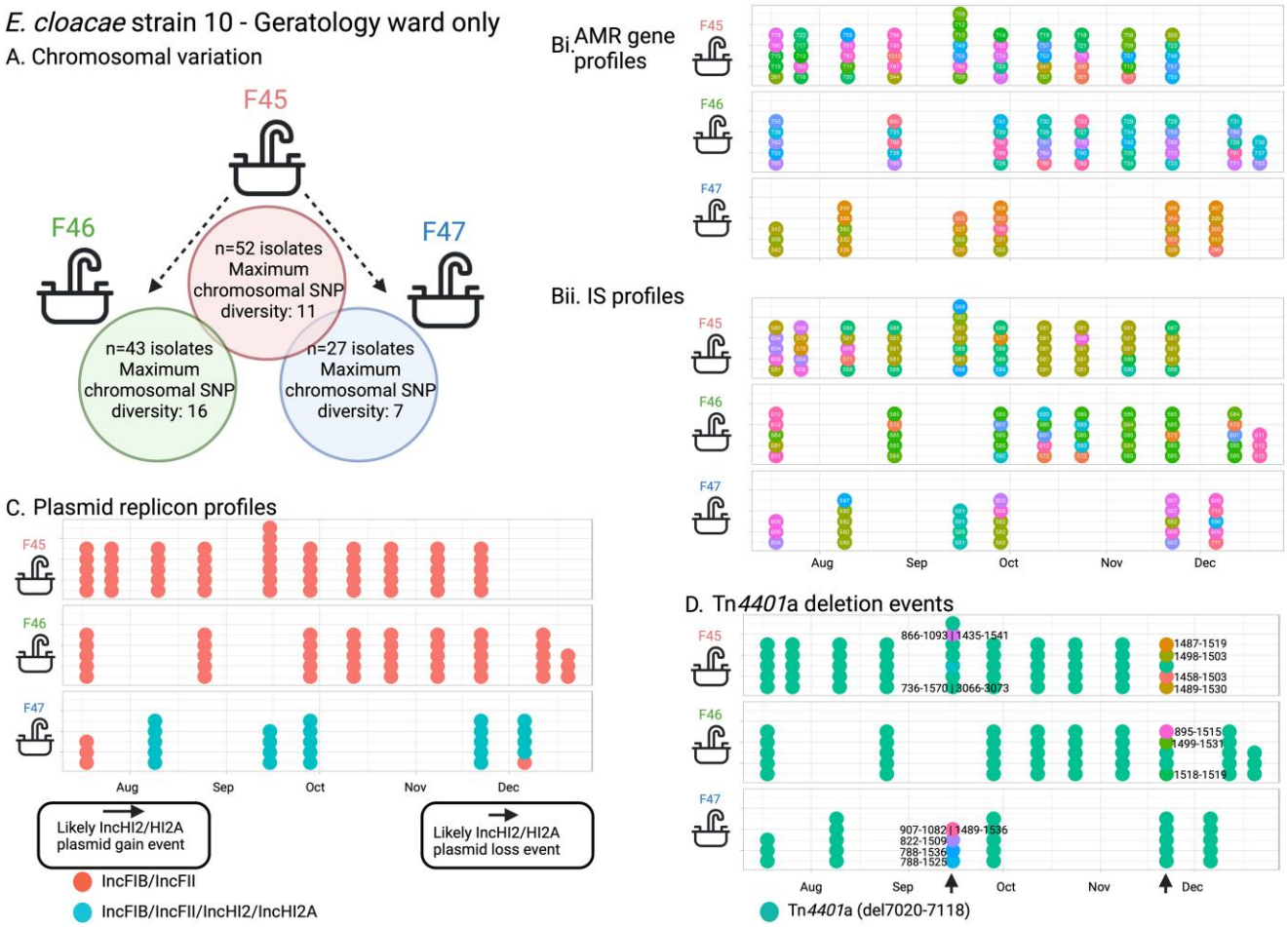

Figure 6. *Enterobacter cloacae* strain 10: features of population structure and transmission dynamics—niche restriction, persistence and genetic turnover. Summaries of (a) chromosomal SNP distances for *E. cloacae* strain 10 across the three sites it colonized; (bi) AMR and (bii) IS profiles; (c) plasmid replicon profiles; and (d) deletions in Tn*4401*a in isolates. Each sequenced isolate is plotted as a dot reflecting within-sample diversity at given timepoints and longitudinally, with colour in panels (bi) and b(ii) reflecting distinct AMR and IS profiles respectively and numbers within the dots representing the numeric identifier for profiles listed in Supplementary dataset 1.

represented two sub-clusters, separated by ~40 SNVs (Figure 8, designated 'cluster 1' and 'cluster 2'; Figure S10), both of which were isolated from patients and wastewater sites across ward settings, including the cardiac unit after plumbing replacement. Within both clusters of isolates there were 10 different Tn*4401*a flanking signatures observed, only 1 of which was seen in the other >1600 isolates sequenced in the study (Tn*4401*a-1_ATTGA_
ATTGA, the most common flanking signature observed overall), and therefore most strongly consistent with the occurrence of an atypical and increased number of transposition events within this *K. pneumoniae* strain. Although all isolates contained IncFIB and IncFII plasmid replicons, evidence of plasmid gain/loss events as demonstrated by changes in plasmid replicon profiles also appeared common (Figure 8 for cluster 1).

Discussion

This work highlights the substantial KPC-E genetic diversity and flux in a *bla*$_{KPC}$ genetic context and in niches that can occur in both human and wastewater reservoirs in healthcare settings over short timeframes. KPC-E dissemination dynamics appeared highly variable, making a generalized framework of transmission, evolution and bla$_{KPC}$ sharing among these species very challenging. Although structuring at the strain level was observed by niche and species (Figure 4), likely transmissions occurred in a non-negligible manner in all directions from patients to other patients and the environment, and from the environment to patients and other environmental sites. The environmental reservoirs and certain species-strain contexts (such as *K. pneumoniae* strain 11) appeared to amplify bla$_{KPC-2}$ transposition events.

Although some environmental sites were persistently colonized with individual strains over months, many appeared to be only transiently colonized. Sites particularly associated with KPC-E colonization included sluices, sluice sinks, mop sinks and shower/bath drains, as supported by other studies,[5] and consistent with these representing a major interface between human faeces and premise plumbing. Toilets were less likely to be KPC-E-positive, perhaps because of regular flushing or particular cleaning protocols; consistent with this, several studies

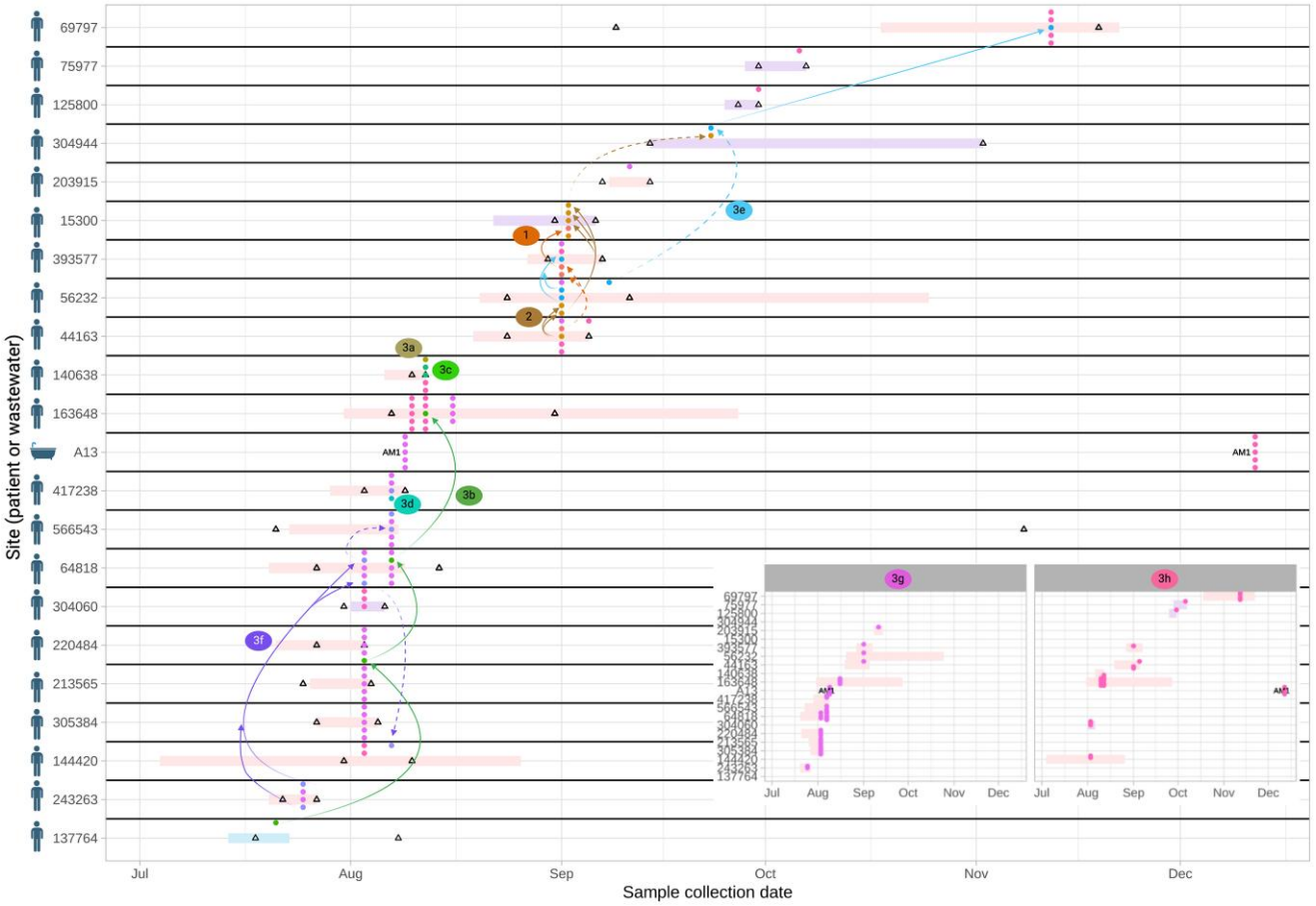

Figure 7. *Klebsiella pneumoniae* strain 9: features of population structure and transmission dynamics—rapid patient-patient transmission. Putative transmission network for sub-lineages of *K. pneumoniae* strain 9, defined by accessory genome clustering (clusters 1, 2, 3a–h). Horizontal bars represent admission episodes to study wards for each patient (pale blue = W45, geratology; pale pink = AM1, acute medicine; pale purple = AM2, acute medicine; A13 represents the only environmental (shower) site involved. Empty black triangles denote CPE-negative rectal screens on patients. Arrows denote possible transmission links (dashed arrows denote events that have no geographical overlap or could be associated with two links based on negative screen, geographical location and timing). For clarity, arrows are not drawn for clusters 3g and 3h, which affected the most patients—these are shown in detail as an inset panel at the bottom right. In some cases patients were colonized by other species-strains contemporaneously (not shown here). (Figure generated in ggplot and www.biorender.com).

have demonstrated that daily bleach application decontaminates these sites.[31,32]

Our study underscores the detailed and dense sampling effort required to understand tracking AMR gene outbreaks across reservoirs, because so much diversity in genetic contexts can be observed as in this study, and this can fluctuate rapidly. This problem has also been demonstrated in other recent work investigating bla_{OXA-48}, where only patient-based sampling was undertaken and most bla_{OXA-48}-associated transmission events were non-delineated.[8] Our study clearly shows that different species and lineages represent different epidemiological risks and have their own behaviours, and a focus on single, clonal strains as mediators of AMR gene transmission becomes less relevant. However, targeting large numbers of single isolates for sequencing is time consuming and resource intensive. One strategy might be to use long-read metagenomics, enabling reconstruction of genetic contexts around AMR genes, or long-read metagenomics of cultured sub-populations of isolates grown on relevant selective media to avoid wasted sequencing effort; this would require further evaluation.

The importance of evaluating asymptomatic carriage, characterizing within-host diversity of strains, considering rapid acquisition/loss of strains over short timeframes, and including environmental sites in transmission networks has been partly demonstrated in previous studies investigating ESBL *E. coli*[10,33] and *Enterococcus* spp.,[34] but generally these features are not jointly investigated because this is resource-intensive and analysis is challenging. However, our study highlights that without this effort, such as in studies that consider only single or infrequent sampling timepoints, and do not investigate asymptomatic colonization or consider within-site diversity,[35] relevant transmission events may easily be missed, leading to incorrect assumptions about transmission networks and therefore potentially ineffective interventions.

There are several limitations to this work. We only evaluated a subset of CPE-positive patients and their samples using genomics

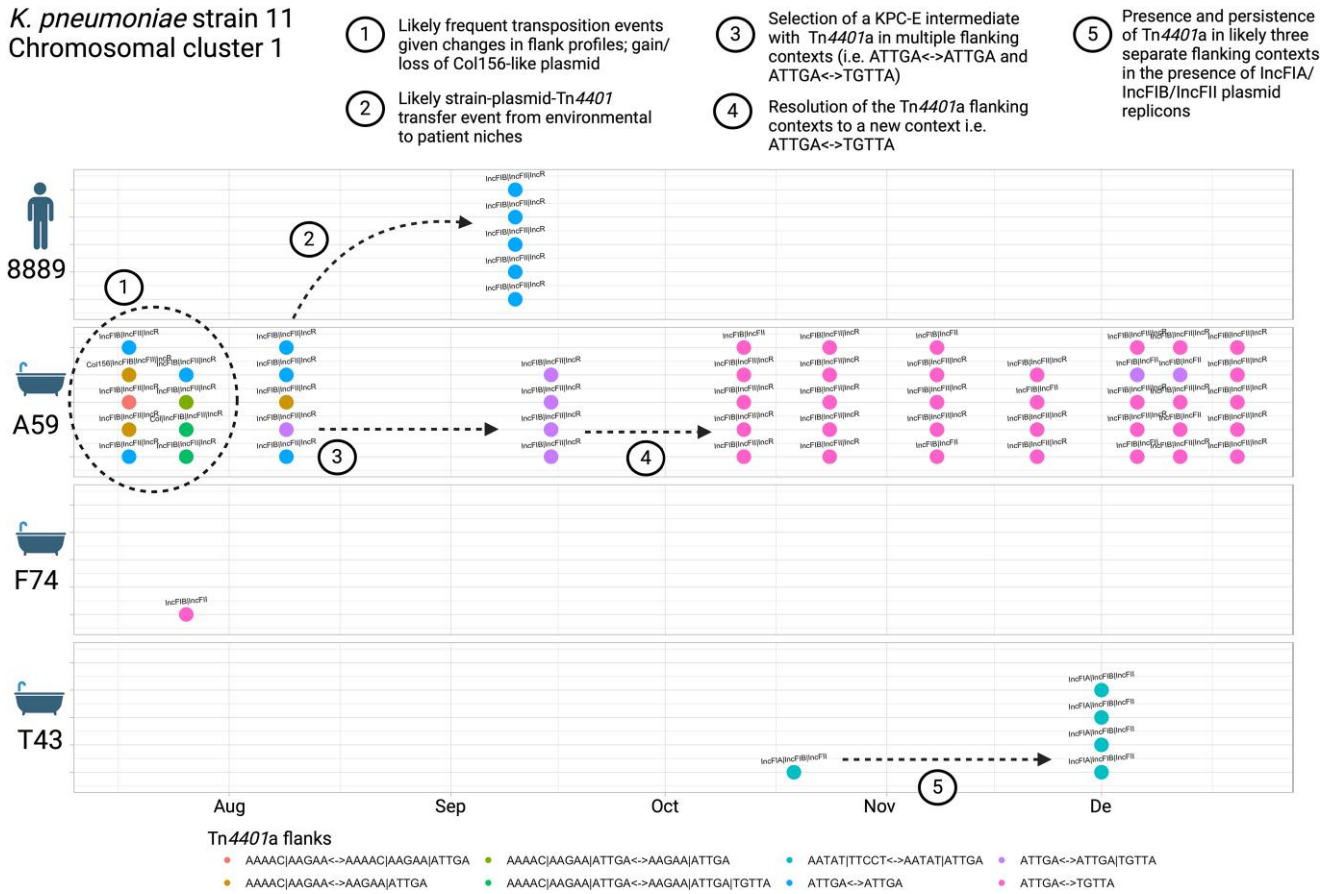

Figure 8. *Klebsiella pneumoniae* strain 11 cluster 1: features of population structure and transmission dynamics—multiple Tn*4401* transposition events. Examples of possible transmission/genetic events are annotated on the figure. Each isolate sequenced is represented as a dot, with colour denoting the TSS profiles observed. Plasmid replicon profiles are annotated as text. (Figure generated in ggplot and www.biorender.com).

(33% and 22%, respectively); we anticipate many transmission events were missed. We were not resourced to perform long-read sequencing to enable plasmid sequence reconstruction, and short-read-based profiling is relatively low-resolution and can be misleading;[21] we therefore avoided a detailed plasmid analysis, which would be important future work. However, the diversity we observed in genetic contexts supporting *bla*$_{KPC}$ was substantial, consistent with high rates of transfer. A further limitation was the absence of metadata capturing relevant selection pressures in each niche, including cleaning protocols for environmental reservoirs and drug prescription data for patients. We focused only on Enterobacterales and may have missed HGT of carbapenemase genes to other bacteria. Our approaches to evaluating some of the genetic changes and transmission events observed were heuristic and manual, partly because the data were highly heterogeneous and complex. The study sampling was undertaken several years ago in 2016–2017, but similar polyspecies, polyclonal carbapenemase gene outbreaks associated with hospital wastewater sites continue to be described,[36] and as such we believe the findings make an important contribution to our understanding of the microbial ecology of the hospital built environment.

Further optimization of methods to evaluate multilevel genetic transmission and within-sample diversity without individual colony-level characterization is needed. Holistic approaches using fully reconstructed chromosome/plasmid assemblies to quantify the diverse types of genomic transmission (including clonal transmission, horizontal transfer at the gene, transposon and plasmid levels) are also required to better understand what facilitates emergence, selection and persistence of different lineages and genetic vectors of bla$_{KPC}$. This information will improve our understanding of how to intervene to limit the transmission of drug-resistant Enterobacterales in healthcare settings.

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The views expressed are those of the authors and not necessarily those of the NHS, NIHR, UKHSA or the Department of Health and Social Care.

Supplementary data

Figures S1 to S10, Tables S1 to S3, Supplementary methods and Supplementary datasets are available as Supplementary data at *JAC-AMR* Online.

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State of the Science Review

Infection control in the new age of genomic epidemiology

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Genomic epidemiology
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With the growing importance of infectious diseases in health care and communicable disease outbreaks garnering increasing attention, new technologies are playing a greater role in helping us prevent health care–associated infections and provide optimal public health. The microbiology laboratory has always played a large role in infection control by providing tools to identify, characterize, and track pathogens. Recently, advances in DNA sequencing technology have ushered in a new era of genomic epidemiology, where traditional molecular diagnostics and genotyping methods are being enhanced and even replaced by genomics-based methods to aid epidemiologic investigations of communicable diseases. The ability to analyze and compare entire pathogen genomes has allowed for unprecedented resolution into how and why infectious diseases spread. As these genomics-based methods continue to improve in speed, cost, and accuracy, they will be increasingly used to inform and guide infection control and public health practices.

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Infectious diseases continue to be one of the leading causes of death worldwide.[1] In contrast with noncommunicable diseases, infectious pathogens can often evolve and spread rapidly, leading to the emergence of novel human pathogens, more virulent forms of existing pathogens, and antibiotic resistance organisms.[2,3] Our infection control practices must continue to evolve and improve in order to combat the ever changing threat of infectious diseases in the health care and population settings.[4,5] Over the last few decades, the new methods in the microbiology laboratory, such as molecular diagnostics, have become central in allowing for the rapid detection and characterization of pathogens.[6,7] The ability to genotype pathogens using various molecular techniques has led to the rise of molecular epidemiology, which has enhanced our ability to detect outbreaks and uncover the events and factors involved in transmission.[8]

Through genotyping, a molecular fingerprint of each pathogen isolate can be generated and compared with others in the same suspected cluster. Isolates with the same or highly similar genotypes, and linked by epidemiologic data, are likely to represent related cases of the infectious disease within a cluster or outbreak. The laboratory methods for genotyping range from determining single nucleotide variants (SNVs) to evaluating repetitive regions of the genome to examining the length of various fragments of the genome. The earlier techniques for fingerprinting infectious agents mostly involved the electrophoretic separation of DNA or RNA fragments, such as examining plasmids, electrophoresis of ribosomal RNA, or restriction fragment length polymorphism (RFLP).[7] Polymerase chain reaction (PCR) can also be used to create fragments of the pathogen genome for analysis through techniques such as randomly amplified polymorphic DNA, arbitrarily primed PCR, or amplified fragment length polymorphism. Other methods amplify repetitive regions of the genome to determine the number of repeats within each region, such as multiple-locus variable number of tandem repeats analysis. Finally, the sequence composition of portions of the genome can be examined by hybridization methods (eg, with DNA probes or microarrays), SNV typing, or DNA sequencing of specific genes (multilocus sequence typing [MLST]).

All of these laboratory genotyping methods support epidemiologic investigations by determining whether any 2 isolates belong to the same strain and are therefore potentially epidemiologically related. The resolution at which these methods can distinguish different strains of a microorganism depend on the species being

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examined, the percentage of the genome being interrogated, and the variability of those regions. These molecular fingerprinting techniques are useful for confirming clusters or outbreaks of an infectious disease. Although the identification of a cluster of epidemiologically and genetically related cases can be enough to intervene or implement measures to prevent future occurrences, additional details about the outbreak, such as the phylogenetic origins and pathogenic potential of the etiologic agent, and delineation of individual transmission events can improve our understanding of the outbreak and therefore better focus interventions and infection prevention measures.

With the recent introduction of massively parallel DNA sequencing technology or next-generation sequencing (NGS), the real-time sequencing of entire pathogen genomes is now possible.[9,10] In contrast with genotyping, where only a small fraction of the pathogen genome is used to infer phylogenetic relationships, the whole genome of the pathogen can be used to resolve the transmission dynamics of an outbreak in much greater detail.[11] Current second-generation sequencing platforms involve fragmentation of the genomic DNA, amplification and tagging of the fragments, followed by massively parallel sequencing by synthesis during which millions to billions of DNA fragments are sequenced in parallel.[12] The many sequence reads are then assembled back into a genome using various bioinformatics algorithms.[13] The multiple pathogen genomes within a cluster of related cases can then be examined for their virulence potential and compared with one another to determine their relatedness at the genomic level. Because the genomes of isolates within a cluster are expected to be highly related, the most common way to compare these genomes is to examine the differences in the SNVs between isolates (when SNVs become fixed within a population they are called SNPs [single nucleotide polymorphisms]).[14] These new sequencing technologies along with the associated bioinformatics algorithms have given rise to the field of genomic epidemiology where whole-genome analysis methods are integrated with epidemiologic investigations to yield the ultimate resolution into communicable disease outbreaks.[15] Armed with knowledge of the entire genome, we can not only better identify and characterize the pathogen responsible for an outbreak so we can better estimate its risk and select the most appropriate interventions, but we can also begin to understand the origins and dynamics of the outbreak.

Genomic epidemiology has been applied to many outbreaks in the last few years and is becoming a widely accepted method to investigate outbreaks.[16] Most outbreaks have been examined retrospectively because the sequencing costs have been relatively high, the time required to prepare the samples and generate the sequences has been lengthy, and the complexity of the bioinformatics analysis poses a challenging problem. However, with improvements in sequencing technology and continuing optimization and standardization of bioinformatics algorithms, genomic epidemiology investigations can now be conducted during the course of an ongoing outbreak to provide real-time guidance for infection control and prevention interventions.[9,17] Genomic epidemiology has played a large role in furthering our understanding of outbreaks in health care settings (health care–associated infections [HAIs]) and larger communicable disease outbreaks at the community, national, and international scales. In this article, we review the evolution from molecular to genomic epidemiology and the technologic and computational advances which have enabled this, and we also discuss the remaining challenges that need to be addressed for the wider adoption of genomics in infection control.

MOLECULAR EPIDEMIOLOGY

Molecular epidemiology is a relatively new discipline that refers to the use of molecular methods in the microbiology laboratory, coupled with conventional epidemiologic tools, to identify potentially linked cases and aid in the investigation of outbreaks. Prior to the 1970s, the laboratory tools for detecting clusters of infectious disease cases were limited to the identification of the causative agent at the genus and species level along with common antigenic features or antimicrobial resistance patterns. Although these traditional methods still play a role in identifying clusters, they have since been replaced by nucleic acid–based methods, which have greatly improved our ability to identify epidemiologically related cases of infectious diseases. Microevolution occurs within all microorganisms through point mutations, genetic rearrangements, or horizontal gene transfer, leading to various degrees of genetic diversity within each species. By finding and measuring those genetic changes that occur at a fixed rate, we are then able to follow the microevolution of an organism over time. Because the timescale of an outbreak is exceedingly short compared with a pathogen's evolutionary timescale, one fundamental assumption is that the degree of genetic diversity of a given pathogen within an outbreak will be less than the genetic diversity within the overall pathogen population. Molecular genotyping methods that are able to reveal these differences in genetic diversity have since become the foundation of molecular epidemiology.

Molecular methods are used to genotype or generate molecular fingerprints of the individual pathogens in a suspected cluster of cases. The choice of methods is dependent on many factors, including the species of the pathogen, the availability of comparative genotypes, and the epidemiologic context within which the method can be applied. Isolates of different pathogen species can be highly related or highly diverse at the genomic level, and the genotyping method must be able to examine the relevant genomic regions to detect the diversity within the given species. Species, which tend to be clonal within the context of an outbreak, will require genotyping methods that target regions of the genome that are more variable but yet exhibit rate of change that is relevant to the timescale of the outbreak. Difficulties can arise when an organism's genome is too monomorphic, in which case, it may be difficult or even impossible to find a genotyping method that is able to show differences between epidemiologically unrelated isolates of that species. Conversely, when a pathogen species is panmictic, with mutations arising through genetic rearrangements or horizontal gene transfer, it may be difficult to interpret genotyping results because epidemiologically related isolates may show different genotypes and even isolates from the same patient may not have identical genotypes. In this situation, it will be necessary to identify genotyping methods which are less affected by these types of mutations or else combine multiple methods to obtain a more complete understanding of this diversity.

The availability of reference genotypes and reference databases, generated with the same genotyping method, is essential for the interpretation of the genotypes generated from a suspected cluster, favoring the use of methods which are already more widely adopted for a given pathogen. The genotyping method must also consider the type of outbreak and the epidemiologic questions being asked: different methods may be needed to characterize an outbreak of a more ubiquitous pathogen versus a rare pathogen, and different methods may be needed to confirm an outbreak versus surveillance for additional related cases. In any case, the tool must be able to clearly demonstrate relatedness between epidemiologically related cases while differentiating unrelated cases. Ultimately, for a genotyping method to be widely accepted and adopted, it needs to be relatively inexpensive, have acceptable turn-around time, be highly reproducible even in different laboratories, and be easy to perform and interpret.[18,19]

There are numerous genotyping methods for molecular epidemiology, and no single method is universally applicable for all

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pathogens and types of investigations.[7] One of the most commonly used genotyping methods is a variation of RFLP called pulsed-field gel electrophoresis (PFGE). In RFLP, restriction enzymes are used to cut the microorganism's genome into smaller fragments which can then be size separated by electrophoresis. Epidemiologically related isolates will have identical or nearly identical RFLP patterns. In PFGE, an alternating electrical current is used to allow for the resolution of large fragments of the genome.[20] PFGE is the gold standard method for most bacterial genotyping because the patterns generated are relatively easy to interpret, highly reproducible, useful for a wide spectrum of bacteria, and often have better discriminatory power than other available methods.[21] Although it is primarily used for cluster investigation in the setting of hospital-based outbreaks, this method has also been extensively developed in the public health arena with a high degree of interlaboratory reproducibility and is the foundation of the public health PulseNet program, which has standardized PFGE methodologies and established molecular fingerprint databases for rapid outbreak response to numerous bacterial pathogens locally, nationally, and internationally.[22] However, both the cost of the equipment required along with the labor-intensive, technically demanding, and tedious procedures are the main limitations with this method. The analysis, although highly standardized, is still prone to some subjectivity, and continuous quality control is required. As well, certain bacteria may not be resolved using PFGE (eg, *Campylobacter* spp, *Mycobacterium* spp). Of recent interest is the limitation of PFGE and other chromosomally dependent methods in characterizing outbreaks from organisms which have acquired plasmid-mediated antibiotic resistance. The emergence of carbapenemase-producing organisms (CPOs) has highlighted the potential limitation of genotyping methods, which only examine the chromosome. Although the reasons are currently unclear, some clusters of CPOs show highly clonal PFGE patterns, whereas others show great diversity, but when plasmid RFLP methods are applied, the clonality of the plasmids identify clusters of highly diverse bacteria harboring the same plasmid and, in some cases, bacteria of different genera and species.[23]

Fragmenting the pathogen genome can also be accomplished by PCR. One example is repetitive-element PCR (rep-PCR), which is the foundation for DiversiLab (bioMérieux, Marcy l'Etoile, France), a semi-automated method, which is used in many hospital settings to investigate clusters and outbreaks of infectious diseases.[24] rep-PCR amplifies the DNA located between conserved repeat sequences in the bacterial chromosome, and the resulting DNA fragments are electrophoretically separated to generate molecular fingerprints. Compared with other genotyping methods, this method is easy to use, quick, and can be applied to a wide variety of microorganisms. The DiversiLab system has been useful in the outbreak detection of organisms, such as *Acinetobacter* spp, *Stenotrophomonas maltophilia*, *Enterobacter cloacae*, *Klebsiella* spp, and *Escherichia coli*, but it is inadequate for *Pseudomonas aeruginosa*, *Enterococcus faecium*, and methicillin-resistant *Staphylococcus aureus* (MRSA).[25] Key challenges with rep-PCR are the limitations with interlaboratory reproducibility and concerns with data security because the DiversiLab database is maintained and stored by the manufacturer.

Similarly, PCR ribotyping has played an important role for HAI outbreaks. This method takes advantage of the variability of the intergenic spacer region between different copies of 16S and 23S ribosomal RNA genes on the bacterial chromosome. PCR is used to amplify these intergenic spacer regions, and the resulting fragments are either resolved by electrophoresis or analyzed by DNA sequencing. These regions often harbor nucleotide differences between clones and are key for strain clustering. Recently, a combination of PFGE and PCR ribotyping has been used to identify and track an epidemic strain of *Clostridium difficile* across the globe. This highly virulent, toxin-hyperproducing strain of *C difficile*, which emerged in North America and Europe in the early 2000s, can be identified through its PFGE and PCR ribotyping patterns, North American PFGE type 1 and ribotype 027.[26]

spa typing is one of the most widely used method for *S aureus* subtyping. It is based on sequencing of the polymorphic variable number of tandem repeats region of the protein A gene (*spa*) of *S aureus*.[27] A high rate of point mutations and a variable number of short repeats contribute to the high diversity of this region of the *spa* gene. Although it has a lower discriminatory power than PFGE, it is less expensive, faster, easier to use, highly reproducible, and has higher throughput and uses an internationally standardized nomenclature, using the Ridom StaphType software (Ridom Bioinformatics, Münster, Germany).[28] As with any single-locus genotyping method, limitations of *spa* typing include misclassification of certain subtypes because of recombination or mutations elsewhere in the genome.

Other sequencing-based methods include MLST in which multiple housekeeping genes are sequenced.[29] This method uses standardized methods for PCR and sequencing and generates sequence types that are unambiguous and easy to share and compare between laboratories. It was initially developed to allow for standardization and comparison of *Neisseria meningitidis* strains, and it was later modified for other pathogens, including *S aureus*.[30] The limitation of MLST is that the housekeeping genes are relatively conserved and may lack the discriminatory power to differentiate certain bacteria. Also, MLST only examines approximately 0.1% of the genome, and the genetic variability in other regions, such as those in the previously mentioned genotyping methods, may sometimes give greater insight into the epidemiologic relationships between isolates in a cluster. Finally, with the increasing use of NGS in microbiology, the MLST sequence type can now also be inferred from pathogen genome data.[31]

NGS

During the last decade, the transition from traditional Sanger sequencing to NGS has been marked with tremendous advances in speed, throughput, and cost savings in DNA sequencing, opening up new opportunities for applications in various fields, including infectious disease epidemiology. NGS collectively refers to a number of recently developed DNA sequencing approaches that combine novel sequencing chemistries with automated instruments, where millions to billions of sequencing reactions can be performed in parallel in a single run.[32] Currently, the second-generation sequencing platforms are the most widely used NGS systems (Table 1). Although the methods for preparing sequencing libraries and the sequencing chemistries vary from one platform to another, these systems are similar in that they require in vitro amplification of the input DNA and that the DNA sequences are determined during the synthesis of the complementary DNA strand, albeit in a massively parallel fashion. The template DNA is first fragmented into short molecules of a few hundred nucleotides long and ligated to synthetic DNA sequences called adapters to create NGS libraries. The DNA fragments are then immobilized on a solid surface (eg, flow cell, bead) and amplified by PCR. Sequencing primers are then used to initiate the formation of complementary DNA. Using various signal-capturing techniques, these sequencing systems are able to detect each nucleotide as it is added to the nascent strand of DNA and therefore determine the DNA sequence. The advantage is that in this manner, millions of short DNA fragments can be sequenced in parallel. However, fragmentation of template DNA is required because the second-generation platforms are only capable of reading a few hundred bases before the signal-to-noise ratio becomes too low because of chemical degradation, synthesis error, and other factors.[33]

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Table 1
High-throughput sequencing platforms for pathogen whole-genome sequencing

Platform (manufacturer)	Max. read length (bp)	Output per run	Reads per run	Run time	Errors	Advantages	Limitations
Second-generation sequencers							
MiniSeq (Illumina)	150 × 2	1.8-7.5 Gb	8-25 × 10^6 (single reads*)	4-24 h	Substitution errors (GGC motifs)	Low-cost desktop system Low error rate Paired-end reads	Short read lengths Higher run cost per sample
MiSeq and MiSeq Dx (Illumina)	300 × 2	0.3-15 Gb	1-25 × 10^6 (single reads*)	4-55	Substitution errors (GGC motifs)	Medium-cost desktop machine Low error rate Paired-end reads	Decreasing read quality toward ends
NextSeq 500 (Illumina)	150 × 2	20-120 Gb	130-400 × 10^6 (single reads*)	11-29 h	Substitution errors (GGC motifs)	Higher-throughput system Low error rate Lower cost per bp Paired-end reads	Short read lengths Longer run time Requires more samples to be cost effective
HiSeq 4000 (Illumina)	150 × 2	125-1,500 Gb	2.5-5 × 10^9 (single reads*)	1-3.5 d	Substitution errors (GGC motifs)	Highest-throughput system Low error rate Lowest-cost per bp Paired-end reads	Short read lengths Longest run times High system cost
Ion S5 with Ion 530 Chip (Thermo Fisher Scientific)	400	6-8 Gb	15-20 × 10^6	4 h	Indels in homopolymeric regions	Low-cost desktop machine Fastest run time of desktop sequencers	Nonpaired end reads Higher error rates than Illumina systems
Ion S5 with Ion 540 Chip (Thermo Fisher Scientific)	200	10-15 Gb	60-80 × 10^6	2.5 h	Indels in homopolymeric regions	Low-cost desktop machine Fastest run time of desktop sequencers	Short read lengths Nonpaired end reads Higher error rates than Illumina systems
Ion PGM with Ion 318 Chip v2 (Thermo Fisher Scientific)	400	0.6-1 Gb	4-5.5 × 10^6	7.3 h	Indels in homopolymeric regions	Low-cost desktop machine	Nonpaired end read Higher error rates than Illumina systems
Ion Proton (Thermo Fisher Scientific)	200	Up to 10 Gb	6-8 × 10^7	2-4 h	Indels in homopolymeric regions	Medium-cost desktop machine Very fast run time	Short read length Nonpaired end reads Higher error rates than Illumina systems
Third-generation sequencers							
PacBio RS II (Pacific Biosystems)	>60 kb (mean, 20 kb)	500 Mb to 1 Gb	55,000	0.5-4 h	Indels	Very long read lengths Very fast run time	High error rates than Illumina systems Requires higher DNA input High system cost
MinION (Oxford Nanopore)	>100 kb (mean, 10 kb)	25-42 Gb	2.5-4.4 × 10^6	1 min to 48 h	Deletions	Portable USB device No PCR amplification required Very long read lengths Very fast run time	Very high error rates Low output High cost per bp

NOTE. Values for each category in each system are based on the manufacturer's specifications as of April 2016.
bp, base pair; *Gb*, giga base pair; *GGC*, guanine-guanine-cytosine; *Indel*, insertion or deletions; *kb*, kilo base pair; *Max.*, maximum; *Mb*, mega bases pair; *PCR*, polymerase chain reaction; *USB*, Universal Serial Bus.
*Double for paired-end reads.

In the Illumina sequencing systems (Illumina, San Diego, CA), a flow cell is used to capture and amplify the sequencing library. The Illumina systems use a technology called sequencing by synthesis, whereby reversible terminator nucleotides labeled with different fluorophores are used in the formation of the complementary DNA strand. After the addition of each nucleotide, a camera takes a picture of the flow cell to determine the specific nucleotide added. Massively parallel sequencing is achieved through hundreds of rounds of nucleotide in addition to the millions (to billions) of DNA fragments on the flow cell.[34] For the ion semiconductor sequencing platforms (Thermo Fisher Scientific, Waltham, MA) beads are used to capture the DNA fragments prior to PCR amplification. The beads, coated with the amplified DNA, are then loaded into microwells on a silicon chip. The ion sensors of the sequencing chip are able to detect the release of a proton as each nucleotide is added to the nascent complementary DNA strand. With millions of microwells on each chip, millions of DNA fragments can be sequenced in parallel.[35]

The third generation of DNA sequencers are capable of single molecule sequencing without prior amplification of the target DNA (Table 1). One platform is the single molecule, real-time sequencer (SMRT; PacBio, Menlo Park, CA).[36] In the PacBio system, the sequencing chip consists of hundreds of thousands of zero-mode waveguides, in which an immobilized DNA polymerase catalyzes the elongation of a DNA fragment with fluorescently labeled nucleotides. The DNA sequences are determined through detection of the fluorescent signal as each nucleotide is incorporated. One of the newest technologies is nanopore sequencing in which DNA strands are translocated through protein nanopores. As each nucleotide passes through the nanopore, the associated disruption of an electrical current across the nanopore allows the system to determine sequence of the DNA. The only system available for precommercial early access is the MinION (Oxford Nanopore, Oxford, UK), which is a pocket-sized device with a flowcell containing several hundred nanopores.[37] Although the error rates of the third-generation sequencers are still relatively high compared with second-generation sequencers (approximately 15%-20% vs <0.1%, respectively), they are able to produce much longer read lengths than the second-generation sequencing platforms (>10 kilobases vs a few hundred bases). The current sequencing capabilities of these systems makes them ideally suited for microbial genomes, especially when combined with data from higher-throughput second-generation platforms.[38]

BIOINFORMATICS

NGS technologies produce significantly more raw data than other molecular diagnostic assays, including Sanger sequencing. The

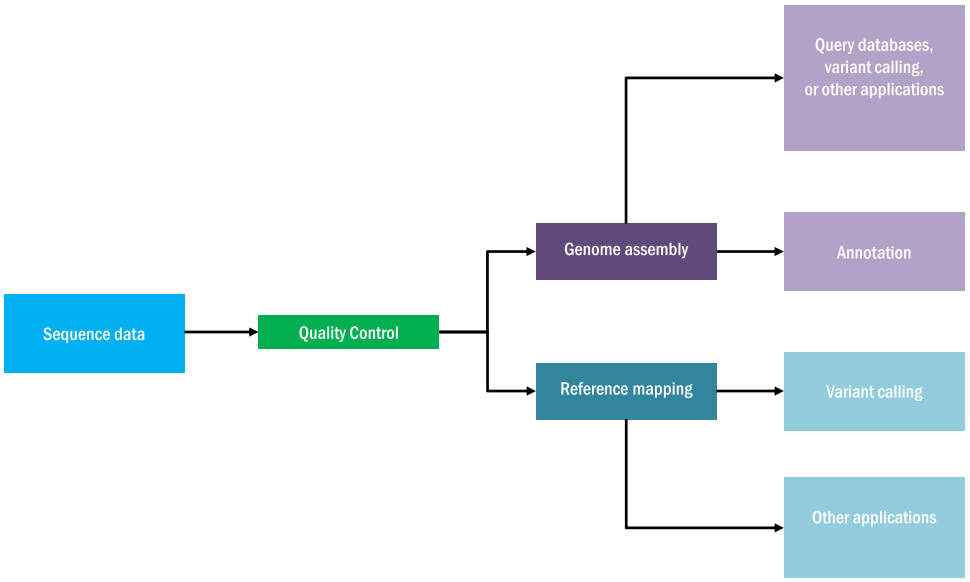

Fig 1. Bioinformatics workflow for processing and analysis of genome sequencing data. Sequence data must first undergo quality control to ensure the quality of sequence data passed on for downstream analysis. Depending on the application, the sequences can be used for genome assembly (either de novo or reference-based) or mapped to a reference. After genome assembly, a draft genome is generated, which can be annotated (ie, gene predictions and their functions), screened against various databases (eg, virulence factors, MLST, antimicrobial resistance), and used for further analysis. Although genome assemblies can also be used to look at single nucleotide variants, reference mapping is commonly used for variant calling. Mapped sequences can also be used to look at genotypes (eg, MLST) or the presence or absence of genes of interest (eg, virulence factors, antimicrobial resistance). *MLST*, multilocus sequence typing.

amount and complexity of the data necessitate the use of bioinformatics analysis to turn the raw sequencing reads into meaningful data. Broadly speaking, the goals of the bioinformatics analyses of pathogen genomes are (1) to clean-up and organize sequence data; (2) to discover genomic variants that can be used to differentiate organisms for identification, genotyping, and tracking of pathogens; and (3) to characterize the genome to computationally predict phenotypic features useful for clinical interventions.

The bioinformatics workflow and some of the available tools are summarized in Figure 1 and Table 2. The first step of bioinformatics analysis is to ascertain the quality of the data. This is especially important in a clinical laboratory. Sequence quality information can be summarized using programs such as FASTQC (Babraham Bioinformatics, Cambridge, United Kingdom; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Because of the high sensitivity of NGS, contamination with nontarget organisms, even when present at low levels, is a significant problem because it can interfere with downstream analyses. Programs such as checkM and Kraken can be used to quickly screen the samples for contamination.[39,40] The presence of contaminants may result in inconclusive genotyping results. In some cases, the contaminant reads can be filtered out using bioinformatics, but otherwise, samples that are contaminated need to be reisolated and resequenced.

In the process of sequencing, fragmented reads containing errors are produced. Redundant and overlapping reads are always generated so consensus sequences can be created to correct for sequencing errors. To reduce redundancy and to reconstruct the genome sequence for downstream analysis, genome assembly is performed either by mapping the reads onto a known reference genome (reference-guided assembly) or by de novo assembly, which means overlapping reads are aligned using computer algorithms to form longer contiguous sequences (contigs) without the use of a reference genome.[56,57] Reference-guided assembly is generally fast and computationally less intense. Burrows-Wheeler aligner and Bowtie

Table 2
Bioinformatics tools for pathogen whole-genome sequencing

Bioinformatics tool	Reference
Quality control	
FastQC	www.bioinformatics.babraham.ac.uk/projects/fastqc/
checkM	Parks et al[39]
Kraken	Woods and Salzberg[40]
De novo genome assembly	
Velvet	Zerbino[41]
SPAdes	Bankevich et al[42]
HGAP	Chin et al[43]
Celera-MHAP	Berlin et al[44]
Reference mapping	
Burrows-Wheeler aligner (BWA)	Zerbino[41]
Bowtie2	Bankevich et al[42]
Annotation	
RAST	Aziz et al[45]
NCBI prokaryotic genome annotation pipeline	www.ncbi.nlm.nih.gov/genome/annotation_prok/
Prokka	Seemann et al[46]
Variant calling	
kSNP	Gardner et al[47]
Cortex_var	Iqbal et al[48]
Databases and other applications	
Virulence Factor Database (VfDB)	Chen et al[49]
Comprehensive Antibiotic Resistance Database (CARD)	McArthur et al[50]
Mykrobe Predictor	Bradley et al[51]
Short Read Sequence Typing (SRST2)	Inouye et al[52]
Ridom SeqSphere+	www.ridom.com/seqsphere/
BioNumerics	www.applied-maths.com/bionumerics
Salmonella In Silico Typing Resource (SISTR)	Yoshida et al[53]
SeqSero	Zhang et al[54]
SerotypeFinder	Joensen et al[55]

Celera-MHAP, celera assembler and min hash alignment process; *HGAP*, hierarchical genome assembly process; *RAST*, rapid annotation using subsystems technology.

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2 are common read-mapping tools.[58-60] The mapping-based approach is fast but not very sensitive; therefore, if the query genome is too divergent from the reference genome, such as a novel strain of a pathogen, it may not properly assemble to the reference genome, resulting in the omission of critical genomic elements, such as novel virulence or antibiotic resistance genes.[57] Therefore, this approach is most suited for common pathogens with many closely related reference genomes available. De novo assembly is preferred for novel genomes. Commonly used assemblers include Velvet and SPAdes for Illumina and Ion Torrent sequences.[41,42] For long, error-prone reads generated from third-generation platforms, the most common assembler customized for PacBio and MinION sequences are HGAP and Celera-MHAP.[43,44] Given the high error rate of third-generation platforms, a polishing step is often needed to improve the quality of these sequences. Sequencing errors are corrected as part of the genome assembly process through the collapsing of redundant and overlapping sequences. The resulting assembled genomes (contigs derived from the consensus sequences) are easier to work with.

Genomic epidemiology depends on the fact that over time, microbes accumulate changes in their genomes. The types of variations include SNVs, small insertion or deletions, large-scale genome rearrangements, gene duplication, gene deletion, and gene acquisition through horizontal gene transfer. WGS (whole-genome sequencing) allows for characterization of a microbe in the most comprehensive manner and provides the highest possible resolution in distinguishing microbial isolates. In the short time frame typical of an outbreak, the focus of variant detection is often on small and frequent changes, such as SNVs. Variant calling algorithms compare mapped sequences (reads or contigs) with the reference genome and identify bases that differ from the bases in the reference genome. In the absence of good reference genomes, SNVs can also be detected from reference-free, raw sequence data using programs, such as kSNP or Cortex_var, that are capable of finding SNVs without a reference genome.[47,48] Irrespective of the method of SNV calling, the candidate SNVs should be filtered for various sources of errors, such as polymerase replication errors during sample processing, base calling errors during sequencing, and SNP calling errors during bioinformatics processing. A standard operating protocol for SNV calling is likely to be species or even subspecies specific because of the different evolutionary forces acting on these genomes.[14] Ongoing validation of these SNV calling methods using control datasets will be needed to improve the accuracy of SNV results for genomic epidemiology. Methods for core-genome or whole-genome MLST, which infer the MLST from genomic data, are being developed and standardized.[31] Several bioinformatics tools, including SRST2, Ridom SeqSphere+, and BioNumerics, have been developed to generate custom core-genome or whole-genome MLST profiles to allow comparison across multiple isolates.[52]

After genome assembly, automated genome annotation engines can be used to predict gene functions, therefore providing a comprehensive picture of the organism's functional capability. Popular automated genome annotation services for microbial genomes include NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and RAST.[45] These online services are free but require uploading of the genomes. Offline annotation tools such as Prokka allow one to carry out rapid genome annotation on a desktop computer.[46] This option, however, requires one to be familiar with Linux-Unix systems and command line interface. One can also take the genomic sequences and query against specialized databases such as the Virulence Factors Database and antimicrobial resistant databases (eg, Comprehensive Antibiotic Resistance Database) to obtain phenotypic predictions of pathogenicity and resistance.[49,50] Novel tools such as the Mykrobe Predictor allow users to take raw reads and search against carefully curated datasets on their own desktops to obtain resistance profiles for *Streptococcus aureus* and *Mycobacterium tuberculosis*.[51] For several enteric bacteria, serotype information can also be derived from WGS data with reasonable accuracy, saving the laboratory from having to carry out labor-intensive and costly serotyping assays.[53-55]

In short, various genotypic and phenotypic features can be derived from WGS data using bioinformatics tools, allowing WGS to provide a 1-stop shop for microbiology laboratories in the near future.

Although most of the bioinformatics tools previously mentioned are not yet user-friendly enough for routine clinical laboratory use, and the computing infrastructure needed is not often available to frontline laboratories, high-quality and user-friendly bioinformatics platforms are being developed to bring bioinformatics analysis capabilities into clinical and public health microbiology laboratories. One example of these efforts is the Integrated Rapid Infectious Disease Analysis (IRIDA) project, a Canada-wide public sector consortium to develop a freely available, genomic epidemiology analysis platform. Another effort is the Genomics Virtual Lab (https://genome.edu.au/), which allows access to bioinformatics tools through a cloud computing environment.[61] Novel algorithms and pipelines are also being developed to allow rapid analysis to be performed on one's desktop, albeit for smaller datasets. In the next few years, with advances in bioinformatics tools and platforms development, microbiology laboratories will be able to routinely analyze WGS data and provide useful reports to other health care professionals.

GENOMIC EPIDEMIOLOGY

The use of whole-genome sequencing to better understand and manage infectious disease outbreaks is arguably one of the more successful applications of NGS. Not only does it provide higher confidence for the confirmation of outbreak cases, but it also allows for more targeted interventions to manage and stop outbreaks. An overview of how genome sequencing could be used to provide higher-resolution data to infection control and prevention for suspected clusters of communicable diseases is outlined in Figure 2. Outbreaks in health care facilities often involve clonal pathogens, and the limited resolution of conventional genotyping methods reduces the ability to discern clusters or transmission dynamics. For example, it is difficult to determine whether temporally separated MRSA cases are sporadic or part of a cluster if the isolates all have indistinguishable PFGE patterns.[62] However, sequencing of the isolates yields higher-resolution, nucleotide-level data that can provide the necessary information to differentiate these cases, and in this example, a genomic epidemiology investigation was able to link 6 cases to a common source.[62] As more outbreaks are analyzed with whole-genome sequencing, the simple rules[63] that we use for interpreting low-resolution genotyping data and whether 2 isolates are likely to belong to the same cluster may no longer apply. With the higher resolution of genome sequence data and the ability to discern single nucleotide differences between genomes, new challenges will arise in determining how much genetic variation can exist within an epidemiologically related cluster. New rules will need to be developed that take into account the existing genetic diversity within each species, its molecular clock, and the characteristics of the outbreak and the questions being addressed by the genomic analysis.

Genomic epidemiology can also tell us about chains of transmission and has been used to track the transmission of HAIs caused by *Acinetobacter baumannii*,[17,64] MRSA,[65,66] and *Klebsiella pneumoniae*.[67] Although this list is not exhaustive, it highlights that during an outbreak, genome sequences can be used to track the chain of transmission between patients, or an environmental source, when supported by epidemiologic evidence. When epidemiologic linkages are missing, genome sequences can also help generate

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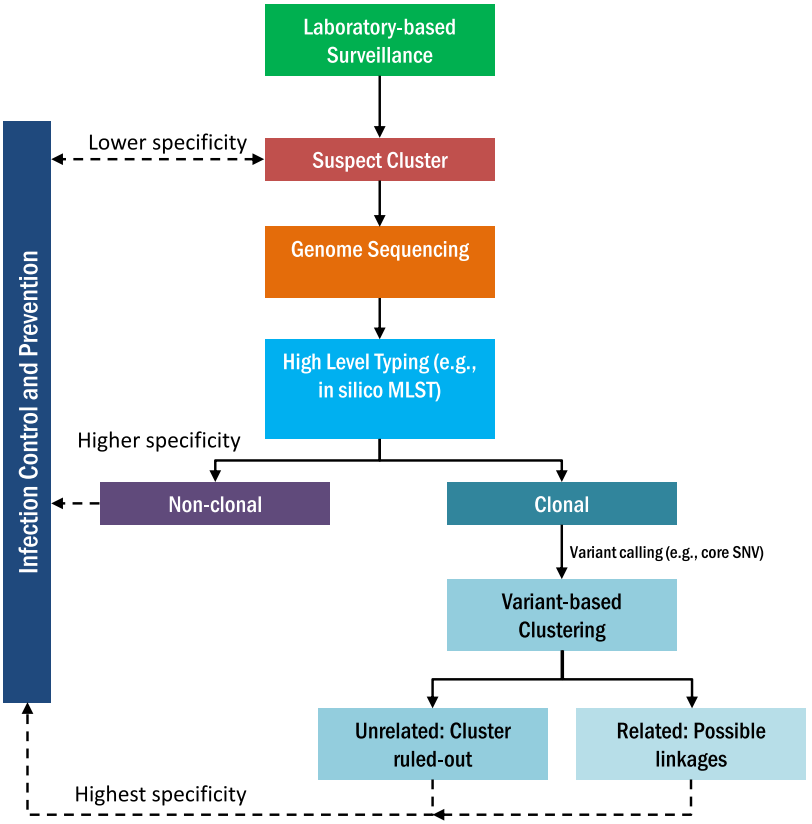

Fig 2. Schematic of a genome sequencing–driven infection control and prevention program. Any increase of a specific infectious disease or pathogen would be communicated between the laboratory and infection control and prevention. If these isolates meet institutional-specific criteria for a potential outbreak, then these clinical isolates (and possibly any environmental isolates) would be sent for genome sequencing. The sequences of each isolate could be grouped based on a high-level typing scheme (eg, in silico MLST) to identify a set of clonal or nonclonal isolates, relative to the other suspect isolates. The clonal isolates would be characterized further by identifying SNVs in the core genome to determine the degree of relatedness between these isolates. Over the course of the genomic investigation, information of increasing specificity would be communicated back to the infection control and prevention team for more targeted interventions and informed policy development. *MLST*, multilocus sequence typing; *SNV*, single nucleotide variant.

hypotheses about the source of an outbreak and sort out which cases are related or unrelated to the outbreak. Although some infection control interventions will not be changed by genomic data (eg, the molecular detection of MRSA or CPO in an individual patient would lead to the same isolation procedures), when sequence data are integrated in outbreak investigations, it can improve clarity and guide other responses and interventions, such as decisions to close a room or ward or the drafting of new procedures and policies to prevent future outbreaks.

The source or reservoir of a pathogen is often unknown. Genome sequences have been used to link outbreaks to environmental or other sources. For example, *P aeruginosa* infections on a burns unit being tracked to the showers[68] and the discovery of a hand-soap dispenser being the source of a persistent *E cloacae* outbreak[69] were both solved by sequencing the genomes of the pathogens. To expand source attribution beyond the confines of a hospital or health care facility, during an outbreak of *Salmonella* enteritidis in a UK hospital, isolates that were epidemiologically unlinked (different wards) and genetically unrelated could be associated with isolates in the community, thereby suggesting that there was not an unknown source of *Salmonella* transmission throughout the hospital.[70] This association with the community *Salmonella* would not have been feasible if the public health laboratory had not been routinely sequencing all *Salmonella* forwarded to their laboratory, or unable to share their data with the investigators.[71] Routine sequencing of health care–associated pathogens could lay the foundation to quickly attribute clones, study changing epidemiology, and surveillance and identify the emergence of new pathogens. This idea has been explored for MRSA in the United Kingdom and Ireland.[72]

The emergence of CPOs has proven to be a formidable foe for infection preventionists. Not only are there few treatment options for CPOs, but also the resistance genes are often part of mobile genetic elements (ie, transposons) that can be further disseminated by transmissible plasmids that have broad species ranges. Clonal outbreaks of carbapenemase-producing *K pneumoniae*[67,73] and *E cloacae*[69] have been investigated using genomic sequencing, including the sequencing of associated plasmids, to understand the transmission dynamics. Because plasmids can spread between species, and transposons can insert into other plasmids, comparing the relatedness of clonal organisms only through their chromosomes is insufficient; instead, linkages between these diverse species can be made through their plasmids, or mobile elements.[74] Again, although infection control interventions at the individual patient level may be similar regardless of the clonality of the pathogen, higher-level decisions and policies at the institutional level may benefit from the additional knowledge from genomic analyses.

Tracking transmission dynamics, however, can be hampered by the diversity of organisms that can be found within a single host.[75] Sequencing of multiple colonies of the same organism isolated from a single person showed that these individuals can carry multiple unrelated types of that species, such as diverse sequence types of MRSA,[76] extended-spectrum β-lactamase–producing *E coli*,[77] or vancomycin-resistant enterococci.[78] SNVs within a single sequence type have been seen in MRSA,[65,78-80] *A baumannii*,[17] and *K*

pneumoniae,[67] and this has been described as a cloud of diversity. These findings imply that a single colony isolated from a single patient may be insufficient to reconstruct the chain of transmission between otherwise epidemiologically linked patients. This diversity is not exclusive to infected patients; asymptomatic carriers can also harbor a diverse set of potential pathogens[79] that can be silently introduced into health care facilities and linked to outbreaks. These organisms may have a unique biology[81] that makes them adept at establishing a foothold in health care settings.

The availability of high-throughput sequencing has also given us immense insights into the ecology and epidemiology of health care–associated pathogens. Although each facility will have its own dynamics, whether driven by policy, population served, or access to resources, genome sequences have been an important tool in understanding these site-specific nuances to better inform infection control practice. Analysis of genome sequences can be used to evaluate the effectiveness of current (or new) interventions on how well they are limiting person to person, or environmental, spread of pathogens.[82] Genomic sequence information can also provide a high-resolution picture of the endemicity and potential unlinked transmission of pathogens, such as *C difficile* within a facility or ward.[83] This can also be extended to look at specific pathogens; therefore, infection preventionists can understand interfacility transmission of organisms, such as vancomycin-resistant enterococci.[78] These types of studies can also be expanded to show the multiple introduction of emerging pathogens (eg, ST258 *K pneumoniae*) into a facility, as opposed to a single circulating clone,[84] or look at the replacement of previously dominant MRSA clones with newer, more-successful clones within hospital settings[80,85] and between continents.[65]

Other important epidemiologic observations have been garnered from the high resolution of genome sequencing. For example, many *C difficile* cases found in a hospital could not be attributed to transmission from other symptomatic patients, and this finding suggests that there is a wide source of *C difficile* diversity in the environment, or that asymptomatic carriers are introducing the organism into hospitals.[86] In another example, genome sequencing was used to demonstrate that there was little transmission of hospital-associated MRSA in the community, but instead the community-associated MRSA strains were able to establish and circulate within health care facilities.[87] This has further implications for admission screening criteria, especially where community-associated MRSA was found to be higher in patients with lower socioeconomic status, who also tend to access health care facilities more frequently.[88]

Despite the improved resolution that genome sequencing offers to outbreak investigation, there are certain barriers to overcome before it can be implemented in real time in acute care facilities. First are technical challenges where current molecular laboratories must possess or have access to genome sequencing equipment, and computational infrastructure and software must be upgraded to be able to store and analyze large bioinformatics datasets. The solutions to these challenges will come with time as sequencing technology continues to improve and new computational strategies are developed. Second, and even more importantly, this genomic information must be interpreted and translated back to infection control and prevention practitioners in a meaningful manner. This will require highly trained staff who will be able to integrate the bioinformatics and epidemiologic data and bridge the gap between genomic epidemiology and real-life infection control. Given these hurdles and the current costs of implementing and maintaining a genomics program, adoption of genomic epidemiology techniques, at least in the short term, may be limited to larger and better resourced academic facilities. These early pioneers in genomic epidemiology will undoubtedly pave the way to wider adoption of these techniques in the future.

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SUMMARY

Genome sequencing has been successfully used to describe unique and detailed insights into the transmission, biology, and epidemiology of many health care–associated pathogens. With improvements to the portability and quality of sequencing, and the acceleration and standardization of analytical pipelines, the routine use of genome sequencing may soon become common place in infection control. The rapid and real-time application of genomic epidemiology methods have been demonstrated recently during hospital outbreaks of *Salmonella*[70] and *A baumannii*.[17] Challenges still remain because laboratories will need to find new ways to deal with not only a new type of data (whole-genome sequences), but also much greater volumes of data than with conventional genotyping. Also, as evident from these exemplars of genomic epidemiology, the sharing of data between clinicians, infection interventionists, and the laboratory is necessary in order to realize the full potential of applying microbial genomics to infectious disease surveillance and outbreak investigations. As such, concomitant advances must also be made in the framework governing the management and sharing of contextual patient and infection control data. Ultimately, data sharing must also happen at the global scale because genomic data are best interpreted through comparison with other genomic sequences, and platforms similar to PulseNet must be developed to facilitate this. With such granular nucleotide-level information, new interpretation guidelines will need to be developed and validated for different organisms and different situations, and infection control professionals will also need to better understand the power and limitations of these new methods. As continued advances are made in sequencing technology and bioinformatics, it will not be long before more hospitals start incorporating genome sequencing in outbreak investigations. With genomic tools to complement existing genotyping and epidemiologic methods, the future of infection control and prevention will see more targeted and successful interventions for outbreaks, which will ultimately contribute to the reduction of HAIs.

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Using Major Outer Membrane Protein Typing as an Epidemiological Tool To Investigate Outbreaks Caused by Milk-Borne *Campylobacter jejuni* Isolates in California

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We describe using major outer membrane protein (MOMP) typing as a screen to compare the *Campylobacter jejuni porA* gene sequences of clinical outbreak strains from human stool with the *porA* sequences of dairy farm strains isolated during two milk-borne campylobacteriosis outbreak investigations in California. The genetic relatedness of clinical and environmental strains with identical or closely related *porA* sequences was confirmed by multilocus sequence typing and pulsed-field gel electrophoresis analysis. The first outbreak involved 1,644 *C. jejuni* infections at 11 state correctional facilities and was associated with consumption of pasteurized milk supplied by an on-site dairy (dairy A) at a prison in the central valley. The second outbreak involved eight confirmed and three suspect *C. jejuni* cases linked to consumption of commercial raw milk and raw chocolate colostrum at another central valley dairy (dairy B). Both dairies bottled fluid milk on the farm and distributed the finished product to off-site locations. Altogether, *C. jejuni* was isolated from 7 of 15 (46.7%) bovine fecal, 12 of 20 (60%) flush alley water, and 1 of 20 (5%) lagoon samples collected on dairy A. At dairy B, *C. jejuni* was cultured from 9 of 26 (34.6%) bovine fecal samples. Environmental strains indistinguishable from the clinical outbreak strains were found in five flush alley water samples (dairy A) and four bovine fecal samples (dairy B). The findings demonstrate that MOMP typing is a useful tool to triage environmental isolates prior to conducting more labor-intensive molecular typing methods.

In the United States annually, an estimated 845,024 infections, 8,463 hospitalizations, and 76 deaths are caused by *Campylobacter* species (1). Preliminary data from the Foodborne Diseases Active Surveillance Network (FoodNet) indicate an overall incidence of 13 infections/100,000 population (2). Two species, *Campylobacter jejuni* and *Campylobacter coli*, cause >90% of reported human infections, although less-common species are considered emerging pathogens (3). Most patients experience a self-limiting gastrointestinal illness that resolves after 2 to 3 days. However, antecedent *C. jejuni* infection is the most common cause of Guillain-Barré syndrome, an acute inflammatory demyelinating polyneuropathy that leads to flaccid paralysis (4).

The majority of *Campylobacter* infections are zoonotic and linked to consumption of contaminated foods of animal origin or untreated water (3, 5, 6). Dairy products are the most common vehicle of transmission implicated in *C. jejuni* food-borne disease outbreaks, whereas sporadic infections are most frequently caused by poultry products (3, 7). An estimated 80% of dairy-related *C. jejuni* outbreaks are from consumption of raw (unpasteurized) milk, but outbreaks caused by failure in processing or postpasteurization contamination have been documented (3, 5, 6, 7, 8, 9, 10). Despite well-documented risks from raw dairy products, there continues to be consumer demand for access to these foods (11).

An increasing need exists to understand the mechanisms of dissemination of *Campylobacter* in the farm environment and to use this information for developing prevention strategies. Farm investigations can be challenging to conduct because of the lag time between the patient's exposure and identification of the food vehicle. Furthermore, the laboratory requirements to support source tracking from the farm can be expensive and labor-intensive. Given the ubiquitous presence of *Campylobacter* in livestock environments, finding a specific genotype or clinical outbreak strain on an implicated farm might be akin to searching for a needle in a haystack. In certain cases, analyzing numerous isolates from environmental samples by using pulsed-field gel electrophoresis (PFGE), the PulseNet standard for molecular subtyping of food-borne bacterial diseases (12, 13), is infeasible for a public health laboratory.

To address these concerns, we endeavored to identify an efficient molecular screening approach that allows triaging of a considerable number of *Campylobacter* isolates with the goal of source tracking the clinical outbreak strain back to the farm environment. We speculated that the DNA sequences of hypervariable regions of the *porA* gene (also referred to as *cmp*), encoding the major outer membrane protein (MOMP) of *C. jejuni*, might be exploited for this purpose (14, 15). As proof of concept, we developed a primer set that amplified a fragment of the *porA* gene (~740 bp) and used phylogenetic analysis to compare the genetic relatedness of strains isolated from human stools with environ-

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mental strains isolated during two *C. jejuni* milk-borne outbreaks in California.

Outbreak 1. In May 2006, a massive outbreak of *C. jejuni* infections involving 1,644 ill California prison inmates with 52 culture-confirmed cases was epidemiologically linked to consumption of pasteurized milk distributed to 11 state facilities (16). An on-site dairy (dairy A) and creamery at one of the prisons supplied the implicated milk to all affected facilities through a vocational program. No milk was distributed to the general public. Refrigerated, retained milk samples were phosphatase negative, indicating no global breach of pasteurization. *C. jejuni* was not recovered from the milk; however, the majority of samples had high aerobic standard plate (9.8×10^7 CFU/ml to 1.4×10^8 CFU/ml) and coliform (350 CFU/ml to $>1.6 \times 10^5$ CFU/ml) counts. By regulatory standards for grade A pasteurized milk, standard plate counts should be $<1.5 \times 10^4$ CFU/ml and there should be <10 CFU/ml coliforms. A quarantine order by the California Department of Agriculture was issued, and >2,900 cases of milk were returned.

Outbreak 2. In November to December 2007, eight culture-confirmed and three suspect cases of *C. jejuni* infections were reported among consumers of commercially available raw milk and raw chocolate colostrum from a California licensed raw dairy (dairy B) with an on-site creamery (17). Cases from five geographically widespread counties had illness onsets clustered tightly within 11 days. Retained or leftover dairy product samples were unavailable for analysis. *C. jejuni* was not recovered from retail raw dairy products from other lots, but routine testing by the California Department of Food and Agriculture revealed a marked increase in aerobic standard plate counts from October (<2,500 CFU/ml) to November (>250,000 CFU/ml) preceding the illnesses. The aerobic standard plate count regulatory standard for grade A raw milk for human consumption was the same as that for pasteurized milk, but there was no coliform standard at the time. The short duration of the outbreak indicated that contaminated raw dairy products were available for only a brief time; thus, no quarantine order was issued.

MATERIALS AND METHODS

Study sites and sample collection. The dairies were located in the central valley, a major inland milkshed in California. Unlike the majority of other central valley dairies, these dairies were relatively small (300 to 500 milking cows), and both bottled milk in a creamery on the property rather than shipping to an off-site processor. Dairy A was part of a state prison vocational program where inmates operated the dairy, grew forage on the property, and supplied milk to other state prisons in northern California. Dairy B was a state-licensed, commercial, organic, pasture-based raw-milk dairy that distributed raw dairy products to retail stores and farmers' markets statewide.

Environmental samples, including bovine feces, wastewater, and bulk tank raw milk (BTM), were collected at dairy A on 21 August 2006. Because of delays in gaining access to the prison dairy herd, our sampling occurred approximately 3 months after the outbreak was reported. Dairy A used a recycled wastewater system for manure management. We revisited dairy A on 21 March 2007 to conduct additional studies of the dairy wastewater system, including the flush alley water and lagoon (ponds).

We visited dairy B on 17 December 2007 to collect bovine feces, milking parlor discharge water, and BTM. Leftover or retained raw milk from the implicated lot was unavailable. Nine retail raw dairy samples with best-by dates between 25 December 2007 and 3 January 2008 produced by dairy B were collected from a health food store. Dairy B did not use a lagoon or flush alley recycled water system.

At dairy A, approximately 2 g of fresh feces collected from the pens was inoculated into *C. jejuni* semisolid aerobic transport medium (SAEM) prepared in 50-ml conical tubes as described previously (18). SAEM functions as both a transport and enrichment medium. BTM from dairy A was collected in 1-liter sterile bottles and divided into 2-ml samples in the laboratory and then inoculated into SAEM. Flush alley water samples were collected at the valve outlet in the stall barn by filling 1-liter sterile bottles and dividing the fluid into 2-ml samples in the laboratory. Lagoon water samples were collected similarly at different points from along the shore. At dairy B, approximately 10 g of fresh feces was collected with a sterile scoop and placed in a sterile Whirl-Pak bag (Wheatherby/Nasco, Inc., Fort Atkinson, WI). Raw milk and discharge water from the mobile milking parlor at dairy B were collected in 100-ml sterile containers. All samples were stored at 4°C in coolers and processed in <24 h.

Culture and identification. Samples were cultured at the U.S. Department of Agriculture/Agricultural Research Service Western Regional Research Laboratory in Albany, CA, by using selective enrichment in Preston broth and plating onto blood-free *Campylobacter* charcoal differential agar (CCDA) medium (19). A 1:10 dilution of fresh fecal material was prepared by first emulsifying 1 g of feces in 10 ml of phosphate-buffered saline and processing for 30 s by vortex; a 1-ml aliquot of the liquid was pipetted into 9 ml of Preston broth poured into vented-cap cell culture flasks. A 1:5 dilution of unfiltered milk and water samples was prepared by inoculating 10 ml of liquid sample into 40 ml of Preston broth also in a cell culture flask. The flasks were placed on their sides in a plastic ziplock bag and grown under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 42°C with shaking (30 rpm) for 24 h. Enriched Preston broth was then spread-plated onto CCDA agar by pipetting 50 μl of liquid onto the plate. Plates were incubated for 48 h at 42°C under microaerobic conditions by using a plastic ziplock bag.

A subset of samples collected at dairy A were cultured at the University of California, Davis, by using selective enrichment in SAEM and plating on Campy-Cefex or CVA agar (BD, Franklin Lakes, NJ) as described previously (18). Approximately 2 g of feces was inoculated into SAEM prepared in a 50-ml conical tube. Milk and wastewater samples (lagoon and flush alley water) were processed by filtering 2 ml of liquid through a 0.22-μm-pore-size membrane and placing the filter in SAEM. Wastewater samples from dairy A were pipetted directly into SAEM without filtration. Conical tubes were mixed by inversion and incubated at 37°C for 24 h. The enriched SAEM was plated onto Campy-Cefex or CVA agar by using a 1-μl disposable loop and streaking for isolation. Plates were incubated for 48 h at 42°C in an anaerobic box containing three commercial CampyPak II gas-generating sachets (BBL Microbiology Systems, Cockeysville, MD).

Plates were checked for growth at 12, 24, 36, and 48 h. Presumptive identification of *Campylobacter* species was based on a Gram stain and examination of a wet mount by using phase-contrast microscopy to identify small, curved, or S-shaped bacteria with darting motility. Isolates were also characterized by hippurate hydrolysis, oxidase, and catalase tests. Multiple (8 to 12) pure colonies of presumptive *C. jejuni* were picked from each plate to increase the likelihood of finding the clinical outbreak subtype and stored on Microbank dry beads (Pro-Lab Diagnostics, Round Rock, TX) at −80°C.

C. jejuni was confirmed by a multiplex PCR, using the lipid A gene *lpxA*, with a modification by using only *C. jejuni* and *C. coli* primers (20). Additionally, suspect *Campylobacter* isolates from dairy B were identified to the species level by using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (21). MALDI-TOF spectra from each isolate were compared with spectra from *Campylobacter* species validated previously by California's laboratory. In-house pattern-matching software was used for species identification by comparison of the presence of multiple ions in each spectrum, including certain ions previously identified as proteins encoded by annotated genes.

Quantification of indicator bacteria in dairy wastewater. Quantification of total coliforms and *Escherichia coli* was determined for flush alley

water and lagoon samples collected during the March 2007 visit to dairy A by using the Colilert QuantiTray 2000 (IDEXX Laboratories, Westbrook, ME) method, according to the manufacturer's recommendations (22). Briefly, duplicate samples (111 ml) of wastewater were diluted 1:10 and 1:100 in Butterfield's phosphate buffer. Colilert reagent containing 4-methylumbelliferyl-β-D-glucuronide (MUG) was added to 100 ml of each dilution, and samples were inoculated into 97-well trays and sealed. After 24 h of incubation at 35°C, wells were scored for yellow color from fermentation of *O*-nitrophenyl-β-D-galactoside; evidence of total coliforms, blue fluorescence from MUG (generic *E. coli*), and the most probable number (MPN) were calculated from the manufacturer's data tables (22). Four control strains (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13882, *E. coli* ATCC 25922, and *E. coli* O157 ATCC 43888) were cultured and tested simultaneously for each assay.

DNA purification, amplification, and sequencing. Genomic DNA for sequencing was prepared from single-colony *Campylobacter* isolates by using the DNeasy tissue kit (Qiagen USA, Inc., Valencia, CA) protocol according to the manufacturer's instructions (23). Alternately, DNA was extracted by boiling single Microbank bacterial storage beads from freezer stocks to resequence *porA* fragments (24). Genomic DNA concentrations were determined by using a NanoDrop microvolume spectrophotometer (Thermo Scientific, Wilmington, DE). PCRs were performed on an MJ Research Tetrad thermocycler with the following settings: 30 s at 94°C, 30 s at 53°C, and 2 min at 72°C (30 cycles). Each amplification mixture contained 50 ng genomic DNA, 1× PCR buffer, 1× PCR enhancer, 2.5 mM $MgCl_2$, 250 μM (each) deoxynucleoside triphosphates, 50 pmol each primer, and 0.2 U *Taq* polymerase. Amplicons were purified on a BioRobot 8000 workstation (Qiagen USA, Valencia, CA).

Cycle-sequencing reactions were performed on an MJ Research Tetrad thermocycler by using the ABI Prism BigDye terminator cycle sequencing kit (version 3.0; Applied Biosystems, Carlsbad, CA) and standard protocols. All extension products were purified on DyeEx 96-well plates (Qiagen USA, Valencia, CA). DNA sequencing was performed on an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA) by using the POP-7 polymer, ABI Prism genetic analyzer data collection, and ABI Prism genetic analyzer sequencing analysis software.

MOMP, MLST, and PFGE typing. Available clinical strains and up to 12 colonies from each positive environmental sample were subtyped. The MOMP primer sequences used for amplification and sequencing were designed from regions of the *C. jejuni porA* gene generating an ~740-bp fragment (MOMP-L, 5′ CT AGT TAA AMT TAG TTT AGT WGC AGC 3′; MOMP-R, 5′ TCT TTW TYA CCR TAG TAT AAA CCA CC 3′). Strains were characterized further by extended multilocus sequence typing (MLST), as described previously (25), and 2-enzyme PFGE analyses, after digestion with SmaI and KpnI, according to the PulseNet protocol (26, 27). MOMP and MLST sequences were aligned by using ClustalW (European Bioinformatics Institute, Cambridge, United Kingdom); phylogenetic analysis of the aligned sequences was performed by using MEGA version 5 (Molecular Evolutionary Genetics Analysis; Tempe, AZ) (28). Sequences were queried against the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/) to assign sequence types (ST). The *porA* allele of a subset of stored strains was resequenced using the Oxford oligonucleotide primers MOMP-1 and MOMP-2, published after our investigation (29); new *porA* allele sequences and trace files were submitted to the PubMLST for assignment of allele and MOMP peptide numbers.

Nucleotide sequence accession numbers. The nucleotide sequences of *porA* (MOMP-L and MOMP-R) were deposited in the GenBank database under accession no. JX967537 to JX967548.

RESULTS

Human strains. Twenty-six patient isolates associated with outbreak 1 were available to the state for PFGE analysis. All isolates had indistinguishable SmaI patterns (DBRS16.0206), which represented only 0.22% of isolates in the PulseNet national *Campylobacter* database. Twenty-four isolates were indistinguishable by using the secondary enzyme, KpnI (DBRK02.0201); two isolates demonstrated a two-band difference from the dominant KpnI pattern. Three patient isolates (RM5846, RM5847, and RM6591) with the dominant SmaI/KpnI pattern were saved by the state and made available for this study for MOMP and MLST typing. Because of the time lag (~3 months) between the original laboratory investigation and this study, the other patient isolates already had been discarded. All three available strains belonged to ST-21, a common subtype in the *Campylobacter* PubMLST database.

Only one of the eight laboratory-confirmed isolates (RM6863) from outbreak 2 was available to us. The other isolates were discarded by clinical laboratories before county and state public health officials recognized the outbreak, which is routine because *Campylobacter* strains typically are not submitted to PulseNet for cluster detection. The SmaI (DBRS16.0006) and KpnI (DBRK02.0011) patterns of this single-patient isolate had not been recorded previously in the national PulseNet *Campylobacter* database. The strain also belonged to a rare MLST subtype, ST-1244. At the time of this study in 2007, only one human ST-1244 isolate was present in the PubMLST database, although not all researchers deposit their sequences.

Environmental investigation. *C. jejuni* was isolated from 7 of 15 (46.7%) bovine fecal samples, 10 of 10 (100%) flush alley water samples, and none of the BTM samples collected at dairy A (outbreak 1) in August 2006. *C. coli* was also isolated from 4 (26.7%) of the bovine fecal samples but none of the flush alley water samples. During the follow-up visit in March 2007 to study the dairy wastewater system, *C. jejuni* was isolated from 2 of 10 (20%) flush alley water and 1 of 20 (5%) lagoon samples. In contrast, *C. coli* was cultured from 9 of 10 (90%) flush alley water and 20 of 20 (100%) lagoon samples during the second visit. The coliform and *E. coli* counts (MPN) for the flush alley water samples were 683,000/g and 546,000/g, respectively. The coliform and *E. coli* counts (MPN) for the lagoon water were 579,400/g and 327,000/g, respectively.

C. jejuni was isolated from 9 of 26 (34.6%) bovine fecal samples at dairy B (outbreak 2) collected in December 2007. *C. jejuni* was not isolated from BTM, retail raw milk, or milking parlor discharge water. Other *Campylobacter* species were also cultured from the bovine fecal samples, including *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. lari*. *C. coli* was also cultured from milking parlor discharge water.

Identification of the clinical outbreak strain in farm samples. Because ST-21 (outbreak 1) was a common sequence type in the PubMLST database, we initially evaluated the uniqueness of the dairy A-related clinical outbreak *porA* sequence by comparing it with the *porA* sequences of 26 representative *C. jejuni* ST-21, clonal complex-21, and related isolates in our strain collection. Sufficient genetic diversity was observed to proceed with screening the farm isolates (Fig. 1).

Phylogenetic comparison of 70 *C. jejuni* MOMP sequences from 17 dairy A samples revealed 10 strains recovered from five flush alley water samples with the same *porA* sequence as the clinical outbreak strain (Fig. 2, MOMP group A3). Of these 10 candidate strains, 8 had indistinguishable SmaI (Table 1, pulsotype 3) and KpnI patterns, compared with the clinical outbreak strain, and belonged to the same sequence type (ST-21). Two strains (RM6199 and RM6200) differed from the clinical outbreak strain by a single band (Table 1, pulsotype 4) and belonged to ST-806, a sequence type closely related to ST-21. The outbreak strain was not identified in 30 lagoon and flush alley water samples

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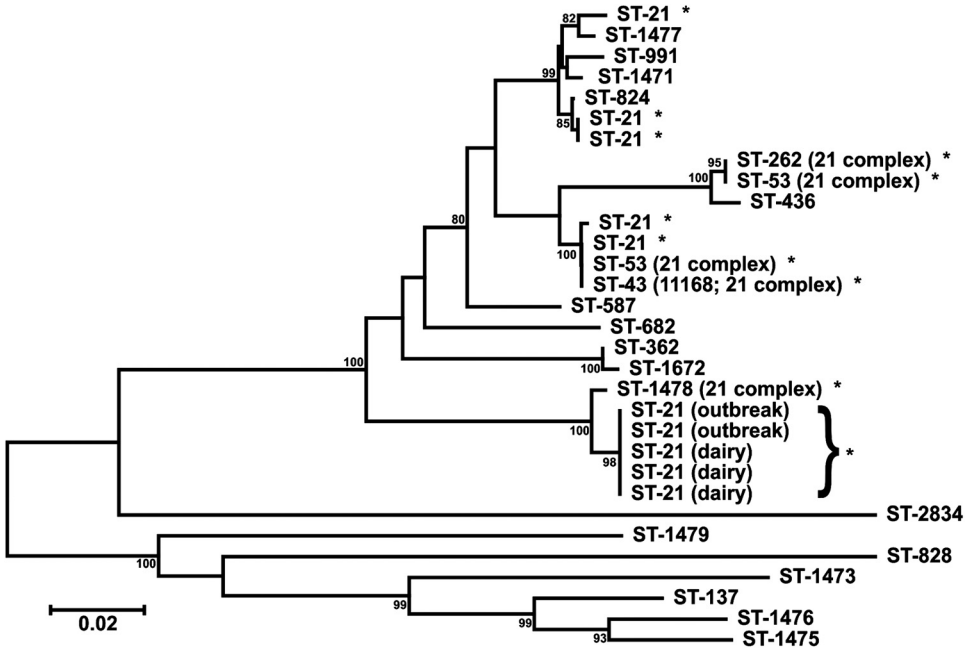

FIG 1 Phylogenetic tree comparing the *porA* sequence of the *Campylobacter jejuni* clinical outbreak strain (sequence type 21) from dairy A with those of related isolates from our strain collection. The sequences of two clinical outbreak isolates (outbreak) and three flush alley water isolates (dairy) identified through major outer membrane protein screening are displayed. *, sequence and clonal type 21 strains. The scale bar represents the number of nucleotide changes per site.

during the follow-up visit in March 2007; however, four wastewater isolates belonged to ST-8, ST-459, and ST-922 (Table 1; Fig. 2).

In December 2007, we used the MOMP screen again to compare isolates from dairy B (outbreak 2) samples with the one clinical outbreak strain available to us. Among 34 *C. jejuni* isolates recovered from 9 bovine fecal samples at dairy B, we identified 20 strains from four samples with *porA* sequences identical to that of the patient strain (Fig. 3, group B1). Of the 20 candidate strains, 17

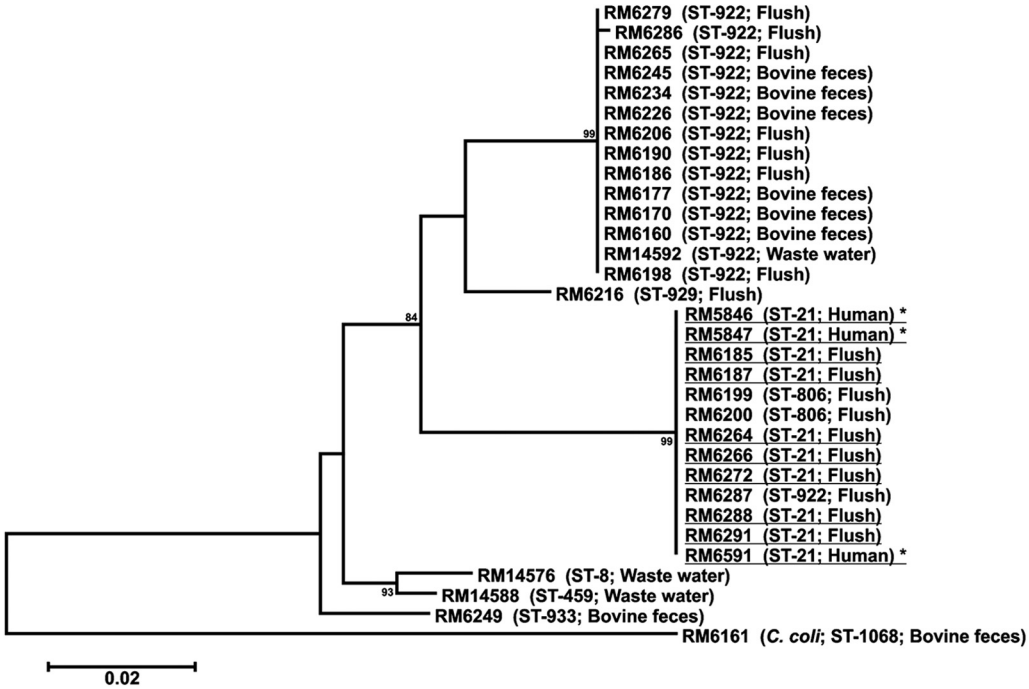

FIG 2 Phylogenetic tree comparing the *porA* sequences of representative *Campylobacter jejuni* and *Campylobacter coli* strains isolated from dairy A environmental samples with that of the clinical outbreak strain (outbreak 1, 2006 to 2007). *, clinical outbreak strain. Dairy strains with the identical *porA* and multilocus sequence typing alleles and indistinguishable SmaI and KpnI patterns are underlined. The scale bar represents the number of nucleotide changes per site.

TABLE 1 Genotypes of *Campylobacter jejuni* strains cultured from environmental samples[a]

Dairy	Source[b]	No. of isolates[d]	MOMP group	Oxford *porA*/MOMP[e]	Pulsotype (SmaI)	MLST ST	No. of MLST alleles							MLST clonal complex
							atpA	*aspA*	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	
A	BF, FW[c]	51	A1	747/692	1	922	1	6	1	2	83	2	3	Unassigned
	FW	3	A1	1524/1398	1	922	1	6	1	2	83	2	3	Unassigned
	FW	1	A2	749/694	2	929	9	17	2	4	62	4	5	257
	FW, H	10	A3	1533/1377	3	21	2	5	1	1	3	2	1	21
	FW	2	A3	1500/1377	4	806	2	5	1	1	3	140	3	21
	FW[c]	1	A4	745/690	5	8	2	6	1	1	3	2	1	21
	L[c]	2	A5	44/41	6	459	1	3	2	3	3	5	9	42
	BF	8	A6	1523/1397	1, 7, 8, 9	933	10	7	1	59	19	10	5	403
B	BF, H	18	B1	1499/1376	10, 11	1244	1	17	1	2	2	225	3	61
	BF	2	B1	1526/1400	10	1244	1	17	1	2	2	225	3	61
	BF	8	B2	48/44	12	22	1	3	3	6	4	3	3	22
	BF	3	B3	1529/1403	13	922	1	6	1	2	83	2	3	Unassigned
	BF	3	B4	1528/1402	14	45	4	1	7	10	4	1	7	45

[a] Genotypes of *Campylobacter jejuni* strains cultured from environmental samples collected during pasteurized (dairy A) and raw (dairy B) milk outbreak investigations, California, 2006 to 2007. MLST, multilocus sequence typing; MOMP, major outer membrane protein; ST, sequence type.
[b] BF, bovine feces; FW, flush alley water; H, human stool; L, lagoon.
[c] ST-8 (MOMP group A4; $n = 1$), ST-459 (MOMP group A5; $n = 2$), and ST-922 (MOMP group A1; $n = 1$) strains were from samples collected in March 2007.
[d] Includes multiple colony picks from individual samples ($n = 112$).
[e] Representative strains from each MOMP group were subtyped by using the Oxford *porA*/MOMP method ($n = 86$). *porA* allele and MOMP peptide numbers were assigned by PubMLST (http://pubmlst.org/campylobacter/).

had SmaI and KpnI patterns indistinguishable from those of the clinical outbreak strain (Table 1, pulsotype 10). Three strains, represented by RM6874 in Fig. 3, indicated a two-band difference from the outbreak strain by SmaI PFGE analysis. The MLST results revealed that all 20 MOMP group B1 strains were ST-1244, including those with slightly different pulsotypes (Table 1).

Genetic diversity of *C. jejuni* and *C. coli* from the dairy environmental samples. Overall, 10 unique MOMP types using our primer set and 15 unique MOMP-MLST-PFGE groups were identified among the 108 *C. jejuni* isolates subtyped in 2006 and 2007 (Table 1). In comparison, resequencing the *porA* fragment of 86 strains by using the Oxford primers (MOMP-1, MOMP-2) revealed 13 MOMP subtypes and a combined 17 MOMP-MLST-PFGE subtypes. Analysis of multiple colony picks resulted in recovery of up to four unique subtypes per sample. The most common MLST sequence type was ST-922, which was also the only sequence type identified for both dairies (Table 1). Fourteen SmaI pulsotypes were identified with ST-933 strains from dairy A showing the most diversity (four pulsotypes; Table 1).

In contrast, the *C. coli* isolates from bovine feces and wastewater samples collected at dairy A during the August 2006 ($n = 42$) and March 2007 ($n = 33$) visits were remarkably clonal; all strains belonged to ST-1068 and had identical *porA* sequences (Fig. 2). Similarly, the *C. coli* strains ($n = 23$) from bovine feces and discharge water collected at dairy B belonged to ST-894 and ST-1068, respectively, and had closely related *porA* sequences (Fig. 3).

DISCUSSION

We used MOMP typing to screen a substantial number of farm isolates collected after two milk-borne *C. jejuni* outbreaks and successfully identified the clinical outbreak strains. This study demonstrates that sequencing the *C. jejuni porA* allele is a prom-

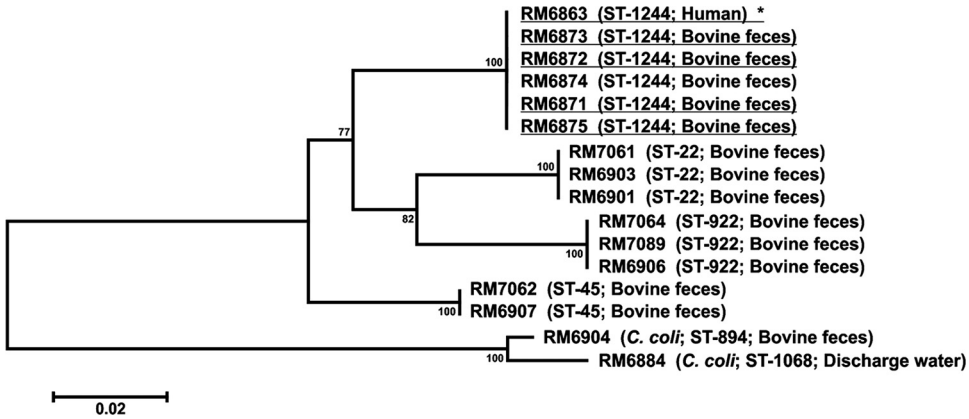

FIG 3 Phylogenetic tree comparing the *porA* sequences of representative *Campylobacter jejuni* and *Campylobacter coli* strains isolated from dairy B environmental samples with that of the clinical outbreak strain (outbreak 2, 2007). *, clinical outbreak strain. Dairy strains with the identical *porA* and multilocus sequence typing alleles and indistinguishable SmaI and KpnI patterns are underlined. The scale bar represents the number of nucleotide changes per site.

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ising target for rapid environmental screening to identify specific clones prior to conducting labor-intensive molecular typing methods following outbreaks.

By implementing a single-allele screening approach, public health laboratories could decrease time and labor costs associated with *C. jejuni* molecular epidemiologic studies in the food supply continuum. Specifically, sequencing of the *porA* gene and construction of a phylogenetic tree can be accomplished in under 24 h compared with MLST and 2-enzyme PFGE confirmation, which may take at least a week to complete in public health laboratories with limited staff and resources. The screen is also amendable to retrospective high-throughput sequencing of strains by boiling single MicroBank freezer stocks to extract DNA directly from the bead, thus eliminating the need to regrow isolates by culture (24). We used the bead extraction technique to rapidly resequence *porA* alleles using the Oxford primer set published subsequently to our original analysis as described below (29). Laboratories could further streamline the screen by developing an allelic discrimination real-time PCR assay to detect single nucleotide polymorphisms in the *porA* sequence specific to the clone (clinical outbreak strain) of interest (30). This approach provides a method for screening a large number of clinical and, possibly, environmental isolates in investigations for rapid bacterial source tracking and selection of strains for next-generation whole-genome sequencing for high-resolution differentiation (31).

In 2008, an extended 8-locus MLST-*porA* typing scheme was published by researchers in Oxford, England (29). The Oxford *porA* product (570 bp, minimum; 738 bp, maximum) overlaps with the fragment we analyzed in this study. Cody et al. (2009) used the Oxford primer set to type a *C. jejuni* strain collection and demonstrated that the *porA* allele has sufficient genetic diversity and stability for use as a molecular epidemiologic tool (32). Results from our study are comparable and show that the Oxford typing scheme discriminated an additional three *porA*/MOMP types.

This study has some limitations that indicate the need for further investigation. For example, and unfortunately for effective trace-back investigations, many clinical laboratories often discard *C. jejuni* isolates after confirmation of species, leaving few human isolates available for analysis. Even in this very large outbreak, isolates were not saved, thus limiting our ability to evaluate the robustness of MOMP typing in discriminating clinical MLST and PFGE subtypes. Our analysis also emphasizes the importance of selecting multiple pure colonies from individual samples during *C. jejuni* outbreak investigations for successful molecular subtyping, as described previously in the human and veterinary literature (33, 34). Indeed, our results confirmed that multiple *C. jejuni* subtypes in individual bovine fecal and wastewater samples are not uncommon (Table 1). However, the regional prevalence of the *C. jejuni* outbreak strains at dairy A (ST-21) and dairy B (ST-1244) was unknown at the time of the investigations. Follow-up studies of multiple dairies in the central valley indicate that ST-1244 is relatively common from bovine sources, including bulk tank milk (M. T. Jay-Russell, unpublished data). Noteworthy is a report by Kwan et al. (2008) of ST-1244 as predictive potentially of strains that are bovine adapted, although further studies will be required to determine whether this is a marker for host-adapted *C. jejuni* (35).

Public health implications. An important goal of this study was to show the utility of MOMP typing to source-track genetically related strains in the context of an outbreak and identify potential on-farm risk factors and prevention strategies. Although *C. jejuni* was not isolated from milk samples during either outbreak investigation, our molecular typing results support strongly the epidemiologic findings implicating milk products produced by the dairies as the sources of both outbreaks (16, 17). *Campylobacter* is notoriously difficult to isolate from milk following outbreaks, possibly due to die-off during the lag time from consumption to recognition of the outbreak, uneven distribution of the bacteria in bulk tank milk, or inadequate culture and isolation methods for milk.

The exact mechanism of contamination at dairy A remains unclear, but it might have been the result of cross-contamination after pasteurization or heavy loads of *Campylobacter* before pasteurization. Based on the ease of recovery of *C. jejuni* from flush alley water, we speculate it is possible that cross-contamination with environmental dairy wastewater during milk processing occurred. Notably, the dairy A pasteurization room was located adjacent to the milking parlor and ~100 m from the dairy lagoon. Moreover, the recovery of *C. jejuni* from flush alley water and lagoon samples at dairy A, approximately 3 months after the outbreak, suggests that the outbreak strain was predominant at this dairy and the lagoon could be an important environmental reservoir. The high numbers of indicator bacteria (coliforms and *E. coli*) identified in lagoon and flush alley water are consistent with the pathogen prevalence. Additional research is needed to define the spread of *Campylobacter* in recycled wastewater systems and strategies to minimize contamination at dairies that use these systems for manure management.

Similar to the results at dairy A, the clinical outbreak strain was cultured from bovine feces during the environmental investigation of the dairy B outbreak linked to organic raw milk and raw chocolate colostrum. Dairy B also had an on-site creamery adjacent to cattle areas. Raw dairy products are not subjected to a "kill step," so it is plausible to suggest that viable *C. jejuni* from bovine fecal material contaminated the milk/colostrum sometime during production or processing. Cross-contamination with environmental waters represents another possible mechanism of contamination. Indeed, a different pathogen, *C. coli*, was isolated from milking parlor discharge water, suggesting the possibility of cross-contamination.

The results obtained by MOMP typing the environmental isolates ultimately facilitated more intensive investigation into on-farm food safety practices at the implicated dairies and were used by state agriculture and public health officials to reinforce the importance of sanitation and education of dairy workers about best practices in dairy production (16, 17). Additionally, the California state legislature passed a bill that became effective on 1 January 2008 requiring no more than 10 coliform bacteria per milliliter in grade A raw milk for human consumption, the same coliform limit required for pasteurized milk (36).

In summary, our study highlights the potential for ubiquitous dissemination of *C. jejuni* in the dairy farm environment and the utility of using MOMP typing as an epidemiologic tool for screening *C. jejuni* isolates to identify specific strains during dairy-related outbreak investigations to support implementation of on-farm prevention strategies. The approach could be applied during investigation of other potential food and water sources where there is a need to prioritize a large number of *C. jejuni* environ-

mental isolates before confirmation by other molecular methods such as MLST, PFGE, or whole-genome sequencing analyses.

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Original article

Clonal or not clonal? Investigating hospital outbreaks of KPC-producing *Klebsiella pneumoniae* with whole-genome sequencing☆

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ABSTRACT

Objectives: Whole-genome sequencing (WGS) is a promising tool for identifying transmission pathways in outbreaks caused by multidrug-resistant bacteria. However, it is uncertain how the data produced by WGS can be best integrated into epidemiologic investigations.
Methods: We tested various genomic analyses to identify clonal groups in two distinct outbreaks of *Klebsiella pneumoniae* carbapenemase–producing *K. pneumoniae* that occurred in Switzerland in 2013 and 2015. In blinded fashion, we sequenced 12 strains involved in the two outbreaks, respectively, and six that were epidemiologically unrelated. We analysed genomic commonalities from conserved genes to plasmid-borne antibiotic resistance genes (ARGs) and contrasted these results with available epidemiologic evidence.
Results: Using WGS, blinded analysts correctly identified the two clusters of strains from the two outbreaks. Nonetheless, the 2015 index strain was found to be slightly different (1–3 single nucleotide variants) from the strains recovered from secondary cases, likely because prior long-term carriage (3 years) by the index patient allowed for genetic mutations over time. Also, we observed occasional loss of ARG-bearing plasmidic fragments in outbreak-causing strains.
Conclusions: Retrospective WGS analysis was successful in identifying clonal groups in both outbreaks. Still, data should be analysed with caution in cases of previous long-term carriage of the studied bacteria.
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Introduction

Whole-genome sequencing (WGS) has become increasingly affordable and is a useful tool in assessing chains of potential cross-transmission [1]. This method consists of sequencing both the chromosome and putative mobile genetic elements of a given organism; it can assess strain clonality via the presence of single nucleotide variants (SNVs). It has thus been used in several outbreak investigations [2–7]. Yet there is no practical consensus on how to use WGS data to determine clonal relationships among strains. Set SNV thresholds have been proposed [8], but these are based on bacterial strains whose relationships were speculative. Indeed, the number of SNVs required to define a distinct strain has varied among studies, ranging from 10 [9] to 22–24 [10]. Moreover, any set threshold can be challenged, given the potential for emergence of clonal subpopulations of resistant bacteria during long-term carriage [11]. SNV characterization itself can be performed with various degrees of granularity: the whole genome (including acquired genetic elements such as plasmids), a selection of conserved genes (whole-genome multilocus sequence typing (wgMLST), which includes only genes conserved within a given

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species) or the specific distribution of short nucleotide sequences (k-mer) in the genome [12].

Enterobacteriaceae producing the *Klebsiella pneumoniae* carbapenemase (KPC) pose a major threat to public health [13]. In 2013 and 2015, two separate KPC outbreaks occurred in Switzerland. In both instances, their paths of transmission were determined via conventional epidemiologic and laboratory methods, and successfully interrupted. We retrospectively performed WGS of both outbreak strains and unrelated (control) strains to assess WGS's concordance with traditional epidemiologic methods on clonality determination.

Materials and Methods

Outbreaks

The 2013 outbreak occurred in St Gallen, Switzerland, and yielded three KPC isolates (B2, B3 and B4); it is summarized in Fig. 1(A) and described in detail elsewhere [14].

The 2015 outbreak occurred at the Geneva University Hospital (HUG), a 1800-bed tertiary-care centre in Switzerland. On 13 January 2015, the index case (HUG1), a 78-year-old Italian man with known KPC-producing *K. pneumoniae* colonization since 2012 (first isolation on 7 March 2012: KPN3 and KPN12, then KPN5 in 2013), was admitted with pyelonephritis and severe sepsis. A KPC-producing *K. pneumoniae* was isolated from blood cultures (KPN7). He stayed at intermediate-care ward 'A' till 29 January, when he was transferred to rehabilitation ward 'B,' from which he was discharged home on 6 February (Fig. 1(B)). Contact precautions, including single-room isolation, were implemented immediately at admission and were maintained through discharge; thus, no specific screening was performed for patients in wards 'A' and 'B.' The following month, three KPC-producing *K. pneumoniae* isolates were identified: on 19 February from patient HUG2 (KPN8 recovered from a thigh haematoma treated with a colistin-based regimen and its colistin-resistant variant KPN13) in ward 'E'; on 8 March from patient HUG3 (urine (KPN9) and faeces (KPN10)) in ward 'D'; and on 13 March from the patient HUG4, who was found to be colonized (positive rectal swab) by KPC-producing *K. pneumoniae* (KPN11) in ward 'F.' KPN7, KPN8, KPN9 and KPN10 displayed >95% similarity in the molecular typing by rep-PCR/Diversilab (bioMérieux, Marcy-l'Etoile, France; Supplementary Fig. S1) and were considered to be the same clone at the time of investigation.

Patients HUG1, HUG2 and HUG3 had shared ward 'A' for 72 hours. Patients HUG2 and HUG3 spent another 24 hours in ward 'A' together 2 days later, while patients HUG2 and HUG4 spent 15 days together in ward 'E.' It was thus considered highly likely that a transmission event occurred between the index patient and patients HUG2 and/or HUG3 in ward 'A,' and that another transmission event occurred in ward 'E' between patients HUG2 and HUG4. The outbreak was contained through the efforts of a targeted task force. Despite active screening of 435 potential contact patients in all concerned wards, no other cases have since been identified.

Microbiologic methods

A total of 18 KPC-producing *K. pneumoniae* isolates were sequenced: the three strains obtained during the 2013 St Gallen outbreak [14], six strains from the 2015 HUG outbreak and three additional strains isolated from that outbreak's index patient during his previous stays in our hospital, six other KPC-producing *K. pneumoniae* control isolates epidemiologically unrelated to the two outbreaks and collected from our institution (unpublished data) or elsewhere in Switzerland [15]. The strains were identified by mass spectrometry (Bruker Daltonics, Bremen, Germany). The antibiotic susceptibility testing was performed by the disk diffusion method on Mueller-Hinton agar media or by an automated microdilution test system for strains B1, B5 and B6 (Micronaut-S; Merlin Diagnostika, Berlin, Germany) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Colistin minimal inhibitory concentrations were

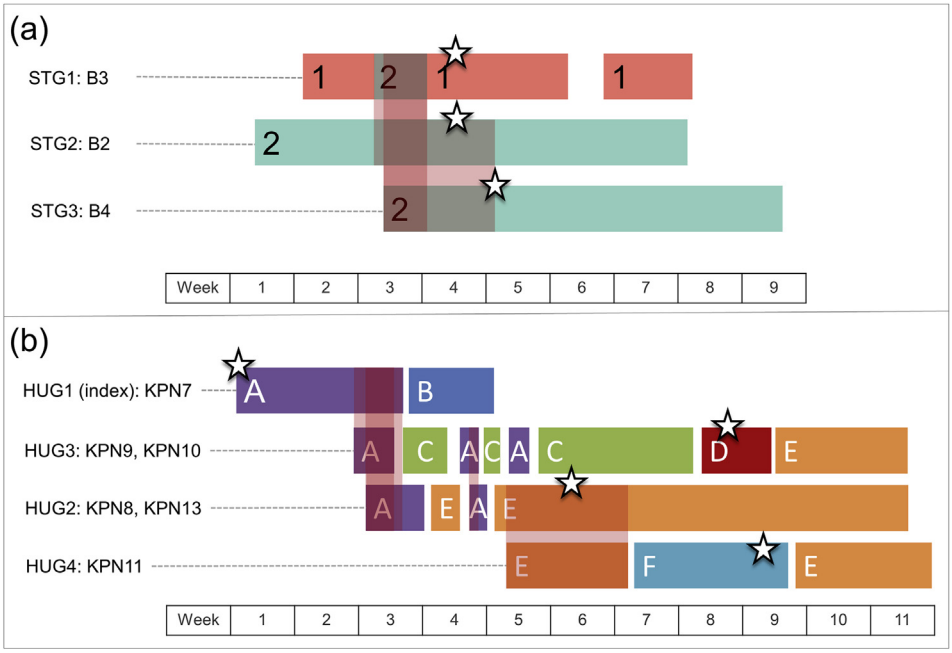

Fig. 1. Schematic representation of patients' hospitalization and first isolation of KPC-producing *Klebsiella pneumoniae* (star). (a) St Gallen outbreak. (b) Geneva outbreak. Wards are represented by coloured rectangles; shaded red area depicts time shared by patients in same ward before isolation of KPC-producing *K. pneumoniae*. KPC, *K. pneumoniae* carbapenemase.

determined by Etest strips (bioMérieux, Marcy l'Etoile, France). Strains were stored at −80°C until sequenced.

WGS and bioinformatic analysis of clonality

Details on the sequencing and bioinformatic methods are provided in the Supplementary Materials. Briefly, the 18 strains were sequenced on a MiSeq device (Illumina, San Diego, CA, USA), and the index strain for the 2015 outbreak (KPN7) was also sequenced on a PacBio RS2 (Pacific Biosciences, Menlo Park, CA, USA) (Supplementary Table S1). Antibiotic resistance genes (ARG) were sought from the assembled contigs from an in-house ARG database comprising 4311 nonredundant genes (Supplementary Table S2). Genes of interest (encoding topoisomerases, porins and others possibly involved in colistin resistance) were compared to the *K. pneumoniae* reference strain NTUH-K2044 (NC_006625.1). In an investigator-blinded fashion (except that KPN7 was known to be the index strain), SNVs were counted at three levels: whole genome including plasmids, whole genome excluding plasmids (i.e. chromosome) and conserved genes (Supplementary Materials).

Results

Blinded assessment of clonal relationships by wgMLST (634 genes)

Fifteen of the 18 strains belonged to ST512, while isolates KPN1, B5 and B6 belonged to ST258. From the wgMLST analysis, we first observed that KPN7 was distinct from other strains and had one SNV difference with the closest cluster that included five strains (KPN8, -9, -10, -11 and -13) (Fig. 2, Table 1). In addition, another cluster of three strains was observed (B2, B3 and B4). The other strains appeared to be unrelated at this level of analysis, with KPN1 and B5 being the most distant strains from KPN7 (Table 1); their involvement in any outbreak caused by KPN7 was thus unlikely.

Blind assessment of clonal relationship by whole genome (including and excluding plasmids)

From a less conservative level, we analysed the SNVs of the common DNA shared by all 18 *K. pneumoniae* isolates, including plasmids (Fig. 3, Table 1, Supplementary Table S3). Index strain KPN7 had three SNVs with the closest similarity to strains KPN8, KPN9 and KPN11. KPN10 and KPN13 were also very closely related to KPN7, with four and five SNVs respectively. Six other strains (KPN2–5, KPN12, B1 and B6) differed from KPN7 by 12 to 45 SNVs at the whole-genome level, including plasmids. We excluded them from the outbreak given the fact that the duration of the outbreak was less than 3 months, and assuming that 12 SNVs could not have appeared in such a short time (Fig. 4 and Supplementary Materials). The same cluster of B2, B3 and B4 was also found here, with no SNV between strains. When excluding plasmids and considering the chromosome only, the number of SNVs did not differ significantly (Table 1).

Analysis of plasmids

Five contigs were identified from long-read sequencing and assembly of the KPN7 genome. The first was 5 423 117 bp (with 5140 predicted open reading frames) and was thereby assumed to be the chromosome of the strain. The other four were plasmids (size, incompatibility group), as follows: KPN7_p1 (208 528 bp, IncFIB/IncFII), KPN7_p2 (114 073 bp, IncFIB/IncFII), KPN7_p3 (44 559 bp, IncX3) and KPN7_p4 (14 027 bp, colRNAI)

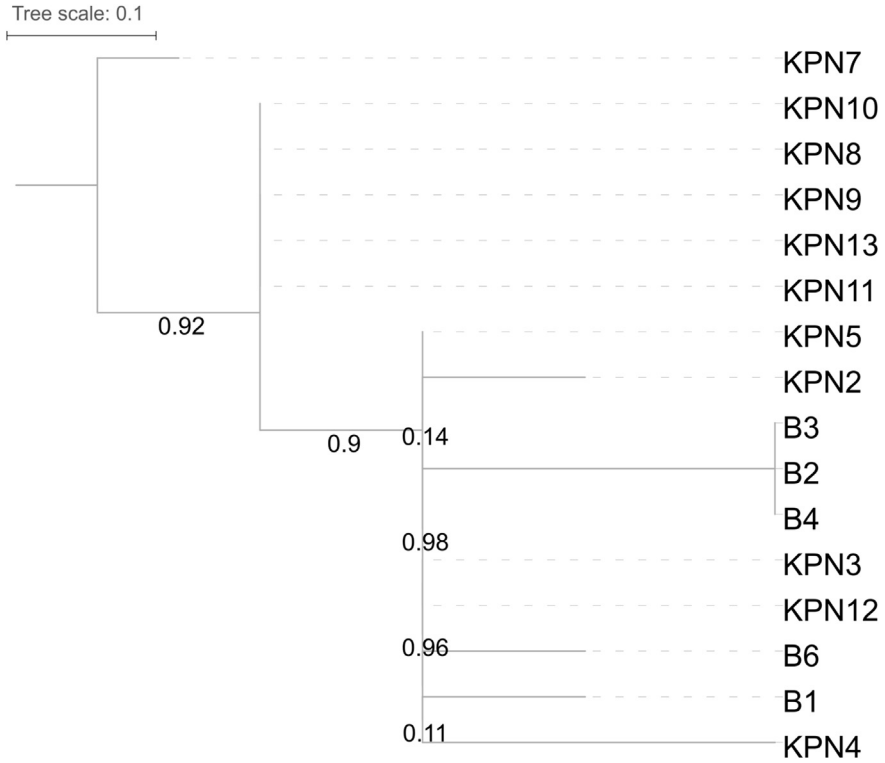

Fig. 2. Phylogenetic tree with bootstrap values obtained by comparison of genes included in *Klebsiella pneumoniae* wgMLST. KPN7 is suspected index strain for outbreak. B5 and KPN1 do not appear on tree because of their high distance. wgMLST, whole-genome multilocus sequence typing.

Table 1
Summary of sequenced strains

Strain no.	Strain	Outbreak	Year	ST	Origin	Date	Patient	Origin	MLST SNVs[a]	Chromosome SNVs	WG SNVs
1	KPN7	HUG	2015	512	Blood culture	20.01.2015	HUG1	Italy	0	0	0
2	KPN8	HUG	2015	512	Collection	02.03.2015	HUG2	Italy	1	3	3
3	KPN13	HUG	2015	512	Blood culture	06.03.2015	HUG2	Italy	1	5	5
4	KPN9	HUG	2015	512	Urine	08.03.2015	HUG3	Italy	1	3	3
5	KPN10	HUG	2015	512	Faeces	12.03.2015	HUG3	Italy	1	4	4
6	KPN11	HUG	2015	512	Faeces	13.03.2015	HUG4	Italy	1	3	3
7	KPN3	No	2012	512	Faeces	13.03.2012	HUG1	Italy	3	21	22
8	KPN12	No	2012	512	Faeces	19.10.2012	HUG1	Italy	3	26	26
9	KPN5	No	2013	512	Wound	06.02.2013	HUG1	Italy	2	12	12
10	B3	St Gallen	2013	512	Blood	NA	STG1	Italy	5	46	47
11	B2	St Gallen	2013	512	Blood	NA	STG2	Italy	5	46	47
12	B4	St Gallen	2013	512	Faeces	NA	STG3	Italy	5	46	47
13	KPN2	No	2010	258	Urine	15.11.2010	HUG5	NA	4	26	26
14	B1	No	2010	512	Blood	NA	CH2	Italy	4	37	38
15	KPN4	No	2012	512	Sputum	20.07.2012	HUG6	NA	5	41	42
16	B6	No	2010	258	Catheter	NA	CH4	Italy	4	45	45
17	B5	No	2009	258	Faeces	NA	CH1	Greece	33	943	944
18	KPN1	No	2010	512	NA	27.09.2010	SION1	NA	32	935	936

MLST, multilocus sequence typing; NA, not available; SNV, single nucleotide variant; ST, sequence type; WG, whole genome.
[a] SNVs were determined with KPN7 as reference.

(Supplementary Figs. S2–S5). The 17 strains displayed similar plasmid content (Supplementary Table S1) with some notable exceptions. KPN10 indeed lacked an 86 kb portion of plasmid KPN7_p1. In the B2/B3/B4 cluster, the KPN7_p2 completeness differed slightly, with 91.1% completeness for B2 vs. 90.5% for B3 and B4.

Analysis of ARGs

All strains displayed similar antibiotic susceptibility profiles, with resistance to all β-lactams and fluoroquinolones but, for the most part, susceptibility to gentamicin, colistin and tigecycline. Still, some differed with respect to their susceptibility to cotrimoxazole (KPN10) and their resistance to colistin (KPN13 and B5). From WGS data, the number of ARGs ranged from six to 13 (Fig. 3, Supplementary Table S4). From the KPN7 cluster, KPN10 lacked the *aadA6, aph(3′)-Ia, mphA, catA1* and *dfrA12* genes (consistent with its apparent susceptibility to cotrimoxazole and the loss of a 86 kb portion of plasmid KPN7_p1). In the B2/B3/B4 cluster, all strains shared a similar antibiotic susceptibility profile and consistently harboured similar ARG content (Supplementary Table S4), yet strains B3 and B4 lacked the *aadA6* and *aph(3′)-Ia* genes found on plasmid KPN7_p1. Further details about resistance determinants are found in the Supplementary Materials.

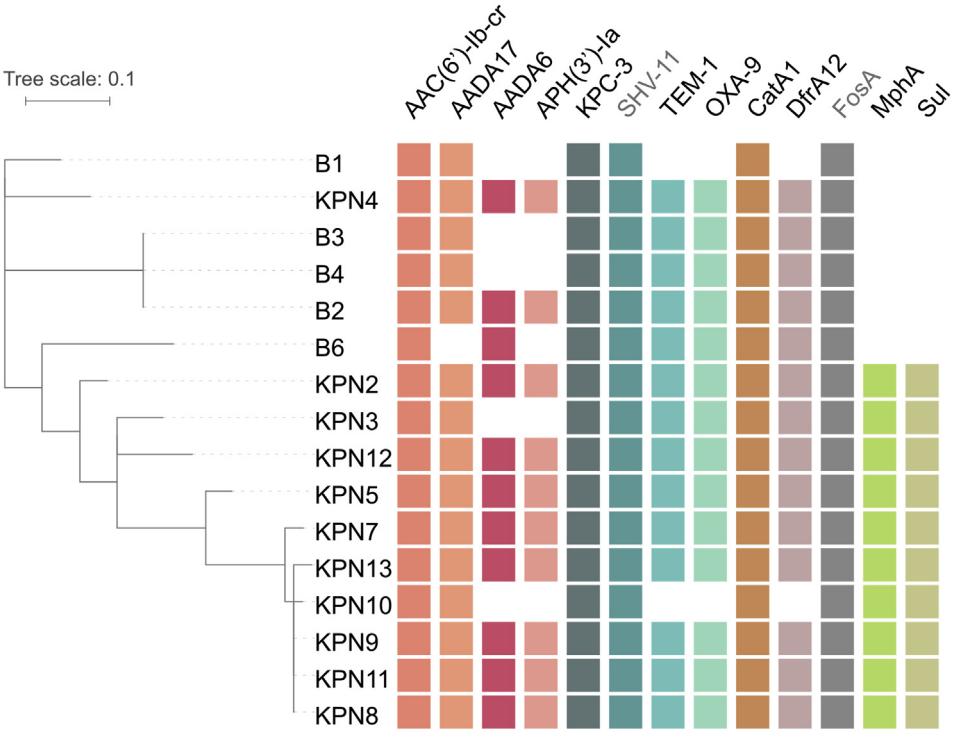

Fig. 3. Phylogenetic tree obtained by comparison of common genomes of all KPC-producing *Klebsiella pneumoniae* and associated ARGs. B5 and KPN1 do not appear on tree because of their high distance. ARGs *SHV-11* and *FosA* (in grey font) are intrinsic to *K. pneumoniae.* ARG, antibiotic resistance gene; KPC, *K. pneumoniae* carbapenemase.

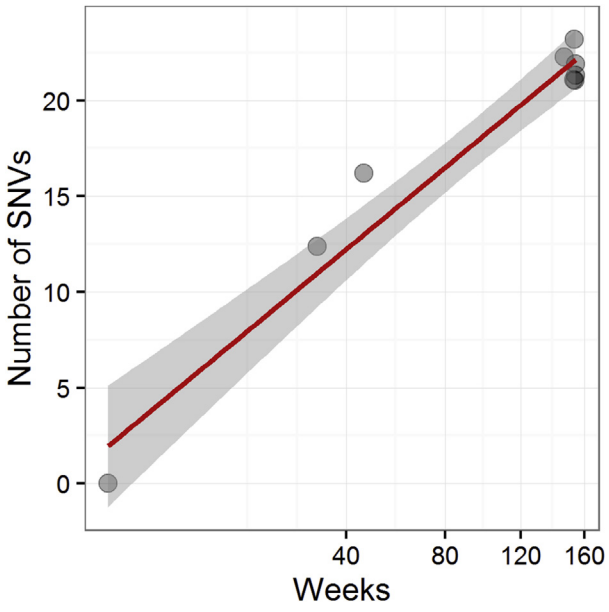

Fig. 4. Number of SNVs observed in KPC-producing *Klebsiella pneumoniae* from index patient HUG1 since first isolation of KPC-producing *K. pneumoniae* in March 2012 (KPN3) and last KPC-producing *K. pneumoniae* recovered in March 2015 (KPN11). Shaded grey area represents 95% confidence interval for number of SNVs at each time point. KPC, *K. pneumoniae* carbapenemase; SNV, single nucleotide variant.

Discussion

Different levels of WGS analysis yielded different information with respect to observed transmission pathways in both outbreaks. In the wgMLST analysis, very few SNVs were found between strains, irrespective of their relationship. Conversely, plasmid analysis revealed a high degree of variability, as illustrated by the loss of plasmid regions between related strains. At the whole-genome level (including plasmids), we were able to identify the two strain clusters described in the two outbreaks. The inclusion or exclusion of plasmids (allowed by the PacBio sequencing of the index strain) had little impact in this context, as relationships among strains did not differ significantly when including or excluding plasmid for the SNV determination. Our results thus suggest that whole-genome analysis (with plasmids) is the most relevant.

Nonetheless, in the HUG outbreak, WGS was nearly too granular: we observed no genome was identical to that of the index strain KPN7, with at least one SNV in wgMLST and three SNVs in whole-genome (plasmids included) analysis in strains KPN8, KPN9 and KPN11, which were otherwise indistinguishable from each other. This suggests that the index strain KPN7 was not directly transmitted; rather, KPN8, KPN9 or KPN11 was the index strain and then evolved into KPN10 and KPN13. Indeed, since 2012 the index patient had likely been carrying several populations of KPC-producing *K. pneumoniae* differing by a few SNVs, as reported in cases of long-term carriage [11]. Perhaps if we had considered other sites for sampling and selected more than one strain from the culture, we might have identified the strain that had been transmitted to HUG2 and HUG3. Currently no specific guidelines recommend the typing of more than one strain per patient. These findings suggest that when typing with WGS, the collection of multiple strains from more than one anatomic site could be justified. Conversely, the strains transmitted from HUG2 (KPN8) to HUG4 (KPN11) were indistinguishable, as were the strains from the St Gallen outbreak. In those cases, the interval between acquisition and transmission was shorter, and thus bacterial populations were likely more homogeneous. Of note, despite the low number of strains analysed, the rate of SNV appearance was higher than that observed in a ST258 KPC-producing *K. pneumoniae* outbreak [11] and a ST15 extended-spectrum β-lactamase-producing *K. pneumoniae* [16]. The dynamics of SNV occurrence will thus continue to be challenging when WGS analysis is used in outbreak settings.

Our study has several limitations. First, the outbreaks we used in the investigation included only a small number of transmission events, which limits the extent of our observations. Second, we could not confirm our assumption that the index patient was carrying various KPC-producing *K. pneumoniae* populations, as initial isolates from the rectal swab were not stored.

Overall, we confirmed that WGS can be used to identify relationships between strains during outbreaks even when prior data are minimal and limited to the index strain. Nonetheless, the discrimination capacity of WGS varies according to the set level of genomic analysis, and previous long-term carriage of an index strain can lead to misinterpretations of strain relationships. Our findings underscore the need for consensus guidelines and cutoffs to support clinical diagnosis and decision making. Indeed, an approach similar to that of the EUCAST consultation (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/2016/EUCAST_WGS_report_consultation_20160511.pdf) for WGS use in antibiotic susceptibility testing would help establish standardized protocols for WGS-derived strain typing.

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Transparency Declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2017.01.015.

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BMJ Open

Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* is a common nosocomial pathogen responsible for significant morbidity and mortality internationally. Patients may become colonised or infected with *P. aeruginosa* after exposure to contaminated sources within the hospital environment. The aim of this study was to determine whether whole-genome sequencing (WGS) can be used to determine the source in a cohort of burns patients at high risk of *P. aeruginosa* acquisition.

Study design: An observational prospective cohort study.

Setting: Burns care ward and critical care ward in the UK.

Participants: Patients with >7% total burns by surface area were recruited into the study.

Methods: All patients were screened for *P. aeruginosa* on admission and samples taken from their immediate environment, including water. Screening patients who subsequently developed a positive *P. aeruginosa* microbiology result were subject to enhanced environmental surveillance. All isolates of *P. aeruginosa* were genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

Results: WGS for 141 *P. aeruginosa* isolates were obtained from patients, hospital water and the ward environment. Phylogenetic analysis revealed eight distinct clades, with a single clade representing the majority of environmental isolates in the burns unit. Isolates from three patients had identical genotypes compared with water isolates from the same room. There was clear clustering of water isolates by room and outlet, allowing the source of acquisitions to be unambiguously identified. Whole-genome shotgun sequencing of biofilm DNA extracted from a thermostatic mixer valve revealed this was the source of a *P. aeruginosa* subpopulation previously detected in water. In the remaining two cases there was no clear link to the hospital environment.

Conclusions: This study reveals that WGS can be used for source tracking of *P. aeruginosa* in a hospital setting, and that acquisitions can be traced to a specific source within a hospital ward.

Strengths and limitations of this study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *Pseudomonas aeruginosa* in a hospital setting.
- We show convincing evidence that transmission has occurred directly from water to patients, but other routes are as likely.
- The main limitation of the study was the sample size, which could be attributable to interventions being carried out during the study.
- Our study focused on a burns unit and critical care unit in a newly built hospital. Modes of *P. aeruginosa* transmission may be different in hospitals with different styles of plumbing and on other augmented care units.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium and an important opportunistic pathogen in the healthcare setting. *P. aeruginosa* particularly affects those with impaired host or mucosal immunity and has a broad range of presentations including respiratory infections in cystic fibrosis and mechanically ventilated patients, bloodstream infections in premature neonates and wounds in burns injuries. Nosocomial *P. aeruginosa* outbreaks are frequently reported and associated with water sources such as taps, showers, mixer valves and flow straighteners, sink traps and drains.[1–10] Other potential routes of transmission include cross-infection, for example, carriage on the hands of healthcare workers, and through contaminated medical equipment such as endoscopic devices.[3 5]

In the UK, the role of water in the transmission of *P. aeruginosa* in healthcare settings has become a matter of urgent concern in response to a recent high-profile outbreak

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affecting a neonatal critical care unit in Belfast in 2012.[11] This source was eventually determined to be sink taps.[11–13] National guidance is now in place detailing enhanced procedures for routine water sampling on augmented care units, with directed interventions such as disinfection and replacement of high-risk plumbing parts required.[14]

Historical phenotypic typing methods for *P. aeruginosa* such as O-antigen serotyping have more recently been replaced by molecular typing methods such as pulsed-field gel electrophoresis (PFGE), variable number tandem repeat analysis, random amplification of polymorphic DNA and multilocus sequencing typing (MLST).[15] These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.[4 16–18] However, such techniques have important limitations for source tracking of infections in hospitals as they sample limited numbers of sites in the genome which may result in false clustering of unrelated strains.[19] In the past 5 years, whole-genome sequencing (WGS) has started to be used to investigate outbreaks in hospitals. WGS is attractive because of its digital, sharable format and ultra-high resolution, which is able to discriminate two isolates differing by just a single mutation. WGS has been successfully used to determine likely transmission chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.[19–21] Benchtop sequencing instruments now offer a cost-effective approach for bringing bacterial WGS to the clinical environment.[22]

In this study, we explore the utility of WGS to determine the likely sources of *P. aeruginosa* in an at-risk population of burns patients. In the UK and US burns patients receive shower cart hydrotherapy as a mainstay of burns treatment.[23–26] A previous hospital audit suggested that up to one-third of such patients became colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may relate to transmission from hospital shower water during therapy. We therefore wished to understand the importance of transmission from water compared with alternative routes such as cross-infection and endogenous carriage.

MATERIALS AND METHODS

Hospital setting

An observational, prospective study design was employed in a burns centre serving approximately 13.7 million people across the Midlands region of England with 300 admissions annually. Opened in June 2010, the burns centre comprises a purpose built 15-bed ward with 11 side-rooms and 2 dual-bedded rooms. Patients requiring mechanical ventilation and organ support are usually treated in two self-contained burns cubicles located within the trauma critical care unit. Despite the observational nature of the study, sampling was carried out during implementation of interim national guidance on control of *P. aeruginosa* issued by the Department of Health. These guidelines were issued in draft form March 2012, and subsequently revised in March 2013. This meant that parallel water sampling and engineering interventions were being undertaken during the period of study. In addition, some enhanced infection prevention measures were also introduced in response to an outbreak of a multidrug-resistant *A. baumannii*.

Study design and patient selection

Patients admitted to the burns unit were eligible for the screening phase of the study if they had burns injuries covering greater than 7% total body surface area (TBSA). Patients were screened as soon as possible after admission after they had given written informed consent. When appropriate, legal consultee advice was sought for patients lacking capacity due to emergency treatment. On admission, recruited patients were screened for carriage of *P. aeruginosa* (wounds, urine and stool) using standard microbiology techniques. Samples were then taken as part of routine microbiology service during the patients stay. Environmental and water samples were taken after the patient was admitted to the burns centre. If during the period of stay *P. aeruginosa* was isolated from a patient sample the patient was recruited into the second phase of the study. In this phase, patients had wound swabs taken at each dressing change as well as twice-weekly urine samples. The patient's environment and water from outlets in their bed space were sampled weekly for the duration of their stay, and after discharge (post-cleaning). Termination of the study was planned after 30 screening patient admissions, or a year, whichever came soonest, after which 10 patients were expected to acquire *P. aeruginosa*. This prediction was based on a previous local audit which suggested about one-thirds of burns patients became colonised with *P. aeruginosa*.

Microbiological and molecular methods

P. aeruginosa isolates were obtained from wound swab, urine, stool, environmental and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide agar and incubation for 24 h at 37°C. Stool samples were cultured overnight in a cetrimide enrichment broth before subculture onto CLED. Identification was confirmed by resistance to C-390 and the VITEK 2 GN identification card. Antibiotic sensitivity assays were performed using the VITEK 2 AST N-210 card (bioMérieux, Basingstoke, UK).

The patient's environment (shower head rosette, drain, shower chair or trolley, bedside table, patient chair, instruments in contact with the patient) was sampled over a 10 cm^2 area by a Polywipe sponge. The sponge was placed in tryptic soy broth incubated for 24 h at 37°C then subcultured onto CLED and cetrimide agar. During water sampling, water was taken from the patient's shower, or tap if a shower was not present. Shower heads were not removed for water sampling. At

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least 200 mL of water was collected into a vessel containing sodium thiosulfate as a neutraliser. In duplicate, 100 mL of water was filtered through a 0.45 µ filter and the filters placed onto CLED plates and cetrimide agar. Plates were incubated at 37°C for 48 h and the number of organisms per 100 mL quantified.

For storage and DNA extraction a single colony was purified from the primary culture plate. When different colony morphologies were observed, a single colony from each type was purified. Additionally, for a randomly selected water sample, 24 colonies were individually picked from one water-filter primary microbiological plate for sequencing. Isolates were stored on Biobank beads at −20°C prior to DNA extraction. Organisms were resuscitated on CLED agar plates and genome DNA either extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight LB broth culture using a Qiagen Genomic-Tip 100G.

DNA extraction and sequencing

Genomic DNA was prepared from single colony picks using the MOBIO Ultraclean microbial kit (MOBIO, Carlsbad, USA). 1 ng input DNA, as quantified by Qubit (Life Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing using the Illumina Nextera XT DNA sample kit as per manufacturer's protocol (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a paired-end protocol resulting in read lengths between 150 and 300 bases. A single additional sample, isolate 910, was chosen as a representative member of Clade 5 for long-read sequencing. DNA from this sample was fragmented using a Hydroshear (Digilab, Marlborough, Massachusetts, USA) using the recommended protocol for 10 kb fragments and further size-selected on a BluePippin instrument (Sage Science, Massachusetts, USA) with a 7 kb minimum size cut-off. The library was sequenced on two SMRT Cells using the Pacific Biosciences RS II instrument at the Norwegian Sequencing Centre, Oslo. C4-P2 chemistry was chosen because it favours long, more accurate reads for *de novo* assembly.

Stool PCR

For simple presence/absence detection of *P. aeruginosa* in stool samples using PCR, a stool sample was collected into a stool collection tube containing stool DNA stabiliser. Total DNA was extracted using the PSP Spin Stool DNA Plus kit (Stratec Molecular). PCR amplification of species specific regions of the 16S rDNA gene was carried out using primers PA-SS-F: GGGGGATCTTCG GACCTCA and PA-SS-R: TCCTTAGAGTGCCCACCCG [12] in the following conditions: 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's using BIOTAQ DNA Polymerase and buffer set. After initial denaturation at 96°C for 2 min, 30 cycles of 96°C for 30 s, 62°C for 30 s and 72°C for 30 s were completed with a final extension of 72°C for 5 min. Products were visualised for size on an 1.5% agarose gel.

Bioinformatics methods

Illumina MiSeq reads from each isolate were adapter and quality trimmed before use with Trimmomatic.[27] Phylogenetic reconstruction of isolates sequenced in this study were combined with data from a global collection of 55 *P. aeruginosa* strains collected world-wide which have been previously analysed by Stewart *et al*.[28] For each of the published strains, 600 000 paired-end reads of length 250 bases were simulated using wgsim (https://github.com/lh3/wgsim) from the complete or draft genome assembly deposited in Genbank. Read sets were mapped against the *P. aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405 using default settings.[29] Single nucleotide polymorphisms were called using VarScan 2.3.6 and filtered for regions with an excessive number of variants. These may represent regions of recombination, misalignments or strong Darwinian selection.[30] FastTree (V2.1.7) was used for phylogenetic reconstruction. This software estimates an approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide evolution with a single rate for each site (CAT).[31] Trees were drawn in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

For *in silico* MLST prediction, trimmed reads were assembled de novo using Velvet [32] with a k-mer size of 81 and searched using nucleotide BLAST against the multilocus sequence database downloaded from the pubMLST website on 5 August 2013 (http://pubmlst.org/paeruginosa/).[33] For Clade E isolates, in order to exhaustively search for discriminatory mutations, a nearly complete reference genome was generated by de novo assembly using Pacific Biosciences sequencing data. Reads were assembled using the 'RS_HGAP_Assembly.3' pipeline within SMRT Portal V2.2.0. Illumina reads from the same sample were mapped to this draft genome assembly in order to correct remaining indel errors in the assembly using Pilon (http://www.broadinstitute.org/software/pilon/). Isolates belonging to each clade were mapped individually against either the PacBio reference (Clade E) or *P. aeruginosa* PAO1 (NC_002516; Clades C, D and G).

Variants (single nucleotide polymorphisms and short insertion-deletions) were called using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.[30] Non-informative positions and regions of putative recombination were removed, the later with a variant density filter of more than 3 SNPs every 1000 nucleotides. Analysing samples in each clade individually maximised the number of variants detected by reducing the likelihood of the position being uncovered by a subset of samples. From these variants fine-grained phylogenetic trees were reconstructed for each clade using FastTree. The scripts used to perform this analysis are available at http://www.github.com/joshquick/snp_calling_scripts. Approximate-maximum-likelihood phylogenetic trees were generated using FastTree and visualised in FigTree. For whole-genome shotgun metagenomics analysis, reads were analysed using the Kraken taxonomic classifier

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software with the supplied *minikraken* database.[34] Reads from the metagenomics data set were aligned to *P. aeruginosa* Clade E as in the previous section and phylogenetic placement was carried out using pplacer in conjunction with FastTree.[35] Sequence data is available from the European Nucleotide Archive for the Illumina data (ERP006056) and the corrected Pacific Biosciences assembly (ERP006058).

RESULTS

Study results

Recruitment lasted a period of 300 days, ending according to protocol after the enrolment of 30 screening patients. In total, we detected *P. aeruginosa* in five patients. Of these patients, three had *P. aeruginosa* detected only in burns wound swabs, one had *P. aeruginosa* detected in their burns wound and in their urine, and one had *P. aeruginosa* in their sputum. One additional eligible patient did not consent to enter the study and was excluded. The average age in the study group was 41 years. Males predominated with a male-to-female ration of 2.3:1. Flame burns were the most common mechanism of injury, followed by scalds and mixed flame/flash injuries. The average burn size of the study group was 12.5% of the TBSA and 27% of patients sustained an inhalation injury. Eight patients required admission to intensive trauma unit (ITU) and the majority required surgical treatment of their burns with excision and skin grafting (80%). A large majority of the study group (83%) received shower cart hydrotherapy as a routine part of their wound management to encourage healing through wound debridement and decontamination. The average length of hospital stay (LOS) was 17 days and taking into account burn size, the average was 1.4 days per % TBSA.

The water and environment in burns and critical care units are frequently colonised by *P. aeruginosa*

A total of 282 water and environmental samples were screened for *P. aeruginosa* of which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from the wet environment and 7/108 (6%) were positive from the dry environment. A total of 86 genome sequences were generated from the 71 positives, as in some cases multiple colony picks were sequenced. Seventy-eight patient samples were screened for *P. aeruginosa* of which 39 (50%) were positive. A total of 55 genome sequences were generated, as in some cases multiple colony picks were sequenced. In total, 141 genomes were sequenced; water and environmental (n=86) and patient (n=55). Genomes were sequenced to a mean coverage of 24.4×, with the minimum coverage of a sample being 14× and highest 64.7×.

When placed in the context of a global collection of *P. aeruginosa* strains, phylogenetic reconstruction demonstrated isolates in our study fell into eight clades (figure 1A). As has been reported previously, there was no strong association between ecological context and position in the phylogenetic tree.[28] Isolates in this study are most closely related to strains from a variety of settings. The majority of isolates (52%) belong to Clade E (figure 1B), whose nearest sequenced relative is the Liverpool Epidemic Strain, a clone often isolated from patients in the UK and Canada with cystic fibrosis.[36 37] Isolates from Clade E were found in the burns unit's water and the ward environment, as well as from two patient's wounds. However it was never detected in the critical care unit. Clade E was detected throughout the study in a total of 10 different rooms (figure 2).

Inferring potential transmission events by WGS

Microevolutionary changes occurring over rapid timescales (ie, days to months) have been used to detect potential chains of transmission in hospital and community outbreaks.[19–21 38 39] The number of distinct mutations between given isolates has been used to infer whether transmission events are likely to have occurred. Such inferences are aided by prior knowledge of mutation rates in similar populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from Clade E isolated from their wounds. These isolates had an indistinguishable genotype from those present in water and the environment of the room they were nursed within (figures 1C and 3). This genotype was detected in the patient's shower water after initial patient screening, during screening of the second patient admission, twice during the second patient's stay and then 127 days later (days 27, 65, 89 and 216, respectively). When water isolates were positive, the genotype was also detected in wet environment sites (shower drain, shower rosette and patient's trolley) on the same days.

Patient 5 was nursed on the critical care unit due to concomitant medical problems. *P. aeruginosa* belonging to Clade G was isolated from sputum during this time. Identical genotypes were detected contemporaneously in the water from the associated sink and sink tap handle (see online supplementary appendix 4).

Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from these patients belonged to Clade C and D, respectively. Neither clade was ever isolated from hospital water. In both cases, identical genotypes were detectable in the environment associated with the patient but these were not detected before or after the patients' stay, indicating that the environment was not persistently contaminated. During the course of patient 3's stay, the dry environment such as the bedside table was contaminated, as was the patient's door handle and shower chair. However, after patient discharge, the strain associated with this patient was never seen again during the course of the study in any location.

WGS permits source tracking of *P. aeruginosa* to individual water outlets

WGS has been reported previously for source tracking, but never for the detection of transmission events from

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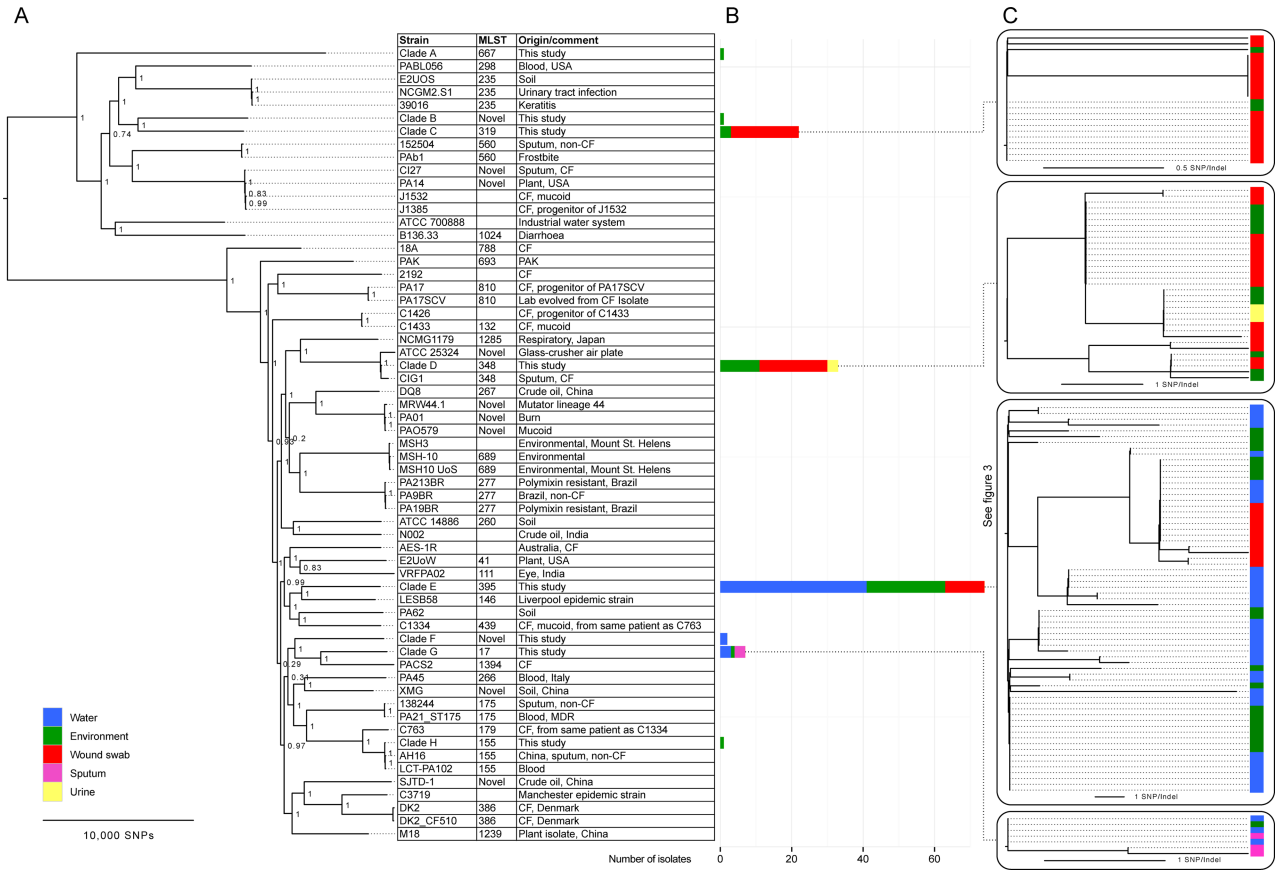

Figure 1 An overview of all samples collected during the study in global phylogenetic context with other sequenced strains of *Pseudomonas aeruginosa* from the set of Stewart *et al*.[28] Samples collected in this study are widely dispersed in the tree, which contains isolates from different environments (A). Bar plots indicate the numbers of each type of sample collected (B). Microdiversity within each clade is shown, with the colour bar indicating the source of each sample (C).

hospital water.[40] Phylogenetic reconstruction within Clade E, the most commonly detected water clone demonstrated additional diversity within this clone, with a total of 46 mutations detected an average genetic distance between isolates of 4.1 mutations (figure 3). The reconstruction demonstrated clear evidence of clustering of genotypes both by room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment (eg, shower rosettes and drains) these genotypes were most often identical to those found in water, indicating that the water was likely the ultimate source of that clone. Genotypic variation was seen between outlets within the same room. For example, tap water sampled from room 11 had a distinct genotype from that sampled from shower water in the same room and this was consistently found over multiple samplings. Notably, isolates from two patients fell within the cluster originating from shower water, indicating that shower hydrotherapy was the most likely source of infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this study set, which both demonstrated geographical clustering, with pBURNS1 only being detectable in isolates from room 8 and pBURNS2 only being detectable in isolates from the shower water in room 9.

Rapid evolution of antibiotic resistance associated with treatment

P. aeruginosa is commonly associated with antibiotic resistance due to a number of predisposing features including intrinsic resistance, a repertoire of efflux pumps and antibiotic-inactivating enzymes including β-lactamases.[41] Three infected patients (2, 3 and 5) received antibiotic therapy, and in each case this was associated with the development of resistance to at least one therapeutic agent. Associated mutations were detected that were either partially or fully explanatory of the phenotype (online supplementary appendix 12).

Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online supplementary appendix 11 for full details). Eight of 21 (38%) tested isolates from this patient were ciprofloxacin resistant. Seven of eight isolates (88%) of the ciprofloxacin-resistant strains were distinguishable from the other isolates by a single SNP in *mexS* (annotated as PA2491 in *P. aeruginosa* PAO1; see online supplementary appendix 1 and 7). This SNP was predicted to result in a non-synonymous amino acid substitution. Disruption of this gene has been shown to cause increased expression of the mexEF-oprN multidrug efflux pump, associated with resistance to quinolones.[42]

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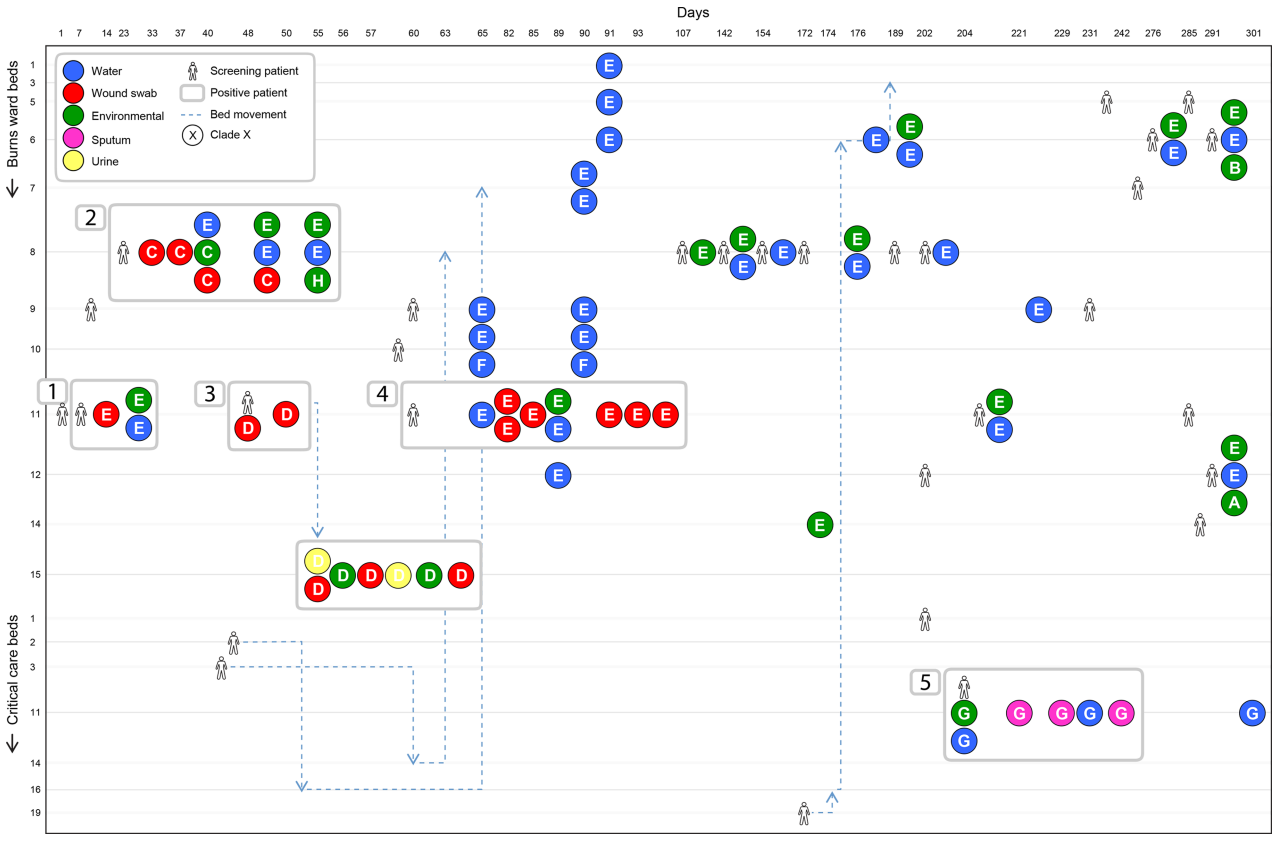

Figure 2 A schematic view of the 300-day study of *Pseudomonas aeruginosa* in a burns centre and critical care unit. Time in days is shown along the x axis with bed numbers in the critical care unit and burns unit along the y axis. Each circular icon indicates a positive isolate of *P. aeruginosa*. The icon's logotype indicates which environment it originated from (wound, urine/sputum, environment or water). The filled colour of the icon indicates the clade it belongs to. Patient icons represent the enrolment of a screening patient into the study and their location. Patient movements around the hospital are noted by dotted lines. The five patients infected with *P. aeruginosa* are denoted by rounded boxes. Boxes are coloured according to the patient number. In the event two or more isolates of the same source and clade were collected on the same day, these have been collapsed into a single circular icon.

Patient 3 was not treated with antibiotics, but isolates associated with this patient demonstrated differences in resistance to timentin and piperacillin-tazobactam. These changes were associated with non-synonymous mutations in *gacA*, the response regulator of the GacA/GacS two-component system and in *lasR*, a transcriptional activator required for transcription of elastase and LasA protease (online supplementary appendices 2 and 8).

Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and colistin. Five isolates collected 10–18 days after initiation of meropenem showed resistance to imipenem and intermediate resistance to meropenem (see online supplementary appendix 3 and 9). The most likely mutation responsible for this phenotype was detectable in two isolates, both of which had a frame-shift mutation in the gene coding for the membrane porin OprD.[43]

Patient 5 had a prolonged stay in ITU and had multiple medical problems including *A. baumannii* infection and was treated with nine antibiotic agents including ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this patient demonstrated the stepwise acquisition of two mutations (online supplementary appendix 4). The first was in *nalC*, a probable repressor of the TetR/AcrR family (online supplementary appendix 10).[44] On inspection of the sequence alignment in this region, a large deletion of 196 nucleotide bases was seen compared to the reference PAO1 strain. This mutation was seen in association with full resistance to piperacillin-tazobactam, ceftazidine, aztreonam, meropenem and intermediate resistance to ciprofloxacin. This deletion is likely to result in over-expression of efflux pumps involving the *mexAB-oprM* operon.[44 45] Ciprofloxacin resistance in a later isolate corresponded to the stepwise acquisition of a second mutation. This mutation is predicted to affect the well-studied DNA gyrase subunit A gene (*gyrA*) which is strongly associated with ciprofloxacin resistance.[46]

Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome metagenomic shotgun sequencing

P. aeruginosa is able to produce and survive in biofilms. Plumbing parts such as flow straighteners, shower rosettes,

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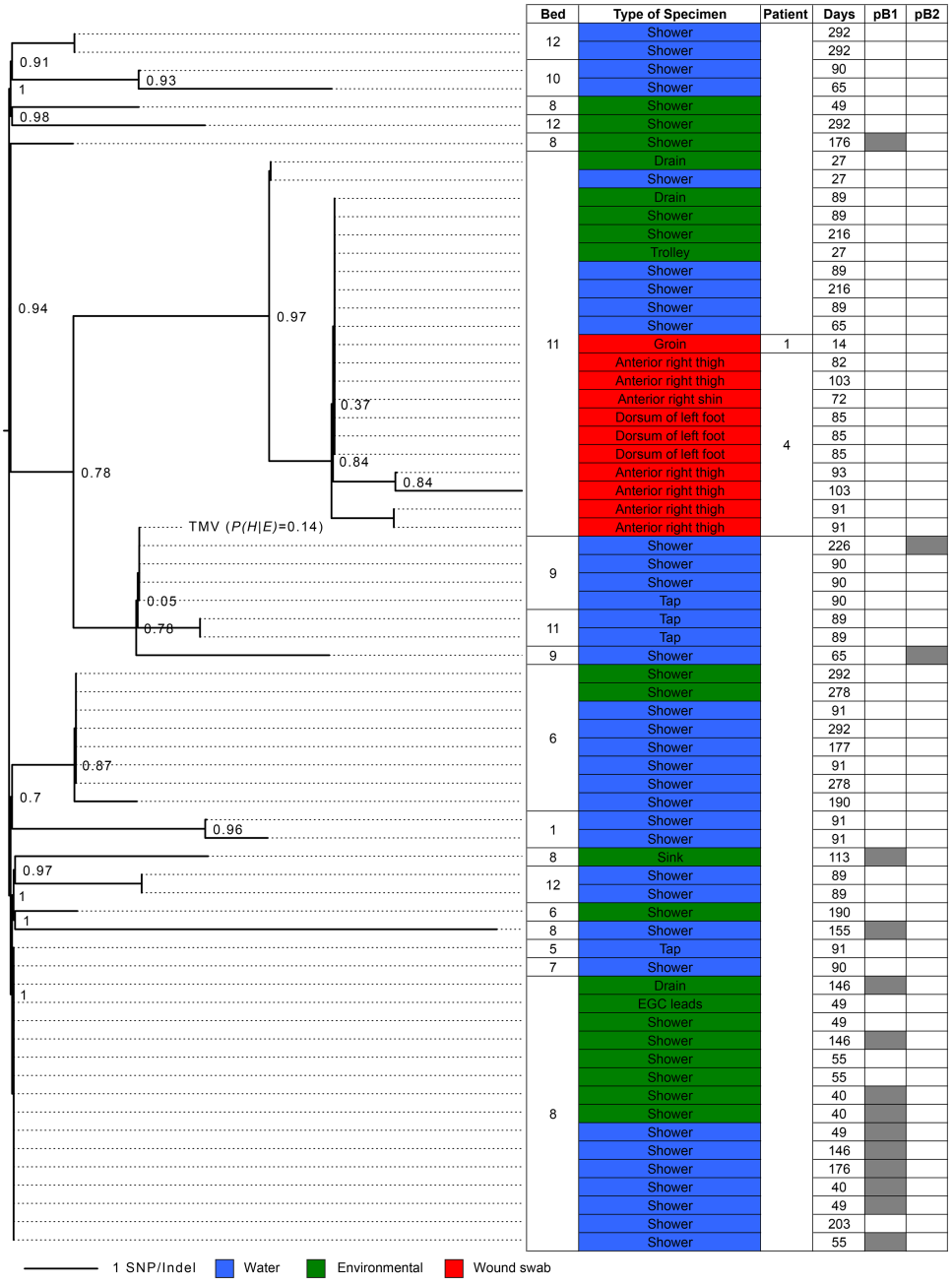

Bed	Type of Specimen	Patient	Days	pB1	pB2
12	Shower		292		
	Shower		292		
10	Shower		90		
	Shower		65		
8	Shower		49		
12	Shower		292		
8	Shower		176		
11	Drain		27		
	Shower		27		
	Drain		89		
	Shower		89		
	Shower		216		
	Trolley		27		
	Shower		89		
	Shower		216		
	Shower		89		
	Shower		65		
	Groin	1	14		
	Anterior right thigh	4	82		
	Anterior right thigh		103		
	Anterior right shin		72		
	Dorsum of left foot		85		
	Dorsum of left foot		85		
	Dorsum of left foot		85		
	Anterior right thigh		93		
	Anterior right thigh		103		
	Anterior right thigh		91		
	Anterior right thigh		91		
9	Shower		226		
	Shower		90		
	Shower		90		
	Tap		90		
11	Tap		89		
	Tap		89		
9	Shower		65		
6	Shower		292		
	Shower		278		
	Shower		91		
	Shower		292		
	Shower		177		
	Shower		91		
	Shower		278		
	Shower		190		
1	Shower		91		
	Shower		91		
8	Sink		113		
12	Shower		89		
	Shower		89		
6	Shower		190		
8	Shower		155		
5	Tap		91		
7	Shower		90		
8	Drain		146		
	EGC leads		49		
	Shower		49		
	Shower		146		
	Shower		55		
	Shower		55		
	Shower		40		
	Shower		40		
	Shower		49		
	Shower		146		
	Shower		176		
	Shower		40		
	Shower		49		
	Shower		203		
	Shower		55		

Figure 3 The high-resolution phylogenetic reconstruction of Clade E isolates. This demonstrates the clustering of genotypes by bed space. Patient associated samples are contained within a room 11 clade. This clade contains water samples from the shower and environmental samples from the shower, drain and trolley. The water samples from the room 11 tap are in a distinct clade, indicating the biofilm within the tap has a distinct genotype to the shower. This suggests environmental contamination was more likely to arise from contaminated shower water than tap water. Details of sampling site, days since start of study and presence of pBURNS plasmids are also shown. The likely phylogenetic position of *Pseudomonas aeruginosa* detected in a biofilm from a thermostatic mixer valve is shown in the clade associated with room 9 and indicated 'TMV'.

flexible hoses, solenoid valves and thermostatic mixer valves (TMV) are particularly at risk of biofilm formation due to factors including surface areas, convoluted designs and inadequate pasteurisation.[47] To confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the burns unit, we obtained a TMV removed by the hospital estates team from the shower in room nine as part of compliance with UK guidelines for managing *P. aeruginosa* in hospitals. On visual inspection, a biofilm was present which was scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted for whole-genome shotgun sequencing. The majority of reads did not map to any known bacterial taxa. The most abundant taxon identified was *P. aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* Clade E reference covered 94% of the 6.3 million base reference genome at a median coverage of

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5×, confirming that reads were correctly classified to this species and not other environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* Clade E reference genome followed by phylogenetic placement of reads demonstrated that it fell into the same clade as previously recovered isolates from the shower or tap in room 9 (indicated on figure 3, and in online supplementary appendix 6).

DISCUSSION

The hospital environment has been intimately linked with *P. aeruginosa* infection for over 50 years yet hospital acquisitions, clusters and outbreaks remain a common occurrence and understanding precise routes of transmission can be difficult.[47 48] Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa* genotypes recovered from patients to specific individual water outlets, we offer compelling evidence of unidirectional transmission from water to patients. Further, by sequencing of a biofilm identified in a TMV from a hospital water system, we can identify the likely common source of genotypes found in water and in the hospital environment.

Our results suggest that use of WGS can reduce ambiguity about potential transmission events in hospitals and consequently inform infection prevention efforts about the direction and sequence of transmission. Typing schemes such as MLST and PFGE are much lower resolution methods and would not be able to provide sufficient information to permit such inferences to be made. It is notable that the burns unit was colonised by a single clone, meaning that it was very unlikely that water outlets at each bed space were colonised as a result of transmissions from the patient or environment. For this to happen would require multiple transmission events from separate patients with the same clone, for which there is no evidence. Instead we speculate that this clone was introduced to the hospital associated with its commissioning. One hypothesis is that particular plumbing fittings, that is, the TMV may have been colonised simultaneously by a clone circulating in water. Clade E (ST395) has been frequently reported associated with water, so this remains a possibility. [49 50] However, it is possible that plumbing fittings are installed 'pre-seeded' with *P. aeruginosa* as has already been proposed by Kelsey.[3 5 47] Investigation of an outbreak in Wales implicated new plumbing parts as a potential source of *P. aeruginosa*. New plumbing components are often tested by companies prior to their supply and it is possible they were contaminated prior to distribution. The limited amount of diversity (average 4 SNPs) seen within this clade is consistent with a single founding genotype coinciding with the opening of the burns unit, based on estimates from a previous study using WGS which reported that mutations accumulate at a rate of approximately one every 3–4 months in a hospital-associated clone.[51] However, our results suggest that our isolates accumulate mutations even more slowly. This may be due to reduced growth rates in nutritionally-poor biofilms.[52]

It is notable that antibiotic resistance to multiple first-line agents developed rapidly in response to therapy. These results underline the importance of selecting appropriate antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic resistance genotypes selected *in vivo* did not show evidence of persistence in the ward environment or transmission to other patients.

Our study has certain limitations. Based on a previous audit, we expected around one-third of patients screened for *P. aeruginosa* would develop colonisation or clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have been related to guidance and engineering interventions being put in place during the study as detailed in national guidance issued while this study was on-going. In addition, infection control policies were revised to address control of an outbreak of a multidrug resistant *A. baumannii* in this same burns unit. Following these interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa* which may demonstrate the importance of national guidance in reducing transmissions.

By focusing on burns patients who receive hydrotherapy, our study population were at extremely high risk of waterborne infection. In other patient groups it may be that alternative routes of transmission including cross-infection or endogenous carriage play a more important role. Our results suggest that our burns unit is endemically colonised with a distinct clone of *P. aeruginosa* that may have been imported coinciding with the opening of the hospital. Other intensive care units, particularly those which have been open for longer may harbour a greater diversity of *P. aeruginosa* as a result of increased opportunities for clones to be imported.

One potential application for WGS in infection control would be to determine whether cases are as a result of water transmission, or represent sporadic clones originating from the wider environment. Despite improved guidance concerning improved engineering infection control practices and the introduction of the water safety group in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In augmented care units such as ITUs, burns units and neonatal wards where *P. aeruginosa* poses a significant risk to vulnerable patients, the increased resolution offered by WGS will justify its use, particularly as the costs continue to fall.

In conclusion, we have identified through WGS clear evidence for transmission of *P. aeruginosa* from specific water outlets to burns patients and offer a forensic-level framework for dealing with outbreaks linked to hospital water. We expect WGS will continue to make inroads into clinical microbiology and become a vital tool for tracking *P. aeruginosa* in the hospital environment, helping inform targeted control measures to help protect patients at risk of infection.

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Contributors MJP, NSM and BO conceived the study. CMW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC, CC and MN processed samples and performed microbiology. NC, CC and JQ did sequencing. JQ, NC, CMT and NJL analysed the data. NJL, NC, JQ, MJP and BO wrote the paper. All authors commented on the manuscript draft.

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Competing interests None.

Ethics approval The study protocol received approval from National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Pacific Biosciences raw data files are available from the corresponding author (Nicholas J Loman, n.j.loman[redacted].

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SYSTEMATIC REVIEW Open Access

Status and potential of bacterial genomics for public health practice: a scoping review

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Abstract

Background: Next-generation sequencing (NGS) is increasingly being translated into routine public health practice, affecting the surveillance and control of many pathogens. The purpose of this scoping review is to identify and characterize the recent literature concerning the application of bacterial pathogen genomics for public health practice and to assess the added value, challenges, and needs related to its implementation from an epidemiologist's perspective.

Methods: In this scoping review, a systematic PubMed search with forward and backward snowballing was performed to identify manuscripts in English published between January 2015 and September 2018. Included studies had to describe the application of NGS on bacterial isolates within a public health setting. The studied pathogen, year of publication, country, number of isolates, sampling fraction, setting, public health application, study aim, level of implementation, time orientation of the NGS analyses, and key findings were extracted from each study. Due to a large heterogeneity of settings, applications, pathogens, and study measurements, a descriptive narrative synthesis of the eligible studies was performed.

Results: Out of the 275 included articles, 164 were outbreak investigations, 70 focused on strategy-oriented surveillance, and 41 on control-oriented surveillance. Main applications included the use of whole-genome sequencing (WGS) data for (1) source tracing, (2) early outbreak detection, (3) unraveling transmission dynamics, (4) monitoring drug resistance, (5) detecting cross-border transmission events, (6) identifying the emergence of strains with enhanced virulence or zoonotic potential, and (7) assessing the impact of prevention and control programs. The superior resolution over conventional typing methods to infer transmission routes was reported as an added value, as well as the ability to simultaneously characterize the resistome and virulome of the studied pathogen. However, the full potential of pathogen genomics can only be reached through its integration with high-quality contextual data.

Conclusions: For several pathogens, it is time for a shift from proof-of-concept studies to routine use of WGS during outbreak investigations and surveillance activities. However, some implementation challenges from the epidemiologist's perspective remain, such as data integration, quality of contextual data, sampling strategies, and meaningful interpretations. Interdisciplinary, inter-sectoral, and international collaborations are key for an appropriate genomics-informed surveillance.

Keywords: Public health practice, Bacterial infections, Next-generation sequencing, Whole-genome sequencing, Genomics, Epidemiology, Scoping review

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Introduction

The advent and continuous improvement of sequencing technologies, especially the shift to next-generation sequencing (NGS), provides many opportunities for the management of infectious diseases. Sequence information can identify a pathogen and its specific characteristics, as well as its relatedness to other pathogens. Compared to Sanger sequencing, NGS technologies allow a faster and cheaper way to sequence large amounts of nucleotides. As such, NGS can be viewed as a tool that makes whole-genome sequencing (WGS) accessible [1]. In contrast to genotyping, where only small parts of the genome are assessed, WGS provides characteristics of the entire genome of the infectious isolates, thereby combining maximal strain discrimination and the ability to link the genotype with clinically and epidemiologically relevant phenotypes [2, 3]. Sequence variations, such as single-nucleotide polymorphisms (SNPs), insertions/deletions, and accessory genes can be identified following bioinformatics analyses [1]. Highly discriminatory subtyping following WGS is accomplished based on either SNPs or allelic variation [5]. With decreasing costs and increasing laboratory and bioinformatics capacities, we are currently transitioning to genomic epidemiology, as whole pathogen genomes are available at the level of the population [3]. Adding genomic data to epidemiological analyses of infectious diseases greatly benefits disease prevention and control [1, 2]. During the last decade, NGS is no longer limited to research settings and is being rapidly translated into public health practice [4, 5].

This review focuses on the applications of NGS to the population-level management of bacterial infections (Fig. 1). This includes the use of WGS to study the relatedness of isolates in order to understand transmission dynamics, to detect and control outbreaks, to monitor trends, and to identify the emergence of new threats. More specifically, this review discusses the applications of pathogen genomics that lead to actionable results from a public health point of view.

Public health activities related to infectious diseases can be classified as outbreak investigations, control-oriented surveillance, and strategy-oriented surveillance [3, 6]. The main objective of an outbreak investigation is

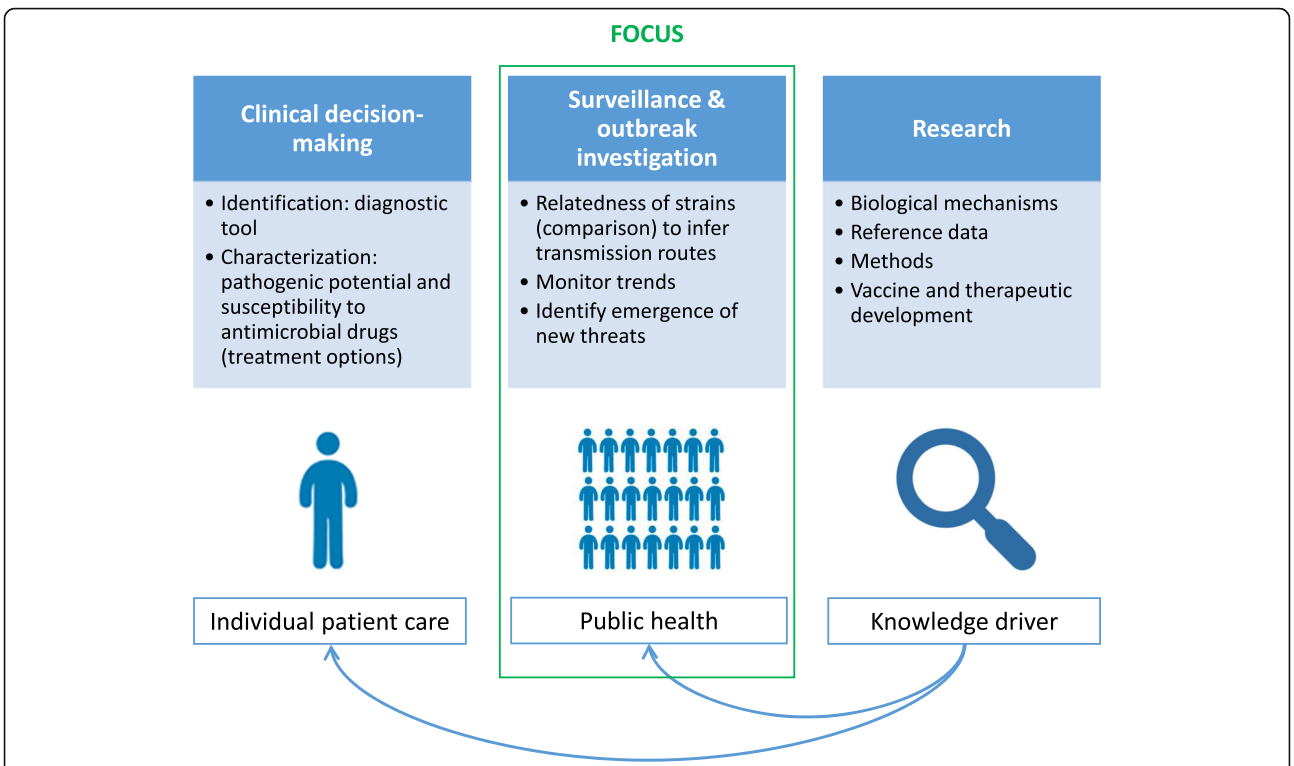

Fig. 1 Focus of the scoping review on pathogen genomics for public health practice. Different domains in the field of infectious diseases require access to the same pathogen genomic data. Whole-genome sequencing (WGS) has the ability to inform and improve individual patient care, by identifying the species, determining its pathogenic potential, and testing its susceptibility to antimicrobial drugs. WGS also provides data for public health surveillance about the relatedness of the pathogen to other strains to investigate transmission routes, monitor trends over time, and allow the identification and control of outbreaks and new threats. Research is a knowledge driver providing reference data, methods, and a deeper understanding about the underlying biological mechanisms to the other domains. The focus of this scoping review is on the use of WGS as a public health tool, i.e., at the level of the population

to investigate the possible source(s) of infection and to implement effective and appropriate control measures to stop its further spread. Outbreak investigations are often hypothesis-driven and a reaction to a sudden increase in the number of cases [7]. In contrast, surveillance is the systematic collection, analysis, and dissemination of data for the planning, implementation, and evaluation of public health programs [8]. Baker et al. [6] differentiate between control-oriented and strategy-oriented surveillance, thereby providing a meaningful way to categorize the applications of molecular/genomic tools for disease surveillance [9]. This framework has also been adopted in the European Centre for Disease Prevention and Control (ECDC) roadmap for integration of molecular and genomic typing into European level surveillance and epidemic preparedness [10]. As defined by Baker et al. [6], the purpose of control-oriented surveillance is "to identify each occurrence of a particular disease, hazard, or other health-related event that requires a specific response, and to support the delivery of an effective intervention". For example, control-oriented surveillance aims at the detection of outbreaks that require a specific response. Early outbreak detection can be achieved by prospectively genotyping as many consecutive cases in a population as possible to identify clusters of clonally linked isolates [3]. Baker et al. state that strategy-oriented surveillance aims "to provide information to support prevention strategies to reduce population risk" [6]. The aim is often to monitor long-term changes in epidemiology over larger geographic and population scales, requiring study designs that have a high degree of representativeness [9]. Strategy-oriented surveillance can for example detect the emergence of strains with enhanced virulence or drug resistance, help to identify risk factors associated with the transmission of specific strains, or predict the effectiveness of control programs such as vaccination campaigns [3].

Collective experience on the use of pathogen genomics for routine public health practice is spread across literature. WGS has been frequently used to aid outbreak investigations and routine surveillance at various levels (i.e., local, national, and international) and in different temporal scenarios (i.e., retrospective and prospective). The aim of this scoping review is to identify and characterize the recent literature concerning the application of NGS for public health practice, by (1) conducting a systematic search of the published literature, (2) mapping the characteristics of the identified studies, (3) describing the range of applications identified, and (4) assessing the added value, challenges, and requirements related to its implementation. The purpose is to provide an epidemiologist's perspective on the use of bacterial pathogen genomics in a public health context to complement previous reviews that focused on technical aspects, bioinformatics, diagnostics, or microbiology (i.e., the perspective of microbiologists, bioinformaticians, and clinicians) [2, 5, 11–16]. This review aims to summarize the experience gained and use it to further advance the implementation of pathogen genomics in routine public health.

Methods

A scoping review methodology was chosen to provide an overview of the nature and extent of the literature on this topic via systematically searching, selecting, and summarizing evidence, rather than a traditional systematic review that often focuses on specific outcomes [17]. This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [see Additional file 1] [18], adapted for use in a scoping review as appropriate, and adhering to the methodology outlined in The Joanna Briggs Institute Manual for Scoping reviews [19]. In addition, the framework outlined by Arksey and O'Malley [20] in their methodological paper on scoping reviews was followed. A scoping review protocol was developed a priori to ensure reproducibility and transparency of the review methods [see Additional file 2].

Eligibility criteria

The inclusion criteria were organized following the PCC (Population, Concept, and Context) elements. Studies had to include at least 2 individuals with a bacterial infection, and NGS had to be applied on the bacterial isolates. Consequently, non-human studies and case reports involving only one patient were excluded, as well as studies focusing on the host genome. Studies had to describe the application of NGS from a public health perspective (i.e., population-level). Therefore, the main study aims had to be within the context of an outbreak investigation, control-oriented surveillance, or strategy-oriented surveillance. Studies focusing on technical aspects, applying NGS solely for individual patient care, and using NGS primarily for research purposes were excluded. Further, only studies applying NGS within a real-life public health setting, as opposed to an experimental setting, and producing an output that can be directly translated into actionable results to benefit public health, were included. This also included proof-of-concept studies mimicking real-life public health situations. Studies published between January 2015 and September 2018 were included to consider the most current activities in this fast-evolving field. A full list of inclusion and exclusion criteria and a decision tree for study selection is provided in the additional material [see Additional files 3 and 4].

Searching

The PubMed search engine was used to identify manuscripts in English published between 1/1/2015 and 4/9/2018. In addition, reference lists of included studies and other reviews were examined (i.e., backward snowballing). Also, forward snowballing was performed by identifying relevant documents that cited the included studies, using the Google Scholar search engine.

Three domains were included in the search using the PubMed search engine: "bacterial infections," "next generation sequencing," and "public health." Each domain had several search terms. Free text search and MeSH term search were combined. The search was pre-tested to determine the most effective balance of sensitivity and specificity in the identification of potentially relevant citations. The ability of the electronic search to capture all relevant primary research was verified by handsearching reference lists from other reviews on the topic. The final search string is reported in the additional material [see Additional file 5]. The initial search was conducted on March 24, 2018, and was updated on September 4, 2018, selecting the date range "March 1, 2018, to September 4, 2018."

Screening

A first screening phase based on titles and abstracts was conducted [NVG], and out-of-topic studies were excluded. A second screening stage based on the full texts was conducted in duplicate by two independent reviewers [NVG, TD] using a standardized eligibility form. If no consensus could be reached between the two reviewers, a third reviewer [NB] helped to resolve the disagreement.

Data extraction

Data extraction was performed by [NVG] using an extraction form that was designed for the purpose of this review through an iterative process [see Additional file 6]. Information regarding the studied pathogen, country, year of publication, number of isolates, sampling fraction and time orientation of the NGS analyses, setting, public health application, study aim(s), and level of implementation was extracted from each included study. In addition, key findings related to the use of NGS were summarized for every study.

Data synthesis

The main characteristics were summarized in tabular form (as per data extraction pro-forma as well as a numerical summary), with an accompanying narrative summary, based on the key findings extracted from every study, describing how the results relate to the review objective and question. The studies were categorized based on the public health application and study aim in order to structure the narrative summary. For the study aim, multiple classifications per article were allowed.

Results

Search results

The study selection process is summarized as a PRISMA flow diagram in Fig. 2. A total of 1549 studies were identified through the initial database search, hand searching, reference checking, and other reviews. The search was updated in September 2018, and an additional 142 articles were identified (of which 19 were included). A total of 275 studies were included in the review.

Study characteristics

Out of the 275 included articles, 164 (60%) were outbreak investigations, 70 (25%) focused on strategy-oriented surveillance, and 41 (15%) on control-oriented surveillance. Almost all studies (274 out of 275) applied NGS technologies in the context of WGS, which will consequently be the focus for the remainder of this review. Table 1 gives an overview of the general characteristics of the included studies. Table 2 gives a deeper insight into the different study aims. The completed extraction form describing the characteristics of all included studies is available in the additional material [see Additional file 7].

Outbreak investigations of food- and waterborne pathogens mainly focused on source tracing ($n = 78$, 48%), in order to identify and eliminate the source as quickly as possible. In case of person-to-person transmission, the outbreak investigations focused on understanding transmission dynamics and reveal the spread of the pathogen in the population, in order to interrupt transmission chains and to prevent its further spread ($n = 85$, 52%). Eleven studies (7%) reported the use of WGS to provide feedback on key phenotypic attributes, such as virulence genes or antibiotic resistance, in order to inform outbreak management. The majority of the outbreak investigations were performed retrospectively ($n = 97$, 59%), i.e., as a proof-of-concept and/or to improve future preparedness by addressing a specific public health problem from the past. Fifty-eight studies (35%) applied WGS in quasi-real time, i.e., directly impacting the ongoing outbreak. In the majority of outbreak investigations, WGS was used on a subset of available samples ($n = 107$, 65%), for example, to further differentiate between isolates assigned to the same subtype as identified by conventional characterization methods. Outbreak investigations using WGS were mainly applied to *Staphylococcus aureus* ($n = 12$) and multidrug-resistant (MDR) Gram-negative bacteria ($n = 27$) in a hospital setting, to *Salmonella* spp. ($n = 33$) and *Listeria monocytogenes* ($n = 13$) during foodborne outbreaks, and to *Mycobacterium tuberculosis* ($n = 14$).

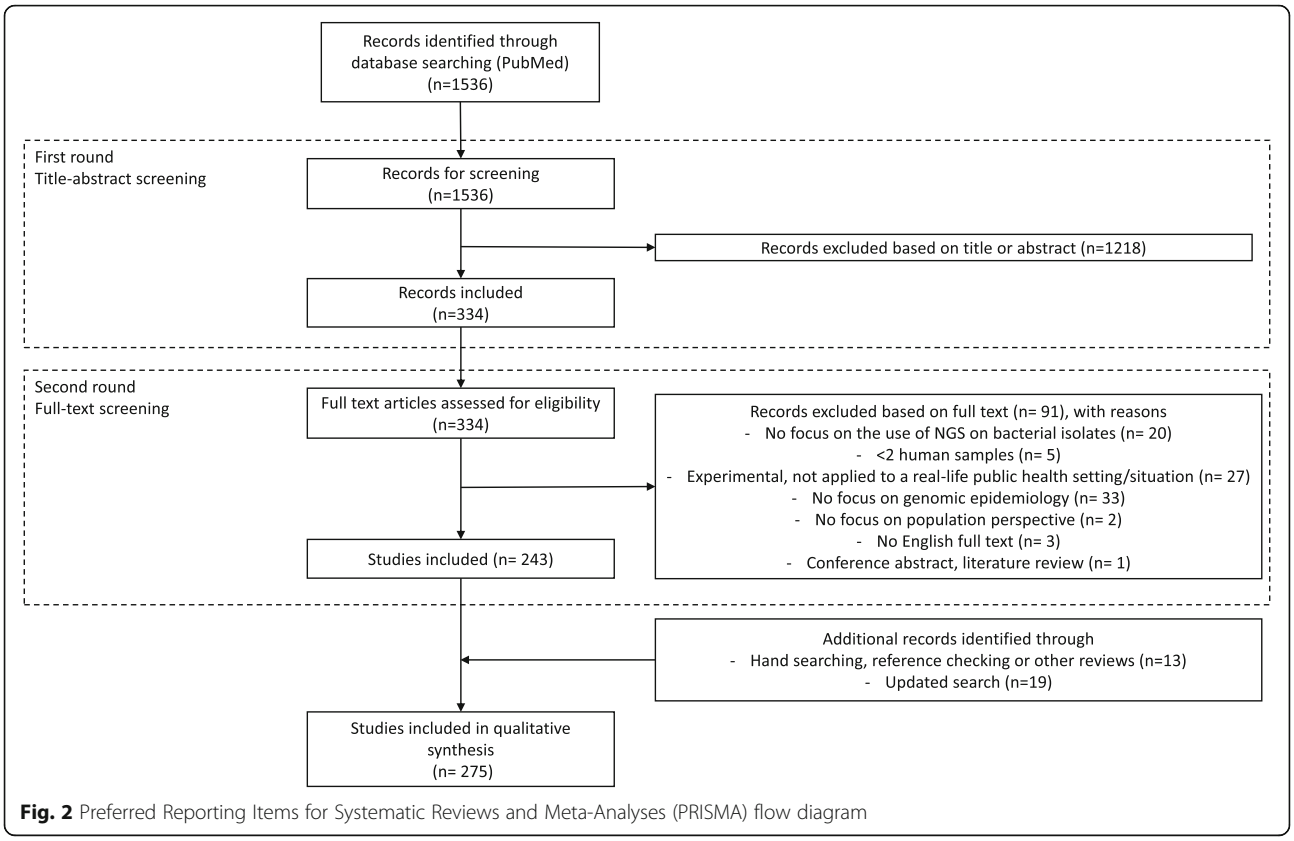

Fig. 2 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram

Classifying studies as control-oriented was mainly based on the fact that these studies aimed to detect events that require immediate action (e.g., early outbreak detection) or that the study was initiated following a specific public health problem. They were performed retrospectively ($n = 18$, 44%), i.e., as a proof-of-concept, and/or prospectively ($n = 25$, 61%). Six studies (15%) reported a nation-wide implementation of prospective genotyping into routine public health practices.

Strategy-oriented studies are in general conducted over larger time periods and geographical areas, in order to better understand the behavior of a certain pathogen within a population, and to plan future prevention and control programs. Twenty-nine studies (41%) applied WGS to assess the impact of prevention and control programs, mostly to evaluate vaccination programs. Genomic-informed strategy-oriented surveillance has also been frequently applied to monitor long-term changes over a larger geographic and population scale ($n = 38$, 54%) to detect the emergence of strains with enhanced virulence, to monitor drug resistance, to detect cross-border transmission events, or to identify zoonotic pathogens. Studies describing the use of WGS for strategy-oriented surveillance were often performed retrospectively ($n = 59$, 84%) on a historical subset of samples in order to answer a specific public health question.

For control- and strategy-oriented surveillance activities, WGS was mainly applied to *S. aureus* ($n = 23$), MDR Gram-negative bacteria ($n = 16$), *Neisseria meningitidis* ($n = 14$), *Salmonella* spp. ($n = 13$), and *M. tuberculosis* ($n = 9$).

Results of individual studies

Outbreak investigations

WGS provides increased resolution for case ascertainment and linking possible sources to these cases during outbreak investigations of food- and waterborne pathogens compared to conventional typing methods where only a small fraction of the genome is used (e.g., pulsed-field gel electrophoresis [PFGE], multiple-locus variable-number tandem-repeat analysis [MLVA], and multi-locus sequence typing [MLST]). The discriminatory power of WGS allows heterogeneous clusters of isolates, often indistinguishable with these previously used typing methods, to be split up into smaller groups of cases that are more likely to originate from a common source [21–31]. The increased resolution of WGS is particularly useful for clonal pathogens or serotypes that show little genetic variation [32–35]. Investigations applying WGS during the course of an outbreak were able to identify the likely source of infection and rapidly implement control measures to stop further spread [36–42]. On

Table 1 Characteristics of included studies (Jan 2015 to Sep 2018: $n = 275$)

Study characteristic	No. of studies (Jan 2015 to Sep 2018)		
	Outbreak investigations ($n = 164$)	Control-oriented surveillance ($n = 41$)	Strategy-oriented surveillance ($n = 70$)
Country[¥],*			
USA and Canada	42	7	16
UK and Ireland	33	13	15
Australia and New Zealand	19	3	7
Germany	14	2	4
Denmark	9	4	0
France	5	2	2
Setting[¥]			
Community	94	23	47
Institutional (hospital, school, nursery, etc.)	73	21	25
Time orientation of NGS analyses[¥]			
Retrospective	97	18	59
Quasi-real time	58	0	0
Prospective	11	25	11
Level of implementation of NGS analyses[¥]			
Proof-of-concept	57	27	8
Used to address a specific public health problem	100	10	61
Implemented into routine public health	14	6	2
Sampling fraction of NGS analyses			
All available samples	57	25	21
Subset of available samples (complementary)	107	16	48
Pathogens[¥]			
Staphylococcus aureus	15	10	13
Multidrug-resistant Gram-negative bacteria	27	4	12
Vancomycin-resistant Enterococci (VRE)	5	6	0
Clostridium difficile	5	1	2
Streptococcus spp.	8	0	6
Listeria monocytogenes	13	4	1
Shigella spp.	1	2	2
Salmonella spp.	33	5	8
Shiga toxin-producing *Escherichia coli* (STEC)	10	4	1
Campylobacter jejuni	5	0	0
Legionella spp.	10	0	1
Mycobacterium spp.	14	6	5
Neisseria spp.	6	0	14
Others	13	1	6

[¥]One study can be assigned to multiple categories
*Non-exhaustive list

several occasions, WGS data combined with epidemiological investigations enabled food authorities to intervene based on strong evidence and the subsequent timely recall of potentially contaminated food [43–45]. Moreover, the digital and universal nature of WGS allows data to be exchanged and analyzed between different countries during multi-national outbreaks [34, 44, 46–49]. However, this requires internationally standardized protocols and

Table 2 Study aims (applications of NGS) of included studies (Jan 2015 to Sep 2018: $n = 275$)

Study aim(s)	
Outbreak investigations ($n = 164$)	
Source tracing	78
Identify transmission routes	85
Inform outbreak management: feedback on key phenotypic attributes	11
Control-oriented surveillance ($n = 41$)	
Understand transmission dynamics (identify transmission networks/clusters)	15
Early outbreak detection	23
Overview of circulating strains to identify the emergence of new threats	12
Strategy-oriented surveillance ($n = 70$)	
Understand transmission dynamics to develop prevention strategies	21
Overview of circulating strains (long-term trends)	38
Impact assessment of prevention and control programs	29
Identification of risk factors and risk groups	6

One study can have multiple study aims

nomenclature, as well as meaningful interpretation guidelines [32]. In addition to rapid source tracing, WGS provides insights into the virulome of certain pathogenic clades [50–53]. For example, real-time WGS is able to generate timely information concerning the presence of virulence genes during a Shiga toxin-producing *Escherichia coli* (STEC) outbreak [51]. WGS was often used to guide nosocomial outbreak investigations. It was reported several times that transmission events that were suspected based on epidemiological data alone or using low-resolution strain typing methods like antibiogram profiles had been disproved by integrating WGS data [54–57]. The ability to quickly exclude a patient or potential source during an outbreak investigation is equally important for infection control purposes as the confirmation of related isolates [39], thereby preventing inappropriate, costly, and ineffective control measures [55, 56].

The most highlighted issue is the fact that WGS, as is equally the case for conventional typing methods, cannot stand on its own and that epidemiological data (including time, place, and exposure data) should complement the WGS results to identify a common source or link cases during outbreak investigations [24, 58–64]. False conclusions could be drawn from WGS data alone since it is possible that epidemiologically unrelated isolates are highly similar at the SNP level [58, 65–67]. Another reported issue was the potential misinterpretation of isolate relationships given the diversity of isolates that can be found within a single host (e.g., following long term carriage) or environmental reservoir. It was stressed by several studies that it is important to account for this "cloud of diversity" by increasing the number of samples taken from the suspected source [55, 66–73]. On the other hand, within-host diversity allows to identify long-term carriers [74].

Control-oriented surveillance

Several public health agencies launched pilot projects to implement WGS in routine practice for control-oriented surveillance purposes, such as early outbreak detection, and evaluated its performance [75–77]. In 2013, a multi-agency collaboration prospectively performed WGS on all available *L. monocytogenes* isolates collected from patients, food, and food processing environments in the USA. Implementation of WGS data into their surveillance activities led to the detection of an increased number of outbreak clusters. In addition, combining WGS data with robust epidemiological information solved more outbreaks compared to before with PFGE [77]. Retrospective comparisons during the transition period from traditional to WGS-based characterization were not considered an obstacle given the possibility to accurately extract traditional typing information for *L. monocytogenes* from WGS data [75]. Also for STEC O157, the added value of implementing WGS as a tool to inform national surveillance was demonstrated as it led to early and accurate outbreak detection [78], as well as the ability to extract information concerning important virulence determinants and monitor the emergence of hyper-virulent strains [79, 80]. Similarly, a prospective trial of sequencing all *Salmonella* Typhimurium isolates concurrently with the conventional MLVA typing technique in Australia demonstrated the higher resolution offered by WGS leading to better source attributions and more targeted epidemiological investigations [81]. However, several challenges related to the interpretation of WGS data remain. As for outbreak investigations, it was reported several times that WGS results should not be interpreted on their own. A single cutoff of the number of SNPs to assess relatedness cannot consistently predict whether isolates are epidemiologically linked [77, 81–83]. However, field data (e.g., demographic data or exposure histories) are only valuable when organized in a standardized format, requiring a more systematic approach to epidemiological data collection [84]. Another implementation barrier reported was the limited capacity of the public health unit to understand and use WGS data, implying an increased need for collaboration and exchange of expertise between microbiologists, bioinformaticians, and epidemiologists [81].

NGS has been applied to monitor antimicrobial resistance of hospital-acquired infections. Genotypic prediction of resistance of *S. aureus* strains seems at least as reliable as routine phenotypic testing. However,

phenotypic prediction based on the genotype cannot replace phenotypic testing, as the present understanding of the genetic basis of resistance and the associated databases are not comprehensive [85]. This limitation was shown during a WGS-based surveillance of antimicrobial resistant determinants in *Klebsiella pneumonia* where the phenotypically determined resistance was higher than the sequenced-based resistance [86].

WGS has proven to be a more reliable tool to predict epidemiological links between tuberculosis cases than the conventional variable number of tandem repeat (VNTR) genotyping that often lead to false cluster identification [87]. WGS as a tool for the identification of tuberculosis outbreaks may be particularly useful in settings where the genetic diversity is expected to be lower such as geographically restricted *M. tuberculosis* populations [88], genetically closely related genotypes imported from a high-incidence region [89], or for highly monomorphic *M. tuberculosis* lineages [90].

Strategy-oriented surveillance

Several studies showed the value of WGS in understanding the impact of vaccination on circulating pathogen populations, potentially resulting in antigenic drift to escape vaccine-mediated immune selective pressure (i.e., strain replacement) [91–100]. Gaining insights into this is achieved by comparing the incidence of infections caused by vaccine targeted serotypes before and after the introduction of the vaccine, and to potentially identify the proliferation of non-vaccine targeted strains. The adoption of WGS methods to monitor pathogen populations during immunization programs has proven to be useful and could potentially identify differential impacts on distinct serotypes [91]. Genomic surveillance provides the required resolution for the development of targeted interventions [92] and to predict the impact of implementing a vaccination program in a given population [101]. The routine use of WGS for surveillance purposes can also inform antibiotic stewardship. One advantage of introducing WGS to inform treatment guidelines is the ability to identify genetically linked resistance that can be co-selected by multiple drugs, as opposed to phenotypic resistance rates that consider each antimicrobial class as a discrete unit [102]. In addition, resistance rates can vary significantly by clone implying that monitoring changes in population structure using WGS is useful to guide antibiotic usage policies [103]. Besides informing vaccination programs and antibiotic stewardship, WGS can reveal a detailed understanding of the transmission dynamics within and between healthcare settings, the community, and individual households, to appropriately direct control programs and decolonization strategies [104–112].

Several studies highlighted the public health benefits of WGS-guided surveillance to monitor the spread of multidrug-resistant isolates and mobile genes, including resistance-carrying transposons and plasmids that are able to transfer resistance between bacterial species [113–118]. The zoonotic potential of clinically relevant multi-resistant bacteria stresses the importance of the 'One Health' approach. In general, WGS-informed surveillance including isolates from different hosts and settings can provide evidence for interspecies transmission and focus control efforts on important reservoirs [119–126].

Reported challenges, issues, and obstacles from an epidemiologist's perspective

Several studies reported challenges, issues, and obstacles (not exclusively) related to the integration of pathogen genomics within the activities of epidemiologists. For example, the inability to link laboratory and contextual data due to missing unique identifiers [127] impedes proper data integration. Further, contextual data was often missing, limited, or unstandardized [30, 34, 38, 49, 62, 71, 84, 94, 108, 128–140]. Regarding the sampling strategy, selection bias might arise when WGS has been performed on a small proportion of cases/isolates, severe cases are overrepresented among sequenced isolates, asymptomatic cases/carriers are excluded, or certain geographical regions or time periods are overrepresented [24, 30, 32, 52, 59, 85, 91, 93, 106, 116, 128, 141–144]. In addition, there might be insufficient statistical power to detect associations due to a low number of sequenced strains [81, 128, 144, 145].

Discussion

Applications

Within the set of studies included in this scoping review, NGS was mainly used as a tool to provide information on the whole genome of the bacterial pathogens. WGS has useful applications in both outbreak investigations and surveillance activities. Outbreak investigations benefit from the increased resolution offered by WGS for case ascertainment, linking cases to the possible sources, defining transmission clusters, and providing rapid feedback on key phenotypic attributes of the involved pathogens. The application of WGS during control-oriented surveillance was mainly aimed at early outbreak detection by accurately defining transmission clusters among circulating strains, unraveling transmission chains to guide targeted interventions, and identifying the emergence of new threats. The use of WGS during strategy-oriented surveillance seemed particularly useful to assess the impact of prevention and control programs, such as vaccination campaigns and antibiotic stewardship.

Level of implementation

WGS has been increasingly used as a typing tool for comparison of isolates during outbreak investigations. Most published studies were retrospective (59%), but an increasing number applied WGS in quasi-real time. For surveillance activities of certain pathogens, there has been a shift from proof-of-concept studies to routine use of WGS. In several countries, public health agencies and regulatory bodies [e.g., Public Health England, US Centers for Disease Prevention and Control (CDC), European Food Safety Authority (EFSA)] have implemented WGS as a routine typing tool for surveillance activities of selected pathogens. Although WGS is (or was) mainly used in parallel with conventional testing in many European countries [76, 81, 82, 87, 146], countries such as Denmark, France, and the UK have already transitioned completely to WGS for certain pathogens [34, 43, 46, 76, 84, 147–150]. Following the results of a survey conducted by ECDC, 20 countries (i.e., two thirds of European Union and European Economic Area countries) were routinely using WGS in 2017 for national surveillance of at least one human pathogen [151].

Added value

WGS has shown superior sensitivity and specificity to identify transmission clusters compared to traditional subtyping methods such as PFGE, MLVA, and MLST that often do not provide the required resolution to discriminate between outbreak-related and sporadic cases [21–31, 59]. Thanks to its greater specificity, WGS allows to reject a false hypothesis of transmission generated by conventional methods, thereby avoiding inappropriate, costly, and ineffective follow-up investigations and control measures [39, 55, 56, 78, 152]. More targeted interventions can save resources at the health protection and local authority level [78]. The major advantage of implementing WGS during surveillance activities or outbreak investigations is therefore inherent in the higher resolution of the WGS output itself. It should be noted that the utility of WGS varies depending on the public health objective (discriminating between closely related individual cases during a point-source outbreak or national surveillance purposes) [153], as well as on the population structure (high incidence settings versus low-transmission settings) [88, 89] and the clonality of the pathogen [32–35, 90]. Also, a stepwise implementation of typing methods has proven to be a useful approach. Conventional molecular methods can serve as a first-level classification to confine possible outbreak isolates. At the next level, WGS can bring deeper and more comprehensive insights [130, 154].

In terms of technical advantages, WGS is a universal test that is applicable to all organisms [155] and has the potential to provide multiple tests in silico (e.g., antibiotic resistance, serotype, virulence genes) from a single assay, thereby replacing several conventional methods and/or providing additional information on the studied pathogen [57, 79, 156, 157]. Therefore, NGS is able to replace current time-consuming and labor-intensive methods with a single, all-inclusive diagnostic test [94, 157, 158]. Moreover, the digital nature and the reliability of WGS data allow exchange and to compare data across countries [44, 46, 47]. The development of shared databases will make it increasingly possible to establish links between sequences from different countries and sources.

Challenges, issues, and obstacles

Definition of a cluster

The main issue reported when using WGS data to detect and to confirm transmission between isolates was the difficulty, if not impossibility, to define with a single SNP/allele threshold how much genetic variation can exist within an epidemiologically related cluster [1, 23, 28, 57, 58, 65, 67, 159]. The number of SNPs within a cluster often depends on various factors, such as the genetic diversity within each species, its molecular clock, evolutionary forces, the nature of the outbreak (point-source, long-lasting, multinational, etc.), the extent of diversity in the background population, within-host diversity, the population bottleneck during transmission, the level of asymptomatic infections, the number of isolates included in the analysis, and the methods used for genomic analysis [1, 25, 67, 74, 81, 83, 147, 160–162]. Many studies stress the fact that we cannot rely solely on genomic information during outbreak investigations or surveillance activities and that epidemiological data describing the temporal and spatial dynamics of infection should always be considered [59–61, 63, 67, 77, 81–83]. Contextual data should therefore be collected carefully and combined with WGS data for a proper interpretation, which is almost seamlessly linked to the challenge of data integration.

Data integration

A useful interpretation of genomic data is highly dependent on the epidemiological and clinical metadata [1, 160, 163, 164]. The integration of laboratory and epidemiological data is often hampered by the incomplete and/or unstructured nature of the contextual data [13, 84, 128, 165]. For example, during a multi-country outbreak investigation, it is important to develop a codebook for uniform and standardized data entry between countries [34, 49]. To maximize the potential of WGS, public health professionals have to identify a minimum set of variables (such as time, place of infection, host characteristics, clinical presentation, and exposures) that should be incorporated within surveillance activities of a particular pathogen [165].

Although WGS data has the potential to support phenotypic predictions of virulence and resistance based on the genotype, phenotypic data will still be needed to identify new resistance/virulence mechanisms and to keep the databases up-to-date [12, 85, 86, 157, 166, 167]. Therefore, phenotypic testing results and clinical data have to be collected in a standardized manner alongside the sequence data to feed the databases from which associations between genotype and phenotype can be observed [160].

More recently, digital streams (also called "Internet of things") are being used as an input for surveillance systems (i.e., digital epidemiology). Examples include search engines, social media, mobile phones, and health trackers. These novel data streams, generated outside public health, could potentially enrich epidemiology by providing information on natural and social phenomena [168].

One Health, the concept of structured collaboration and coordination between human, animal, and eco health systems, has become an emerging focus due to the increased understanding of how animal and ecological reservoirs significantly influence human health [169]. Therefore, the management of infectious diseases requires sampling from different hosts and sources. As indicated by Rantsiou et al., the development of WGS is currently not at the same level in the food industry as compared to public health agencies [170]. The outputs produced by the different sectors should remain comparable at any time to ensure the linkage of isolates.

An overview of data integration is presented in Fig. 3.

Collaboration between the different stakeholders

Following the previous section addressing the importance of data integration, it is clear that the switch to WGS requires an increase in multi-disciplinary working [9, 81, 148, 171]. In particular for data interpretation, expertise in bioinformatics and in biological, epidemiological, and microbiological sciences needs to be combined. Infectious disease epidemiologists implementing WGS data into their routine workflow might need training in genomics as well as skills in analyzing high-dimensional data sets. In addition to interdisciplinary and inter-sectoral (One Health) collaboration, the implementation of WGS should be coordinated at an international level as infectious diseases do not respect national boundaries [49].

Sampling frame

As for any type of epidemiological study, genomics-informed surveillance activities should be based on robust sampling strategies defining the required sample size and the number of samples needed from the different sources. The sampling framework will vary

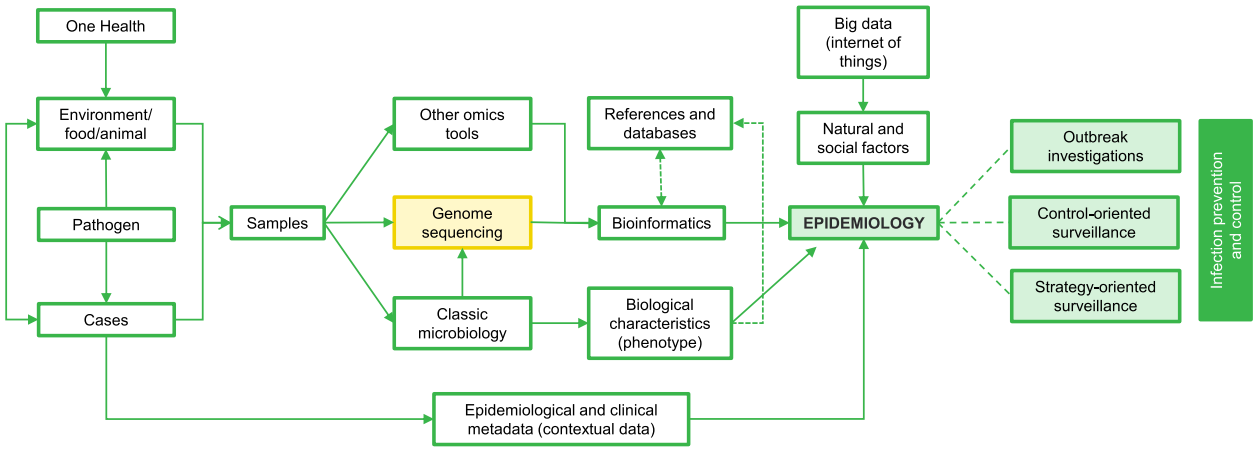

Fig. 3 Integration of multiple data types. The anticipated workflow of infection prevention and control includes the following: (1) samples are obtained from cases infected with a certain pathogen, as well as from other sources such as the environment, food, and/or animals following the One Health approach; (2) pathogens are isolated, and information concerning the biological characteristics is obtained through classical microbiological testing. Phenotypic tests are still required to feed databases and confirm genotype-phenotype associations. Culturing steps (isolation) are often preceding genome sequencing; however, sequencing directly from clinical samples is also possible using culture-independent methods (metagenomics); (3) high-throughput sequence data is generated (other -omics technologies such as transcriptomics, proteomics, and metabolomics can complement the genomic information); (4) relationships among isolates and specific characteristics are inferred based on sequence information obtained through bioinformatics tools; (5) to come to a meaningful outcome (i.e., transmission chains, cluster identification, source tracing, key phenotypic attributes), the genomic evidence is combined with epidemiological metadata (time, place, exposures, etc.) from field epidemiological investigations, clinical data obtained through the healthcare system, biological characteristics obtained through classic microbiological methods, and big data on natural and social factors. Finally, infection prevention and control measures can be conducted on the basis of this aggregated information

depending on the type of public health application (e.g., investigating an explosive outbreak often requires dense sampling including multiple samples from single sources, as opposed to strategy-oriented surveillance requiring a representative coverage of the population) [171]. Selection bias can be introduced when the subset of samples selected for WGS is not representative [128, 163]. In order to efficiently assemble a representative sample, it is often needed to develop a stratified sampling scheme according to time, place, and person, or to perform normalizations to maintain original sampling fractions.

Translational research and information overload

Genomic information must be interpreted and translated in a meaningful manner into both immediate public health action and longer term prevention programs [2, 57, 172]. Moving from low-resolution typing methods to WGS-based typing will lead to an increase in the detected number of hazards. Not only will the sensitive nature of WGS increase the number of clusters detected [76, 77], it will also provide additional information on the presence of resistance genes, virulence genes, etc. It will be important to filter out the hazards that are truly relevant from a public health point of view (i.e., separate the "signal" from the "noise") and that subsequently require the initiation of a public health action.

Limitations of the scoping review

A possible limitation is the fact that only studies published after 2015 were included. However, NGS is a fast-evolving technique, and we were mainly interested in its state-of-the-art applications. Another potential limitation is that only one database (PubMed) was searched. However, hand searching and reference checking were applied to partly account for this. Still, it is likely that additional publications exist outside this search. Some work will have been missed, but the aim was to have a systematic overview of the field rather than an exhaustive capture of every single published article. An encountered difficulty during the selection process was the subjective nature of the inclusion and exclusion criteria, mainly in terms of classifying the studies according to their context (public health, research, or diagnostics). We tried to account for this by screening the full texts by two independent reviewers. Further, data extraction was based only on the data provided in the individual studies and, if available, supplementary information. Although the data extraction was performed using a standard procedure, it is possible that some information was misinterpreted. Given the large number of included studies, it was opted not to contact the investigators to retrieve additional data.

Future perspectives

Currently, the most common strategy to integrate WGS into routine public health surveillance is to add genomic typing to conventional surveillance activities where an increased resolution is considered necessary, i.e., on a selected subset of isolates. As costs will drop, microbiology laboratories will potentially implement WGS in their routine workflow. Following this scenario, the use of WGS is foreseen for all clinical isolates and the number of isolates sequenced would no longer be driven by predetermined study designs [157, 163]. This way, sequence data gathered for diagnostic purposes can be accumulated for public health activities. In addition, large-scale research into genotype-phenotype associations from routinely collected data will be possible [157].

Conclusions

This scoping review addresses the current state and potential of implementing pathogen genomics for routine public health practice. Main applications include the use of WGS data for (1) source tracing during outbreak investigations, (2) early outbreak detection, (3) unraveling transmission dynamics in order to implement targeted interventions, (4) monitoring drug resistance, (5) detecting cross-border transmission events, (6) identifying the emergence of strains with enhanced virulence or strains with zoonotic potential, and (7) assessing the impact of prevention and control programs, such as vaccination campaigns. The main reported added value of WGS by the included studies is the superior resolution compared to the conventional methods, and consequently being able to accurately confirm or rule out transmission events. However, it should be emphasized that WGS cannot stand on its own and should be integrated with other data types. High-quality epidemiological data and study designs are needed to realize the full potential of WGS. Collaborations between infectious disease epidemiologists, public health practitioners, microbiologists, and bioinformaticians are key for a successful genomics-informed surveillance.

Additional files

Additional file 1: PRISMA checklist. Completed PRISMA checklist indicating page number in manuscript of relevant content. (PDF 409 kb)

Additional file 2: Protocol. (PDF 939 kb)

Additional file 3: Inclusion and exclusion criteria organized according to the Population-Concept-Context (PCC) principle, as outlined in the Joanna Briggs' Manual for Scoping Reviews. (PDF 499 kb)

Additional file 4: Decision tree used during screening to distinguish between research, individual patient care, and public health practice. (PDF 275 kb)

Additional file 5: PubMed search string. (PDF 176 kb)

Additional file 6: Extraction form listing the characteristics that are extracted from the included studies. (PDF 179 kb)

Additional file 7: Characteristics of included studies.(XLSX 105 kb)

Abbreviations

CDC: US Centers for Disease Prevention and Control; ECDC: European Centre for Disease Prevention and Control; EEA: European Economic Area; EFSA: European Food Safety Authority; EU: European Union; MDR: Multidrug-resistant; MLST: Multi-locus sequence typing; MLVA: Multiple-locus variable-number tandem-repeat analysis; NGS: Next-generation sequencing; PFGE: Pulsed-field gel electrophoresis; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; SNP: Single-nucleotide polymorphism; STEC: Shiga toxin-producing *Escherichia* coli; VNTR: Variable number of tandem repeat; VRE: Vancomycin-resistant Enterococci; WGS: Whole-genome sequencing

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Authors' contributions

The protocol was developed and drafted by NVG, together with HVO, AR, BD, and NR. The screening of the articles by title and abstract was performed by NVG. The screening of the full texts of the articles was performed by NVG and TD. NB screened the articles in full text for which no consensus could be reached. NVG drafted the manuscript, and feedback was provided by all other authors. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset(s) supporting the conclusions of this article is (are) included within the article (and its additional file(s)).

Ethics approval and consent to participate

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Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories

Methods for the Examination of Waters and Associated Materials

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The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories

Methods for the Examination of Waters and Associated Materials

This booklet contains details of practices and procedures that should be adopted within laboratories.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other booklets include

Part 1 - Water quality and public health
Part 2 - Practices and procedures for sampling
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and most probable number techniques
Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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Glossary

CI	confidence interval
COSHH	Control of Substances Hazardous to Health
E. coli	*Escherichia coli*
MPN	most probable number
MPR	most probable range

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this booklet are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods is available from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

January 2002

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1999 (SI 1999/437). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

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Practices and procedures for laboratories

1 Introduction and scope

The microbiological analysis of water and associated materials involves the use of selective procedures and media. In addition, the nature of the organisms being isolated and enumerated can present challenges to analysts. It is, therefore, important that the media used by a laboratory are prepared, and the procedures conducted, in such a way that the results reflect the quality of the water being tested and that the data generated are reliable. It is essential that a laboratory is able to demonstrate that results produced are fit for the purpose for which they are to be used. This can be achieved by implementing an appropriate programme of quality assurance. Statutory regulations define the performance criteria of methods for compliance purposes for the monitoring of drinking water supplies. Methods should be capable of establishing, within acceptable limits of deviation and detection, whether the sample contains numbers of selected groups of micro-organisms which contravene prescribed values. Depending on the test being used, it is necessary to be able to demonstrate the presence (or absence) of particular micro-organisms or class of micro-organisms in a given sample volume, and to estimate their numbers. The detection of small numbers of organisms is particularly important. Any effective quality assurance programme should, therefore, cover the whole process from sample collection to reporting and interpretation of results. The programme should also include a system of internal quality control, and participation in an appropriate proficiency testing scheme.

Any laboratory where the analysis of water and associated materials is undertaken should operate a quality system. The main functions of such a system are to define the processes that have been put into place to ensure that results are reliable and have been performed to recognised procedures by properly trained staff using suitable equipment. A good quality system enables analytical data to be audited and provides documentary evidence that data generated are accurate and precise. A quality system also provides the basis for documenting structures for the laboratory and staff, equipment and associated service and calibration, and methods that the laboratory uses. The quality system also acts as a reference system for any documentation relating to the laboratory and its operation.

The quality system will depend on the content of a number of documents, each of which is inter-dependent on other documents for its correct function. This booklet describes the basic requirements of a quality system, coupled with criteria for equipment and materials, which enable the reliable analysis of water and associated materials to be undertaken. Guidance on basic analytical procedures, and statistical considerations concerning results, is also given, together with protocols for comparing methods prior to adoption of a new method within a laboratory.

2 The quality manual

Most quality systems begin with a quality manual that defines the laboratory and its policy towards quality. The manual should be broad in its approach, covering all aspects that are considered important. It should also be simple in that it is easy to read and be understood by all members of staff, and easy to maintain in the ever-changing circumstances of the laboratory. In broad outlines, the manual should contain a quality statement, details of the laboratory in terms of location and staff structure, and how it will set, maintain and check quality standards. In addition, the manual is a useful document for incorporating health and safety policies, safe working procedures and environmental policies.

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The quality manual should define records that the laboratory should keep and maintain, the nature of measurement calibrations, the format of analytical procedures, internal and external quality assurance, and resulting remedial action which should follow in the event of a failure in documented procedures.

Every laboratory should have an organisation chart showing staff posts and associated role profiles in day to day tasks, and importantly, the chain of accountability and reporting. The post responsible for the quality assurance programme should be clearly defined and each member of staff should have a well-defined job description outlining responsibilities. A record of staff training should be maintained and regularly updated. In addition, there should also be a defined plan for provision of cover for work when staff members are absent.

The reporting system should enable results which require immediate remedial action to be communicated without delay to appropriate persons. Records of laboratory results should be kept for as long as is necessary to comply with requirements for archive and audit trails, and should include, for example the date, place and time of sampling, and the members of staff undertaking the sampling and analysis.

The quality assurance programme can only be successful if all constituent parts are well documented. The manual should provide valuable source material covering all activities of the laboratory and should only require periodic review.

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3 Laboratory records

The laboratory should have a system for the purpose of recording data relating to equipment, measuring devices, quality control and environmental monitoring. The following are considered to be important components of a laboratory quality system.

3.1 Staff training records

Staff training records should show appropriate training for each documented method where training has been given. In addition, the record should show training in the use of major items of equipment. There should also be documented evidence that training has been both adequate and successful. This may involve staff analysing external quality assurance samples where their data can be compared with data from other analysts or laboratories. Alternatively, spiked or raw water samples containing low numbers of target organisms, may be used provided that replicate samples are also analysed by a fully trained member of staff. In order to demonstrate confidence, an appropriate number of replicates should be tested using the full analytical procedure. Details of the comparisons of the test results could also be documented in the training record. Training records can also be used to store documentary evidence of additional training, for example, courses, conferences, workshops etc that the analyst has attended.

3.2 Analytical procedures

There should be appropriate documentation of all analytical procedures in current use. Such documentation should be controlled, for example with an appropriate issue number, date of issue, appropriate pagination and known documented circulation. Where methods are revised, the original method should be stored for possible future reference. A master copy of all methods should be kept in a secure place and relevant copies issued to the laboratory. Such copies need not contain all the methods and may include only those applicable to a particular section (for example, a drinking water microbiology section). It may be useful to store

validation data with the appropriate method, together with the protocol used for conducting the validation exercise.

3.3 Equipment lists and service records

All major items of equipment should be catalogued and include data on, for example, the date of purchase, the name of the supplier, the frequency of servicing and calibration, and, if appropriate, the location of instruction manuals. The type of equipment typically covered may include, for example, incubators, water baths, autoclaves, refrigerators and microscopes. Service records of such equipment may also be stored and include reports prepared by the service engineer and details of any calibration carried out on the equipment. Details of equipment faults, repairs and the upgrading of any equipment may also be stored.

3.4 Laboratory calibration records

Laboratories should have well documented procedures for the calibration of all equipment that involves, for example, recording weight, volume, temperature or time. Calibration standards for such equipment may include certified standards, for example thermometers, and should include a certificate that traces the calibration to national standards. Alternatively, uncertified standards that have been calibrated by an accredited laboratory may be used. Certified standards need not be used routinely, but could be used to calibrate uncertified working standards on a regular basis. Once calibrated, these standards should themselves be re-calibrated as appropriate to national standards.

3.5 Environmental monitoring

Because of the ubiquitous nature of most of the microbes of interest, it is essential to ensure that any organisms that are detected have originated from the original sample and have not been introduced inadvertently during sampling or subsequent analysis. Laboratories should consider appropriate monitoring of the environment, both related to the sampling procedure and the analysis within the laboratory, to ensure that any micro-organisms detected do not adversely contribute to any result.

4 Materials used in a microbiology laboratory

Chemicals used in a microbiology laboratory should be of analytical grade quality. Reagents and chemicals should be stored and used in accordance with manufacturer's instructions and discarded if the expiry date, ie the date by which the reagent should be used, has passed. Reagents and chemicals are usually supplied with hazard assessment and toxicity data. Records of these data should be kept, and any specific hazard documented in each method and COSHH assessment[(1)]. Chemicals and reagents should always be handled with care and any spillages that occur should be cleaned up immediately.

4.1 Media

Manufacturers can supply most microbiological media as dehydrated formulated preparations. Media that are supplied as dehydrated powders should be stored in a cool dry place, and containers labelled clearly with the date of receipt, and the dates when containers are opened. When a container is opened for the first time, the laboratory should allocate an expiry date to the formulation relative to its potential deterioration. Manufacturers may provide details of expiry dates and storage conditions. Whilst details of the preparation and sterilisation of media are usually provided by the manufacturer, these should also be documented in the

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analytical method (see section 8.1). Most powdered media are hygroscopic. Hence, after the containers have been opened and media removed, the lid should be tightly secured to reduce further absorption of moisture. Over a period of time, some media absorb excessive amounts of water and solidify. This usually results in discoloration of the medium and denaturation of the nutrients or selective properties. Any such medium should, therefore, be discarded, even if the expiry date has not passed.

Media are prepared by weighing out the appropriate amount of material and adding distilled, deionised or similar grade water (see section 4.4). Many media contain selective chemicals and where these are supplied as powders, appropriate containment measures should be taken. Media, as broth, dissolve readily in cold water. Those that contain agar do not completely dissolve in water without heating. Agar is an extract of seaweed and is usually added to media to a concentration of between 1.0 - 1.5 % depending on the purity of the agar. Agar only dissolves completely in water if the solution is heated to boiling point. Agar solutions solidify or set at approximately 42 °C. Any broth medium may be solidified by the addition of the appropriate amount of agar.

4.2 Preparation of media

The appropriate weight of the dehydrated formulated preparation, or ingredients as listed in the method, is added to the appropriate volume of water. Whilst it is often not essential to have to adjust the pH of the medium, in certain circumstances it will be necessary to do so. Adjusting the pH should be carried out by the addition of small amounts of an appropriate concentration of hydrochloric acid or sodium hydroxide solution until the required pH value is achieved. This is often carried out before sterilisation takes place. For example, when membrane lauryl sulphate broth is used for the enumeration of coliform bacteria in water, it should have a pH of 7.4 ± 0.2 after sterilisation. Sterilising the solution by heating may cause breakdown of the lactose in the medium and result in a lowering of the pH. It may, therefore, be necessary to raise the initial pH of the medium by 0.2 - 0.4 units before sterilisation, in order to achieve a final pH of 7.4 ± 0.2 after sterilisation. The pH of a medium should be measured as soon as practicable after it has reached room temperature. Media should not be allowed to stand at room temperature for significant periods of time (ideally, not more than 2 hours) before testing, as the pH may change over time.

All dehydrated media should be completely dissolved before being dispensed and sterilised. Failure to do so may result in charring of some of the media ingredients during the sterilisation process. Some media, for example membrane enterococcus agar used for the enumeration of enterococci in water, do not require sterilisation because they are sufficiently selective to prevent the growth of heat resistant organisms. Heating is required only to dissolve the agar and excessive heating may adversely affect the medium. Such media should be cooled quickly after heating to minimise breakdown of heat sensitive constituents.

A small amount of prepared medium can be used to check the final pH of the medium and to determine growth and selectivity characteristics before the remaining bulk of the medium is used.

Typically, media are sterilised by autoclaving at 115 °C for 10 minutes or 121 °C for 15 minutes. It is important that media should not be overheated. Media should not be autoclaved more than once. Sterilised media should be removed from the autoclave as soon as practicable after sterilisation is complete and due care should be taken as media may be super-heated and boil rapidly. Once removed from the autoclave, the tops on the containers may be tightened and the media allowed to set. Solidified media may be stored in the dark at room temperature

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(no greater than 25 °C), should be subjected to quality control tests and used only when the tests have shown that the medium gives satisfactory performance. A prepared medium should be allocated an expiry date and this should be stated in the method. Media can be melted by heating in steam at normal atmospheric pressure or by using a microwave oven on low power.

Before a medium is poured into Petri dishes or tubes, it should be cooled, for example in a water bath or incubator, to approximately 50 °C prior to the addition of any supplements. Media should not be left standing at 50 °C for long periods of time. Media at temperatures above 50 °C should not be poured into Petri dishes, as this may lead to excessive condensation in the Petri dish during the setting process and subsequent storage. When the medium is poured into a Petri dish, a lid is added and the medium is allowed to solidify. When the medium has solidified, the Petri dish should be inverted and stored at a temperature of between 2 - 8 °C in such a way as to prevent excessive drying. This may be achieved by storing the Petri dish in an air-tight container. When the medium in a Petri dish shows excessive drying, the Petri dish should be discarded. The preparation of media should be planned to ensure adequate supplies are available, yet minimise discarding unwanted media.

Freshly prepared media should not be left on benches for any length of time, particularly if these are exposed to sunlight. The action of sunlight on media produces peroxides and other toxic substances that inhibit the growth of certain bacteria. When a medium has been prepared, it should be transferred to a dark environment as quickly as practicable. Media that show obvious signs of contamination or deterioration should be discarded.

Media that have been stored at a temperature of between 2 - 8 °C should be dried before use by leaving at room temperature (no greater than 25 °C) for 2 hours or at 37 °C for 30 minutes. The medium should be poured into a Petri dish to a depth (typically, 3 - 7 mm) sufficient to ensure the medium does not dry out during incubation. During the pouring and subsequent cooling of agar media in Petri dishes, a film of moisture is often left on the surface of the solidified agar. The incubation of an agar medium that has not been dried may result in the spread of bacteria, particularly motile bacteria, across the agar, with the result that colonies may not be isolated on the agar. This may hinder subsequent sub-culture and render them meaningless. When large numbers of agar Petri dishes are being dried, the drying time may need to be extended, or a small amount of drying agent (for example self-indicating silica gel) added to the drying chamber to absorb excess moisture.

4.3 Quality control of laboratory media

Each batch of prepared medium should be identifiable, for example marked with a batch number. For complex media requiring aseptic additions after sterilisation, each bottle of medium may need to be treated as a separate batch. Where commercial media are used, the manufacturer's batch number should be recorded. When a medium has been prepared it should be labelled with its batch number and expiry date. Details of the sterilisation should be available for all media that require autoclaving and these should be recorded together with results of any time cycle checks, for example autoclave temperature charts. The signature of appropriate staff can also be included in these records as a means of demonstrating a suitable audit trail to show correct preparation of the media.

Once preparation is complete, a small aliquot of the medium should be checked for pH. The medium should be within ± 0.2 pH units of that stated in the method. If the medium is outside the defined pH limits it should be discarded. The pH of the medium should not be adjusted after sterilisation because of the risk of introducing microbial contamination. Such effects may not be immediately obvious but may become significant during storage.

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When the sterilised medium has been poured into Petri dishes, a representative number should be checked to ensure that they are satisfactory. These Petri dishes should be incubated at a temperature and time appropriate for the medium and examined for any contaminant microbial growth. Where liquid media are prepared bottles or tubes should be similarly checked. In addition, the broth may be inoculated onto an appropriate nutrient agar medium and incubated at an appropriate temperature to assess effective sterilisation. Media should also be checked to establish that they support the growth of the target organisms for which they have been prepared, and differentiate or are selective against non-target organisms. Appropriate organisms should be identified and tested with each medium. A list of typical organisms is given in Table 4.1.

Table 4.1 Examples of bacterial strains used for quality control of media for water microbiology

Bacterial strain	Reference strains	Media or reagents
Escherichia coli	NCTC 9001 NCIMB 11943	membrane lauryl sulphate broth membrane lactose glucuronide agar negative control for oxidase reagent
Enterobacter aerogenes	NCTC 10006	membrane lauryl sulphate broth
Enterococcus faecalis	NCTC 775	membrane enterococcus agar
Clostridium perfringens	NCTC 8237 NCIMB 6125	tryptose sulphite cycloserine agar
Pseudomonas aeruginosa	NCTC 10662 NCIMB 8295	pseudomonas agar positive control for oxidase reagent non-target control for membrane lauryl sulphate broth
Salmonella dublin	NCTC 9676	Rappaport-Vassiliadis enrichment broth xylose lysine desoxycholate agar

NCIMB = National Collections of Industrial and Marine Bacteria
NCTC = National Collection of Type Cultures

4.4 Quality of distilled and deionised water

In most circumstances, tap water is unsuitable for the preparation of laboratory media. Tap water may contain unwanted organic carbon, and inorganic and metal ions. These substances may be inhibitory to bacteria, and important components of the medium may change or precipitate out of solution.

Water used for preparing media can be produced in a variety of ways. Distillation should be carried out in Pyrex glass vessels. A single distillation process may not be sufficient to remove all organic material likely to be present in tap water, unless the water is first deionised. Deionisation will remove many of the organic and inorganic compounds found in water, will also minimise scale formation in the distillation apparatus and can be used to produce good quality water. Whilst ion exchange resins will remove inorganic ions from water, carbon filtration will remove organic material. Reverse osmosis can be used where substantial amounts of water are required.

Once produced, water will readily absorb substances, for example carbon dioxide and ammonia, from the atmosphere and the container in which it is stored. Electrical conductivity

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measurements are often used as a measure of the ionic content of water. The electrical conductivity of water suitable for bacteriological analysis should, ideally, measure less than 3 μS/cm. Electrical conductivities of water of less than 0.5 μS/cm are generally achievable using deionising or double distillation sytems. Single distillation systems may produce water with electrical conductivities of up to 5 μS/cm. The electrical conductivity of the water should be measured regularly, depending upon use, and the results recorded. Water stored in large containers may contain sufficient nutrients to allow microbial growth. The bacteriological quality (for example the enumeration of heterotrophic bacterial content, see Part 7 in this series) of the water should, therefore, be monitored regularly, depending upon the rate of production and its usage. Distilled or deionised water is not sterile, and may contain low numbers of organisms per millilitre. Where bacteriological counts are found to be excessive, for example over 1000 per ml in freshly produced water, or over 10000 per ml in stored water, then consideration should be given to changing the manner in which the water is produced and stored. Consideration should also be given to cleaning and disinfecting the container on a regular basis.

If water is stored for long periods, it may be appropriate to determine chlorine residual, total organic carbon and ammonia levels. Respective levels should be less than 0.02, 0.5 and 0.05 mg/l.

5 General laboratory equipment

It is important that all equipment is kept clean, is well maintained and is clearly identified so that appropriate records can be traced quickly. Any spillages should be cleaned up immediately. Cleaning, servicing and maintenance should be carried out to a standard following manufacturer's instructions. See also sections 3.3 and 3.4.

5.1 pH meters

pH meters are designed to measure the hydrogen ion concentration at ambient temperature (ie 15 - 25 °C). When not in use, pH electrodes should be stored according to manufacturer's instructions. The pH meter should be calibrated according to manufacturer's instructions and this may involve a daily operation using 2 or more freshly prepared buffer solutions in the appropriate pH range. The results of calibration data should be recorded. The response of electrodes (for example slope and millivolt output) should also be checked regularly. Flat-tip membrane electrodes or spear-tip electrodes are suitable for measuring pH values of solid media, simply by touching the surface or spearing the agar. Flat-tip membrane electrodes may require the filling solution to be replaced at regular intervals, according to the manufacturer's instructions, as electrolyte often leaches from the end of the electrode. Particular attention should be paid to rinsing the electrodes after use, as a build-up of organic material can severely inhibit electrode response.

5.2 Incubators

Incubators are available in many shapes and sizes with, or without, fan assistance. Fan-assisted incubators provide a more even temperature distribution inside the cabinet. The inside of the incubator should be made of stainless steel (for easy cleaning) and there should be a glass or Perspex door inside the main door (to help minimise temperature loss but allow viewing of the incubator contents or thermometers when the main door is opened). The temperature of the incubator should be measured at regular intervals. The minimum number of readings that should be taken include one at the beginning of the working day, before cultures have been removed, and one at the end of the working day, particularly with

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temperature cycling incubators. Continuous temperature monitoring (with associated alarm systems) of the internal environment should be considered as it provides a complete appraisal of incubator performance, particularly for incubators with temperature cycling. Continuous monitoring allows realistic assessments of temperature fluctuations within incubators. Whether incubators are, or are not, fan-assisted, it is important that an even temperature distribution is established within the incubator. This can be achieved by placing thermometers, or temperature recording devices, in different parts of the incubator over a period of time, for example one week, and recording the temperatures at regular intervals. The temperature profile of the incubator should show no significant differences wherever the thermometers are placed. The temperature distribution may also depend upon the manner in which the incubator is loaded. For example, stacking Petri dishes in columns of greater than six dishes may affect the temperature distribution, and result in the temperature profile of individual dishes being different. Correct incubator temperature control is vital for the satisfactory performance of microbiological enumeration and detection. The temperature control of incubators is described in section 5.8.

5.3 Pipettes and pipettors

Many laboratories use sterile, plastic disposable 1 ml and 10 ml pipettes for routine microbiological purposes. These pipettes deliver the measured volume between the graduation and the tip of the pipette. Any pipettes that are damaged, or broken, should therefore be discarded. Volumes are usually dispensed with the aid of a pipette bulb and pipettes are plugged with non-absorbent cotton wool to prevent contamination of the contents of the pipette and the bulb when pipetting samples and cultures. A representative number of pipettes from each batch should be checked to confirm delivery of correct volumes.

Automatic pipettors and pipette tips can be used to dispense fixed or adjustable volumes of liquids. This is achieved by air displacement using a manually operated or electrically powered piston within the pipettor. There is a risk of the pipettor barrel or piston becoming contaminated and, therefore, plugged pipette tips should be used. A pipette tip of the correct size for the pipettor should be used in accordance with manufacturer's recommendations. Loosely fitting tips may leak, may not deliver the correct volume or may fall off the end of the pipettor when being used. Automatic pipettors should be used, stored when not in use, and calibrated when appropriate according to manufacturer's instructions.

Pipette tips can be purchased sterile, packaged either as individually wrapped, or in small convenient numbers. Pipette tips can also be placed into suitable containers and sterilised by autoclaving at 121 °C for 15 minutes. If containers are wet on removal from the autoclave they should be dried, by placing them in an incubator, before being used. Should the outside of the pipettor become contaminated during use it should be disinfected, by wiping with 70 % ethanol or 2-propanol, prior to continued use.

New pipettors should be calibrated before use, and at suitable intervals, according to manufacturer's instructions. The volumes chosen should represent the range of volumes for which the pipettor is likely to be used. For each volume chosen, the data are recorded and used to calculate mean volume dispensed, standard deviation and coefficient of variation. Ideally, the coefficient of variation should be less than 1 % and the bias should be less than 2 % of the volume chosen. Details, for example dates and staff undertaking calibrations, should be recorded and stored for each pipettor.

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5.4 Refrigerators and freezers

The temperature of refrigerators used for the storage of media, reagents and samples should be between 2 - 8 °C. Each refrigerator should contain a calibrated thermometer which is used to record the temperature on a regular basis. Where possible, media and reagents should be stored in separate refrigerators or compartments, and should not be stored in such a manner that the temperature of the compartment is adversely affected. Samples should not be stored in the same refrigerator as media. Modern refrigerators and freezers are usually available as frost-free items, but older equipment may require regular defrosting. The use of a sodium bicarbonate solution, to wash down the inside of refrigerators and freezers, helps reduce the growth of fungi.

Freezers usually operate at a temperature of - 20 °C, but cabinets that operate at a temperature of - 70 °C are available. As with refrigerators, the temperature of freezers should be checked regularly.

5.5 Anaerobic jars

Anaerobic jars are used to encourage the growth of anaerobic and micro-aerobic bacteria. They usually comprise a polycarbonate jar with a close fitting lid held in place by a clamp. Before incubation, oxygen in the jar is removed by generating hydrogen and reacting the two gases (to form water) with the aid of a platinum catalyst. Hydrogen can be generated following the addition of water to a sachet of sodium borohydride. Great care should be taken to ensure the correct size of sachet for the appropriate volume of jar is used. The efficiency of the platinum catalyst is adversely affected by moisture, and therefore the catalyst should be dried after use by placing it in an oven at a temperature of between 80 - 100 °C for 30 minutes. Alternatively, the catalyst can be placed in a sealed container with a suitable desiccant, such as silica gel, and stored in an incubator at a temperature of between 37 - 44 °C between periods of use. As an alternative technique, oxygen can be removed from the jar by absorbing the gas using a selection of chemicals. Anaerobic jars should be cleaned after use, or disinfected if necessary.

Cultures should be stacked loosely in the jar. The caps of screw-topped containers should be loose. After loading the jar, the appropriate conditions are established, together with a means of establishing whether the conditions have been attained. This can be achieved using anaerobic indicator strips, or the inclusion of two bacterial cultures, one, which is aerobic, and another, which is micro-aerobic or anaerobic. The correct incubation of materials is only achieved if the indicator strips or bacterial cultures show that suitable internal atmospheric conditions have been achieved.

Anaerobic and micro-aerobic systems based upon small plastic bags and gas generating systems are commercially available. These are suitable for the incubation of small numbers of cultures. Before use, new batches should be performance tested with appropriate anaerobic or micro-aerobic organisms.

5.6 Autoclaves

The principle of sterilisation by autoclaving is based on moist heat transfer. Autoclaving is used to sterilise media, bottles and other equipment used in the bacteriological examination of waters. Heat is applied in the form of steam, under pressure in the absence of air. Steam may be generated in a boiler that is separate to the sterilisation chamber. Alternatively, steam can be generated by the direct heating of water in the bottom of the chamber. Where steam is

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generated in a boiler separate to the sterilisation chamber, air is displaced more quickly than it is when steam is generated in the bottom of a chamber. Hence, the medium heats up and cools down faster. Sterilisation is timed from the moment when materials in the autoclave attain the appropriate sterilisation temperature. In order for correct sterilisation to take place, it is essential that steam penetrates the load, and that the heating time is not adversely affected by overloading the autoclave, both in terms of large numbers or volumes of objects in the autoclave.

Autoclaves vary in complexity, and range from simple pressure cooker systems to complex microprocessor-controlled machines, capable of a variety of sterilisation cycles. The autoclave cycle comprises an initial heating period, a period of free steaming (where air is purged from the chamber), a further heating period (where the contents are raised to the sterilisation temperature), a holding period at the sterilisation temperature, and finally a cooling period. Guidance on use, performance and construction of laboratory autoclaves is given elsewhere[2, 3].

It is important that the correct time and temperature are achieved during each sterilisation cycle and that these are monitored and recorded. The internal temperature of the autoclave should be established and verified during a sterilisation cycle. This can be achieved, for example using a multi-point thermocouple calibration procedure conducted on an appropriate load within the autoclave. Calibrations should be carried out following installation, repair or modification to the autoclave and all data recorded and stored. Thermocouples should be calibrated to national standards before calibration procedures are undertaken.

Details of each cycle of the autoclave should be recorded, together with the contents of the autoclave. A temperature cycle or sterilisation time-temperature record provides an audit trail to show correct sterilisation has taken place. Individual loads can, in addition, be marked with heat-indicating tape to demonstrate that they have been subject to a heat process.

Autoclaves should be serviced at regular intervals according to manufacturer's instructions. For autoclaves where steam is generated by the direct heating of water in the bottom of the chamber, the water should be replaced frequently. Failure to do may result in corrosion within the chamber, particularly the heating elements.

Contaminated material and media should not be autoclaved together. Furthermore, bottles of media should not be filled completely, and caps or stoppers should be loose fitting. Failure to loosen the cap or stopper may result in the bottle exploding. It should not be possible to open autoclaves until the sterilisation cycle is complete and the temperature has cooled down to a designated safety level. Although the temperature inside the autoclave may register, for example 80 °C, the temperature of the contents may remain above this. It is important, therefore, that when the sterilisation cycle is complete, the autoclave is opened carefully and that appropriate safety equipment is used when the contents are removed.

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5.7 Water baths

Water baths may be used to incubate certain cultures or keep agar media in a molten state until ready for use. They usually comprise a stirrer or circulating pump with a heating element and thermostat. When water baths are used to incubate cultures, the water should be distilled or deionised, always be stirred or circulated within the bath and switched on only when the water has reached the recommended level. When not in use, water baths should be stored according to manufacturer's instructions and should be drained at regular intervals and wiped with a suitable disinfectant (for example 70 % ethanol or 2-propanol) before being refilled.

When in use, the temperature of the water should be measured regularly using a calibrated thermometer.

5.8 Thermometers (or temperature recording devices) and temperature control

Thermometers may be of the mercury-in-glass or alcohol-in-glass type and are available in a wide variety of temperature ranges and sizes. They may be available calibrated to national standards or be un-calibrated. Certified and calibrated thermometers require re-calibration and certification every 5 years. Certified thermometers can be used to calibrate working reference thermometers that may subsequently be used to calibrate working thermometers used to measure temperatures within the laboratory.

When thermometers, or temperature recording devices, are used, they should be capable of measuring a given temperature within a specified tolerance. For a temperature of between 20 - 40 °C, for example in incubators, the maximum fluctuation around the given temperature should be ± 1 °C. In these circumstances, thermometers capable of measuring within a tolerance of ± 0.5 °C can be used, but those measuring to within ± 0.2 °C would be better. For regulatory drinking water compliance purposes, it may be more appropriate to use thermometers that measure to within ± 0.1 °C. For incubators set at 40 °C or above, the maximum fluctuation around the given temperature should be ± 0.5 °C and thermometers should measure to within ± 0.1 °C. In practical terms, if dual temperature incubation is used (for example, incubation at 30 °C and 44 °C) then two thermometers may be required.

With such sensitive equipment and in order to prevent heat loss when the temperature is measured, thermometers can be placed in suitable plastic or glass containers filled with an inert liquid. Suitable liquids comprise glycerol, liquid paraffin or polypropylene glycol. These liquids stabilise temperature measurements and prevent a sudden drop in the temperature when the thermometer and container are removed from the incubator for reading. Bottles containing thermometers should be placed in the incubator in locations reflective of the incubated samples or materials. Mercury-in-glass thermometers are fragile and may, if broken, present a health hazard. Consequently, they should be placed inside protective cases that do not interfere with the temperature measurements. Thermometers should not be used if the mercury or alcohol column is broken.

Working thermometers should be calibrated at regular intervals and any errors compared with the reference thermometer should be no greater than the tolerance of the reference thermometer. Electronic temperature recording devices (digital thermometers and thermocouples) are now available. These can be used to monitor temperatures continuously and data can be stored, retrieved and inspected. It is essential that such devices be regularly calibrated against certified thermometers or temperature recording devices.

5.9 Balances

Balances should possess a sensitivity that is appropriate for the substance being weighed. They should be clean and serviced regularly, and calibrated to national standards at pre-determined frequencies. The preparation of media and reagent requires careful and accurate use of balances and it is essential that balances are located in suitable positions away from sources of excessive vibration, temperature variation and air movements.

Balances used for general purposes, for example top pan balances, should be accurate to ± 0.01 g. Where greater accuracy is required, for example analytical balances used for weighing amounts of less than 1.0 g, balances should be accurate to at least ± 0.0001 g. A

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range of calibrated weights, appropriate for the balance in use, should be available for checking the accuracy of the balance. This should be undertaken on a regular basis, for example weekly or daily, depending on use. Balances not within specified tolerances should not be used until re-calibrated.

5.10 Microscopes

Microscopes can be used to examine material and facilitate the classification of micro-organisms. The microscope performs efficiently only if it is serviced regularly depending to its usage, is aligned correctly[4], is protected from environment contamination and is used according to manufacturer's instructions. Details of service records should be recorded and stored. When not in use, the microscope should be protected with a dust cover to prevent optical surfaces from becoming covered in dust, which might affect its use. In addition, the optics and stage should be cleaned with lens tissue after use. When microscopes with ultra-violet illumination are used, the period of use should be recorded and bulbs replaced at appropriate frequencies. When ultra-violet bulbs are replaced, safety gloves and eye protection should be worn as these types of bulbs can explode during replacement. Direct human contact with bulbs should be avoided so as not to etch the glass.

Microscopes can be calibrated using an eyepiece graticule and stage micrometer. The graticule is fitted inside the eyepiece and, generally, is marked in a scale of 0 - 100 units. The slide micrometer is usually marked with a 1 mm scale with 0.01 mm divisions. Calibration is normally performed by assessing the number of graticule units that equate to 1 mm on the slide micrometer. When a microscope is used for taking measurements of biological material, calibration should be undertaken on a regular basis.

5.11 Microwave ovens

Microwave ovens can be used to heat liquids, and melt agar quickly and easily before it is dispensed. However, certain precautions need to be taken when microwave ovens are used. When bottles of liquid are heated in a microwave oven, the liquid sometimes becomes super-heated and tends to boil, especially if the bottles are shaken when they are removed from the oven. Using low power for longer periods of time will minimise the risk of liquids becoming super-heated. Also, when bottles are removed from the oven they should not be shaken. Sealed containers can explode within the oven. Bottle caps or stoppers should therefore be loose before the bottles are placed into the oven, and bottles should not be removed from the microwave oven as soon as the heating process is complete, but should be left to cool down. If bottles of liquid are heated in a microwave oven, they should only be half full.

Microwave ovens should always be clean and any spillages that occur should be cleaned up immediately. Microwave ovens should be checked regularly for radiation leakage and to ensure that doors are well sealed.

5.12 Glassware

All items of glassware, such as pipettes, flasks and Petri dishes etc, used in the preparation of media or handling of samples should be of suitable quality. They should also be free from inhibitory substances and be adequately cleaned and sterilised before use. In many instances, pre-sterilised plastic items can be used. The accuracy of volumetric equipment should be traceable to national standards.

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6 General laboratory environment

The laboratory environment where microbiological examinations of water are undertaken should comply with guidelines[5] for category 2 containment. Guidelines include provision of sealed non-absorbent floor surfaces, work surfaces that are impervious and resistant to chemicals, and separate hand-washing facilities that are close to the exit of the laboratory. In addition, laboratory cupboards should be labelled with their contents and lighting for all purposes should be adequate. The laboratory environment should be cleaned frequently and in particular, work surfaces should be cleaned and disinfected often. If micro-organisms in a category 3 containment level are intentionally sought and isolated (for example S*almonella typhi*) then separate and additional requirements[5] need to be complied with.

Whilst laboratory-acquired infection is rare, staff should be adequately trained in the prevention of infection, not only to themselves but also to other colleagues. Training should include the understanding of risks from micro-organisms associated with ingestion, inhalation and skin absorption. Further guidance is given elsewhere [5, 6, 7].

7 Laboratory staff

All laboratory staff should have training records that should show relevant education and experience that have been acquired, see also section 3.1. Failure by an analyst to perform satisfactory should lead to a thorough investigation including both the adequacy of, and response to, the training. Wherever possible, staff should be encouraged to broaden their understanding and to make contact with people from similar organisations. It is important that staff should understand the principles of the tests carried out and the reasons why they are carried out.

In addition to receiving training in analytical techniques, laboratory staff should be made aware of the required hygiene practices that are so important in minimising the risks of infection when handling samples or cultures. These include basic requirements for wearing laboratory coats, the need for hand washing and personal hygiene and the disinfection of laboratory work surfaces and cleaning up of spillages. New employees should be made aware of these practices which should be observed and maintained at all times.

8 Analytical techniques

All analytical techniques should be fully documented, see also section 3.2. This includes giving descriptions of the micro-organisms, the preparation of media, the test procedures and any confirmatory tests that may be required. Each method used in the laboratory should be held in a reference file and be available to all staff. Details of procedures for preparing suspensions of test organisms (for validation purposes) and the practical details of how validation trials are conducted should also be recorded. Methods should also include reference to organisms used as positive and negative controls that are used in the isolation procedures and confirmatory tests, as well as quality control tests for assessing media.

As new methods are developed, test procedures will be replaced. Thus, it is important that new test procedures are properly validated and their performance assessed against the old test procedures. New test procedures should only be adopted after they have been shown that they are equivalent to, or better than, the old test procedures. Details of, and data generated for, the new test procedures should be fully documented. Details how to validate new test procedures are given in section 9.

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8.1 Standard operating procedures

Details of laboratory methods can be described in standard operating procedures that should include particulars of the scope of the method, the equipment required, the preparation of media and reagents, full analytical procedures and the calculation and reporting of results. A suitable format for a standard operating procedure is given in section 8.1.1. See also Parts 4 - 10 in this series as indicated on page 2.

8.1.1 Format of a standard operating procedure

1. Introduction - This section gives a brief discussion of the organism for which the method is designed. Details of its significance to drinking water in terms of water quality, indicator value, pathogenicity and occurrence are also, generally, given.

2. Scope - Details of the type of waters that can be analysed are given.

3. Definitions - The organism is defined with specific regard to the method being described.

4. Principle - Brief details of the method are given.

5. Limitations - Brief details of those circumstances where the method is not suitable are given.

6. Health and safety - References to relevant COSHH information and special hazards associated with the method are noted.

7. Equipment - Details of equipment and special apparatus specific to the method. Reference to standard equipment requirements (and performance criteria) in general guidance to laboratories section.

8. Media and reagents - Details are given of all reagents and media that are employed in the method, together with instructions for their preparation and storage and, where appropriate, whether commercial formulations and kits are available.

9. Analytical procedure - This section gives details of the procedures that need to be carried out. The section is often sub-sectioned as follows:-

9.1 *Sample preparation* - guidance is given on volumes, special storage conditions prior to analysis, and pre-treatment or dilution preparations.

9.2 *Sample processing* - Details are given on the technique (ie, membrane filtration, MPN inoculation, pre-enrichment etc) including incubation conditions.

9.3 *Reading of results* - Details of how results are read and recorded are noted (including colony counting, biochemical tests etc.).

9.4 *Confirmation tests* - Details of any biochemical, serological or other tests used in confirmation tests are referred to.

10. Calculations - Details of the procedures required for the calculation of results are presented.

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11. Expression of results - Information is given on the terms and units used for the reporting of results.

12. Quality assurance - Information on media, reagents and specification of reference organisms is described.

13. References - Technical and allied references relevant to the method are given.

8.2 Methods for the isolation and enumeration of indicator and other organisms

Two procedures for isolating and enumerating organisms are commonly used in water microbiology. These involve membrane filtration and the multiple tube or most probable number (MPN) technique. The media and incubation conditions differ with both methods according to the organism being sought. In practice, for most conventional testing of clean waters, the membrane filtration procedure is the most widely used, as it is simple to conduct and can be applied to a wide range of organisms.

8.2.1 Preparation of samples

The volume of sample submitted to the laboratory should be sufficient to ensure that all routine examinations can be carried out. Any excess sample that is not required may be stored in a refrigerator until the initial examination has been completed. This sample can then be discarded or, if required, used for additional or repeat tests in the event of unexpected high counts or possible mishaps. The enumerated counts obtained for these additional or repeat tests should either be used with caution and additional comment, or not reported, as they may not be comparable to those of the original sample examination obtained before storage.

To facilitate mixing, an air space should be present in the sample bottle. The sample bottle is inverted rapidly several times to ensure adequate mixing. If ten-fold dilutions of the sample are required, they can be prepared at this stage. Sterile solutions of quarter-strength Ringer's solution or maximum recovery diluent are suitable for preparing dilutions. Known volumes of sterile diluent solution are measured out (for example 90 ml or 9 ml) into sterile dilution bottles or tubes. Alternatively, volumes of diluent, pre-sterilised in screw-capped bottles can be used. In these cases however, it should be recognised that some bottles may suffer a loss of diluent on sterilisation or storage. Volumes should, therefore, be checked and any bottles showing obvious signs of incorrect quantities must be discarded. Whilst the bottle is held in one hand, the stopper or cap is removed with, and retained in, the other hand. A dilution of the original sample is then made, by transferring one volume of sample to nine volumes of diluent. Using a fresh, sterile pipette each time, the process is repeated as often as is necessary to ensure the correct dilution range has been prepared. Each prepared dilution is carefully and thoroughly mixed before additional dilutions are prepared. A sufficient quantity of each dilution should be prepared to enable all tests to be carried out.

8.2.2 The multiple tube (or MPN) technique

In the multiple tube technique, measured volumes of sample, or diluted sample, are added to a series of tubes containing a liquid differential medium. It is assumed that on incubation, each tube with one (or more) target organism will exhibit growth in the medium, and produce characteristic changes in the medium. Provided that some of the tubes exhibit no characteristic growth (ie the results are negative) and some of the tubes exhibit characteristic growth (ie the results are positive) then the MPN of organisms in the sample can be estimated from probability tables. Counts are typically expressed as the MPN of organisms per 100 ml

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of sample. Confirmation, that positive results are due to the growth of the targeted organism sought, can be obtained by subculture to confirmation media.

The multiple tube method is particularly suitable for the examination of sludges and waters containing sediment.

8.2.3 The membrane filtration method

In the membrane filtration method, a measured volume of the sample, or diluted sample, is filtered through a membrane filter, typically composed of cellulose-based, or similar, fibres. The pore size of the membrane filter is such that the targeted organisms to be enumerated are retained on or near the surface of the membrane filter, which is then placed, normally face upward, on a differential medium, selective for the targeted organisms sought. The selective medium may be either an agar medium or an absorbent pad saturated with broth. After a specified incubation period, it is assumed that the targeted organisms retained by the membrane filter will form colonies of characteristic morphology and colour. The growth of non-target organisms is inhibited, or if present, can be distinguished by their colonial appearance. The colonies of the target organism sought are counted and the result, taking into account any dilutions made, is typically expressed as the presumptive number of organisms per 100 ml of sample. The presumptive count may then be confirmed, by sub-culturing all, or a representative number, of colonies formed.

The membrane filtration apparatus consists of a base supporting a porous disc. The filter funnel, which may be graduated, is secured to the base, for example by means of clamps, screw-threads or magnets. The filtration apparatus is connected to a vacuum source. For the examination of large numbers of samples, multiple filtration units may be used. The filtration apparatus should be sterilised on a regular basis between batches of analyses. Spare funnels as required can be disinfected by immersion in boiling distilled water for at least one minute between samples. After disinfection, each funnel should be placed in a stand and allowed to cool before use. Alternatively, a fresh pre-sterilised funnel may be used for each sample. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should be processed after non-polluted samples.

Membrane filters, typically 47 mm in diameter, with a nominal pore-size of 0.45 μm retain most of the bacteria commonly enumerated in water. A pore size of 0.2 μm is, however, necessary for the isolation of species of *Campylobacter* and some environmental bacteria (for example *Vibrio* species). The use of membrane filters with grid-marks facilitates counting of colonies. Where there is a need to filter large volumes of sample (for example 500 ml of river water) which may block standard 47 mm diameter membrane filters, then a large volume filtration apparatus may be useful. Membrane filters of 90 mm or 142 mm diameter and appropriate porosity can be housed in stainless steel filtration units and the samples passed through the filter using a suitable pump.

Periodically, it is necessary to check that membrane filters are suitable for the targeted organisms being sought. Quality assurance is important and membranes should be free from toxic substances that inhibit bacterial growth. When membrane filters with grid-marks are used, bacterial growth should not be inhibited or stimulated along the grid-marks. Membrane filters should be pre-sterilised before use and should not be re-used. Membrane filters should not be used beyond their shelf life date.

Absorbent pads, for use with broth media, should be at least the same diameter as the membrane filters and approximately 1 mm in thickness. The pads should be made of high

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quality paper fibres, and be uniformly absorbent and free from any toxic substances that inhibit bacterial growth. Absorbent pads need not be sterile if they are of the appropriate quality. This should be verified for each batch of pads prior to use. If necessary, pre-sterilised absorbent pads are available, or pads can be sterilised by autoclaving at 121 °C for 20 minutes, either in containers or wrapped in waterproof paper or metal foil.

8.2.4 Advantages and limitations of the membrane filtration method

An advantage of the membrane filtration technique is the speed with which results can be obtained. For example, presumptive coliform and *Escherichia coli* (*E. coli*) counts and individual colonies for confirmatory testing can be available after 18 hours incubation. In addition, there is considerable saving in labour and in the amount of media and glassware required when compared to traditional MPN techniques. Furthermore, false-positive reactions that may occur with some media in the multiple-tube technique are less likely to occur with membrane filtration.

The membrane filtration technique is unsuitable for use with waters of high turbidity. In these circumstances, the membrane filter may become blocked before sufficient water has been filtered. Also, the accumulated deposit on the membrane filter may inhibit the growth of the organisms being recovered or enumerated. A membrane filtration technique may be unsuitable for use when waters are examined that contain small numbers of targeted organisms in the presence of large numbers of non-targeted bacteria that are also capable of growth on the medium used.

8.3 Statistical considerations

Whilst bacterial counts from a sample can yield valuable information, it is important to assess the reliability of this information. The expression “bacterial count” is used to imply the count of organisms being enumerated, for example coliform count; the same principles also apply to the counting of viruses or other pathogens. In terms of the assessment, there are two main factors to be considered. Firstly, what can be inferred from the sample regarding the level of bacterial contamination in the water source from which the sample was taken; ie the variability or fluctuation in bacterial content of the water? Secondly, how accurate and precise was the actual count for the sample using the method chosen by the laboratory? These two sources of variability are often confused, leading to misunderstandings in the interpretation of sample counts. Generally, in water microbiology, the first sample variability, the bacterial content at the water source, is the more important[8]. This type of variability should be clear to all staff with an interest in the submission of samples for analysis, the interpretation of results and any subsequent actions. The second source of variability, the accuracy and precision of the count, is generally of more concern to laboratory staff.

8.3.1 Estimating bacterial counts within the water source

The examination of a single sample gives an indication of the bacterial count of the sample at a particular location in the catchment area, or supply, at a particular time. The location where a sample is to be collected should be carefully chosen, and thus, a sample should be typical of the sampling area. The actual volume of water sampled may not however possess identical characteristics, with respect to microbiological quality, as those present in adjacent volumes of water. Indeed, only a very small volume of water is examined in the laboratory compared with the volume of source water in question. The confidence interval (CI) for the bacterial density in a body of water cannot, generally, be estimated from a single sample. A mathematical description of the distribution of bacteria is required before a range, such as a

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95 % confidence interval, can be estimated. The only situation where a single sample can give such an estimate is when the bacteria are distributed randomly, and in this case, the appropriate mathematical description is the Poisson distribution which has a single statistical parameter, ie the mean, μ, has the same value as the variance. However, there is at present no evidence that bacteria are ever randomly distributed in any part of a water system.

There tends to be enormous variation in the microbiological quality of untreated waters[8]. For treated waters, contamination may be intermittent and organisms may be present as aggregates, often on particulate matter, rather than evenly or randomly distributed. Thus, samples from the same sampling point, even when taken closely adjacent in time, can show large differences in bacterial counts[9]. The statistical parameters describing the distribution of bacteria may change over time and, therefore, a series of single samples collected at different times cannot be used to estimate confidence intervals for the bacterial content of the source of water at any one time. They should be used instead to indicate trends over time.

8.3.2 Estimating the accuracy of counting bacteria from a single sample

Differences between results from the analyses of a single sample can arise for several reasons. For example, from inadequate sampling procedures and from prolonged delays or unsuitable storage conditions that occur between sampling and commencement of analysis. These types of differences will not be considered here, neither will differences caused by accidental contamination. Two possible sources of error that will be considered are those caused by the effects of dilution and the method of counting the organisms. Both these sources are inherent in laboratory methods and are manifest in the second source of error outlined in section 8.3.

8.3.2.1 Potential imprecision due to dilution of the sample

Samples of treated water should not need to be diluted before examination. Samples of heavily contaminated, untreated water may, however, require dilution in order that a suitable count can be enumerated. If the multiple tube technique is used, then some tubes, but not all, should exhibit characteristic growth of the target organism. If the membrane filtration technique is used, then the membrane filter should not be overgrown with target organisms, or indeed, non-target organisms.

It is usual practice to report the bacterial count of targeted organisms, as the number of organisms per 100 ml of sample. With undiluted waters, 100 ml of sample is examined by the membrane filtration technique, and 105 ml by the MPN technique, for example in the 11-tube series of 1 x 5 ml, 5 x 10 ml and 5 x 1 ml. If the sample requires dilution (prior to any additional dilution inherent in the multiple tube method) and this dilution is, for example 10-fold, then only 10 ml (or 10.5 ml) of the original sample will be examined. The count obtained is then multiplied by the appropriate dilution factor, and the calculated count per 100 ml is now an estimate of the number of organisms contained in 100 ml of sample and a confidence interval can be calculated using random distribution theory[10]. Some examples are shown in Table 8.1.

The variability introduced by dilution is likely to be relatively small compared with the variability in bacterial density in a water source, where numbers are sufficiently high to require dilution of the sample before examination. Confidence intervals, as shown in Table 8.1, should not be stated when results are reported, as quoting such intervals may cause misunderstandings and be taken as a statement about the likely bacterial density in the water source. Table 8.1 illustrates the additional imprecision introduced into the bacterial count when dilutions are used and volumes smaller than 100 ml are examined.

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Table 8.1 Estimated count per 100 ml and 95 % confidence intervals for the number of organisms reported in 100 ml of sample, where a sub-sample is examined, following dilution

Number of organisms found in sub-sample	10-fold dilution		100-fold dilution	
	EC	CI	EC	CI
10	100	50-180	1000	480-1830
50	500	380-650	5000	3750-6640
100	1000	820-1200	10000	8190-12200

EC = estimated count
CI = 95 % confidence interval

8.3.2.2 Other imprecision of laboratory methods

Samples of treated waters should not contain indicator organisms, and the presence of very small numbers of such organisms in samples of water are capable of being detected, and enumerated with good precision, by methods described in this series, see page 2. Untreated waters, however, may yield moderate or high bacterial counts, and in these situations, the accuracy with which the count is made should be considered.

The overall bias and precision of a method should be established by primary validation[11] and comparison of results obtained using a reference method. A protocol for undertaking such a comparison, together with examples, is described in section 9.

8.3.2.3 Multiple tube (or MPN) method

In the multiple tube method, a series of sub-samples is taken from the original sample, and processed to ascertain which of the sub-samples show the presence of the targeted organism. A mathematical formula, based on laws of probability, is then used to estimate the MPN of organisms present in the sample[12, 13, 14]. These various mathematical approaches and the principles involved in the estimation of bacterial densities by dilution methods have been reviewed[14, 15] and tables have been developed[16, 17] which give greater detail. However, in practice, the full extent of the tables are rarely used[18, 19].

A confidence interval was often published with the MPN that demonstrated the variability of the count, not the variability of the water source, although it has often been confused with the latter. For this reason, confidence intervals of this sort should not be reported.

Modern computers now enable the determination of the probability of counts associated with each dilution series to be quantified exactly[20, 21]. While the latest calculation of the MPN shows little discrepancy with previously published values, these new calculations have highlighted two issues. Firstly, the variability of previously published confidence intervals. Secondly, for moderate or high bacterial density, the multiple tube method does not give a clear MPN. There is a “most probable range” (MPR) of counts, all of which are almost equally likely to be as correct as the MPN. All calculations are based on the assumption that the organisms present in the water are evenly or randomly distributed and the importance of thorough mixing of the sample cannot be over-emphasised. Although the multiple tube method is very sensitive for the detection of small numbers of indicator organisms, the MPN is not an exact value unless very large numbers of tubes are examined, as is the case with recently developed multi-well MPN techniques. Apparent differences between results should, therefore, be interpreted with caution. It should be appreciated that variations in bacterial

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numbers in the water source may be very much greater than any variability introduced by the multiple tube method.

8.3.2.4 Membrane filtration method

If a sample of water is filtered and the membrane filter incubated, and then every relevant colony on the membrane filter is counted, and every colony is tested and confirmed, then the presumptive and confirmed counts are as precise as the method allows. No further statistical imprecision need be considered. If the sample is diluted prior to filtration then the count becomes an estimate of the bacterial density in the undiluted sample, as already described. If the presumptive count is obtained from only a segment of the membrane filter then variability is introduced comparable to that introduced by diluting the sample, assuming that the segment is typical of the whole filter.

8.3.3 Confirmation of isolated organisms

Confirmatory tests of the presumptive colonies present on a membrane filter should be carried out. When multiple colonies are present, different approaches can be adopted when consideration is given to the number of colonies that should be tested for confirmation. If the aim is to estimate the count of relevant colonies, then consideration should be given to the variability that is introduced when only a fraction of the total number of colonies present is tested for confirmation. The colonies should be chosen at random and the number tested should provide a sufficient level of accuracy. This may require sub-culture of all the colonies on a membrane filter when fewer than ten presumptive colonies are present. However, this may not be practicable and may not be necessary, especially in the case of highly specific methods where all the colonies are expected to confirm as positive.

Alternatively, if the aim is to demonstrate the presence or absence of the targeted organism, then a different approach may be chosen, provided there are no microbiological contra-indications. The presence of the organism is demonstrated as soon as one colony is tested and a positive confirmation is made. Hence, a laboratory may choose to examine fewer colonies, initially, than when the aim is to estimate the count, rather than demonstrate presence or absence. However, if the colonies that are chosen and tested for confirmation, and positive confirmations are not obtained, then the sample cannot be assumed, at this stage, to be free of confirmed organisms. This is because other colonies on the filter, which have not been chosen for confirmatory testing, may, if tested, prove positive. Hence, other colonies from the membrane filter should be tested until at least one positive confirmation is obtained, or all colonies have been tested and no confirmation has been shown. This sequential testing is acceptable only when refrigerated storage of the membrane filter is not detrimental to the survival and/or recognition of the relevant organism.

If all presumptive colonies are tested to confirm their nature, then no further imprecision (other than that due to the method) is introduced when the presumptive count is converted into a confirmed count. If only some of the colonies are tested by confirmatory methods, then further imprecision is introduced into the confirmed count. For example, if a presumptive count is made by counting all the typical colonies, N, on a filter then it is common practice to make confirmatory tests on some, but not all, of these colonies, unless N is small. If n is the number of colonies tested, and x is the number of colonies that are confirmed as the target organism, then the confirmed colony count is estimated as xN/n. For example, if 60 colonies were observed on the filter, and 20 colonies were selected at random for testing, and 15 of these colonies were confirmed, then the estimated confirmed count would be
15 x 60 / 20 = 45.

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It is assumed that the "n" colonies are selected at random, or by some other procedure which ensures they represent a typical sub-sample of the "N" colonies. It is further assumed that all the "N" colonies are equally likely to be from the relevant organism group. The conditional probability that y is the true count, given that x colonies have confirmed can be calculated from:

$$P(x \mid y) = {}^{y}C_{x} \,.\, {}^{N-y}C_{n-x} \,/\, {}^{N}C_{n}$$

The 95 % CI for the confirmed count can be found by observation of the probabilities for all possible values of y, using the observed value of x. The CI will exclude "end of range" high and low values of y, such that their cumulative conditional probabilities sum to less than or equal to $0.05^{(10)}$.

As an example, if 10 colonies are observed (N) and only two colonies are tested (ie n = 2) then Table 8.2 shows the complete range of probabilities.

Table 8.2 Range of probabilities, if 10 colonies are counted and only two colonies are tested for confirmation

Given that y =	the probability that		
	x = 0 is	x = 1 is	x = 2 is
0	1.000 }		
1	0.800 }	0.200 }	
2	0.622 }	0.356 }	0.022
3	0.467 }	0.467 }	0.067 }
4	0.333 }	0.533 }	0.133 }
5	0.222 }	0.556 }	0.222 }
6	0.133 }	0.533 }	0.333 }
7	0.067 }	0.467 }	0.467 }
8	0.022	0.356 }	0.622 }
9		0.200 }	0.800 }
10			1.000 }

The brackets (}) show the range of y values which should be included in the 95 % CI for the true count. It should be noted that if one of the two colonies tested confirms (ie x = 1) then all the possible values of y are within the 95 % CI because all the probabilities exceed 0.05.

In general, if only a small number of the total colonies are tested, then the CI can be very wide. They also tend to be wider if a substantial number of colonies turn out not to confirm. This may not be of concern in cases where a positive result may mean a breach of any regulations. Some examples are shown in Table 8.3.

Table 8.3 Variation in the 95 % CI with variation in the proportion of tested colonies confirmed

Colonies observed (presumptive count) ie N	Number tested, ie n	Number confirmed, ie x	Confirmed count	95 % CI
10	2	0	0	0-7
10	2	1	5	1-9
10	2	2	10	3-10
14	7	5	10	6-12
50	10	5	25	9-41

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With treated waters, where the vast majority will yield zero or very few presumptive colonies, then to improve accuracy, as many colonies as possible should be tested by confirmatory methods. For untreated waters, it may be worthwhile considering the use of the presumptive count rather than introduce the additional variation which accompanies confirmation of some but not all of the colonies. It should be noted that the practice of confirming a maximum of 10 colonies can still introduce potential significant variation, especially if the presumptive count is large and some colonies fail to confirm. However, a balance should be made between the benefits of improved accuracy and the capacity of the laboratory to undertake confirmatory tests for large numbers of colonies.

8.3.4 Comparing results with prescribed limits

Typically, prescribed microbiological limits for drinking waters concentrate on the presence or absence of indicator organisms and pathogens. Therefore, the potential problem of how to compare actual counts enumerated and estimated counts calculated need not be addressed with respect to potable waters.

8.3.5 Reporting results

The report should be a clear statement of the findings. A further statement on sample error, to qualify these findings, should not be necessary for routine samples. The sampling strategy should be designed with the aim of acquiring an adequate level of information. If it is necessary that a report for a special or unusual sample warrants a statement on accuracy and precision, then a clear distinction should be made between the variability of the water source and the variability inherent to the laboratory method.

Absence of organisms or immeasurably high counts should be reported according to the following criteria:

No organisms detected. A water sample in which no relevant organisms are detected should be reported as “none found in the volume of sample examined”. It should be noted that in microbiological terms there is no equivalent to the chemical concept of “limit of detection”. An expression such as “less than 1 per unit volume” has no meaning.

Overgrowth of membrane filter or all multiple tubes positive. This means that the analysis has failed to estimate the true count either because of insufficient pre-dilution or the presence of high numbers of non-target organisms. With the multiple tube method it is customary to report this, in the appropriate units, as “> 180” for the 11 tube series or “> 1800” for the 15 tube series, but recognising that the count could be very much higher. With membrane filtration and other methods the report should be “count too high to be estimated at the dilution used”.

8.4 Internal quality control

To ensure that a laboratory is capable of isolating, accurately identifying and enumerating micro-organisms in a sample, and to avoid contaminating samples with extraneous micro-organisms, it is necessary to undertake a system of internal quality control. This consists of submitting quality control samples to the usual isolation, enumeration, identification and confirmatory procedures normally used with real samples. Quality control samples should contain micro-organisms similar to those normally sought, and where appropriate non-target organisms, as well as samples that are sterile. If the procedures function satisfactorily, such micro-organisms will be detected, or in the case of sterile samples, no micro-organisms will

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be found. The control procedures should be undertaken in parallel with each batch of samples examined. This may necessitate several positive and negative control samples, and blank samples being set up each day, with separate quantitative testing schemes to check enumeration. See also section 8.5.

Positive control samples contain target organisms that produce typical colonies or positive reactions on isolation media and in confirmation tests. Negative control samples contain non-target organisms that do not produce colonies or positive reactions, or produce atypical colonies, on isolation media and in confirmation tests. Blank control samples are usually sterile samples used to test the integrity of the analytical procedure.

Control organisms should, wherever possible, be derived from freeze-dried, wild-type reference strains, obtainable from reference sources, and reconstituted and diluted with quarter strength Ringer's solution, or similar appropriate diluent, to contain suitable numbers of organisms. However, care should be taken in the selection of these organisms as some have been shown to give atypical results when compared to genuine wild-type organisms. It is good practice to minimise the number of sequential sub-culture operations as the biochemical characteristics of some strains may change on repeated culturing. The use of natural waters, known to contain relevant organisms, may also be suitable as analytical control samples. All confirmatory tests should include positive, negative and blank control samples.

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8.5 Quantitative internal quality control

In addition to qualitative checks with positive, negative and blank control samples there should be checks on the enumeration procedures[22]. Two approaches can be considered and involve the use of appropriate reference materials and the use of split sub-samples from a source known to contain the target organism.

If automatic counting instruments are used these should be tested and calibrated against known values.

Quality control or Shewhart charts are used extensively in the water industry for demonstrating statistical control of laboratory chemical procedures. This practice can be extended for demonstrating microbiological control. However, the natural random variation in the number of organisms present in sub-samples of the same sample means that there can be a wide scatter of results between sub-sample analyses, which is to be expected. Many more samples are required for microbiological examination compared with chemical analyses in order to detect real "out of control" situations. Even then, these situations would better be described or classified as probably out of control rather than definitely out of control. Hence, for microbiological purposes, "guidance charts" are used with appropriate response lines, rather than action or warning lines that trigger remedial action or further investigation.

8.5.1 Reference materials

Guidance charts can be plotted using regular counts enumerated on samples taken from a batch of suitable reference material that may be commercially obtained or internally prepared. The usual practice is to plot the results sequentially over a period of time.

If the reference material does not possess a statement of certified mean and variance values, then these values should be estimated from a suitable number of replicated analyses. These analyses should be carried out under conditions that ensure the values are "in control" or assumed to be "in control". The chart is plotted using the values determined or constructed

from these data using transformed data, for example, square root or log count data. Response lines are then drawn on the chart at appropriate intervals. Appropriate response lines may be located at ± 2 standard deviations of the mean (equivalent to upper and lower "warning" limits) and at ± 3 standard deviations of the mean (equivalent to upper and lower "action" limits). Regular samples of the reference material are then processed with routine samples and the counts plotted sequentially. Documented action should follow if values are recorded that fall outside the range of the response lines. The following guidance is often used as a basis for action[22].

(i) One count falls outside an action limit:
(ii) Two out of three successive counts exceed the same warning limit:
(iii) Nine consecutive counts fall on the same side of the mean:
(iv) Six consecutive counts show a trend that continuously rises or falls.

It should be noted that the original estimates of the mean and variance values may not be totally reliable and may need to be further studied, especially if action is triggered repeatedly because response limits are exceeded and remedial action does not identify appropriate causes. In addition, the quality of the reference material may need to be questioned.

If possible, the counts for the reference material should be enumerated without prior knowledge of the mean and variance values. A guidance chart that does not exhibit some degree of variation in counts (in line with random variation) may be indicative of operator bias.

8.5.2 Split samples

Quality control checks for consistency in enumeration can also be made using split samples. Split samples comprise a sample divided into 2 sub-samples, each of which is analysed with each batch of routine samples. The use of split samples should involve samples that are known to contain target organisms. The duplicate sub-samples can be considered as two halves of a single sample, and the results can be plotted on a chart containing appropriate response limits.

Because of the manner in which micro-organisms are distributed in water, the examination of split samples can result in significant variation in the counts enumerated. For example, if the count reported for the first sub-sample is 5, then the 95 % CI for the count of the second sub-sample will be 0 - 14. The CIs for the count of the second sub-sample, given the count observed in the first sub-sample are given in Annex A. Thus, it may be expected that duplicate sub-samples will give counts outside of the 95 % CI, on 5 % of occasions, ie once in every 20 samples. Procedures should be adopted within the laboratory to deal with situations that occur too frequently (ie greater than 5 % of occasions) where sub-samples give counts outside of the 95 % CI. The count for the first sub-sample should be recorded on a control chart, together with the corresponding CI for the count of the second sub-sample (obtained from table A1 in annex A). The count of the second sub-sample is then recorded alongside these figures. If this count falls outside the range of the CI, then this fact should be recorded. If, over a period of time, the count of the second sub-sample falls outside the range of the CI on more than 5 % of occasions, then investigations should be carried out to determine the cause[23].

Alternatively, a more approximate statistical approach can be used with paired counts using the Index of Dispersion chi-squared test[15, 24]. For paired split samples, the formula for calculating the Index of Dispersion, D, is:

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$$D^2 = (x_1 - x_2)^2 / (x_1 + x_2)$$

To construct a guidance chart, the median is plotted, as are values of the 99% and 95% confidence level limits, ie for p = 0.05 and p = 0.01 (ie 3.841 and 6.635 respectively, each with 1 degree of freedom). These values are approximately equivalent to 2 and 3 standard deviations, and act as appropriate "response" limits. The calculated values of D^2 obtained for split samples should be equally distributed on either side of the median line.

Laboratories using split sample internal quality control should carry out analyses regularly, and plot the results on guidance charts. Each sub-sample should be treated as separate samples and analysed in the normal, routine manner. The sub-samples should be randomly positioned in the incubator, and these positions should be changed frequently when different batches of samples are examined. If possible, counts should be enumerated in such a manner so as to ensure that the sub-samples are not recognised as being connected. If the variation between the counts of the sub-samples is significantly less than would be expected, then operator bias may be suspected.

8.6 External quality control

Laboratories should participate in an appropriate inter-laboratory external quality control scheme that involves the examination of samples distributed by an external organisation. The laboratory's results can then be compared with those obtained by other participating laboratories and provide an independent assessment on the quality of the laboratory's performance. It is essential that the samples distributed by the scheme organiser are treated and analysed in exactly the same way as routine samples, and that appropriate action is taken when results fall outside of the expected range.

It should be noted that the purpose of external quality control samples is to assist individual laboratories assess their own capabilities to undertake selected analyses and to correct any deficiencies which may be present. They should not used for the purpose of determining whether one laboratory performs better or worst than another participating laboratory.

9 Comparison of methods

9.1 Introduction

Methods for drinking water bacteriology should be capable of serving their intended purpose, ie to detect and/or quantify target organisms or groups of target organisms with adequate precision and accuracy. In addition, the validation of microbiological methods is important and a recent report[(11)] describes various procedures for carrying this out.

In certain countries methods are prescribed, in other countries, they are not. If alternative methods are used in place of statutory, regulatory or laboratory accredited methods, they should be of "equivalent or better" performance. Methods, capable of achieving a certain performance are published by a variety of sources, including those shown in the Table 9.1.

Table 9.1 Sources of methods

International Organisation for Standardisation (ISO)
European Committee for Standardisation (CEN)
Individual national standards organisations (for example BSi, DIN, AFNOR)

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Standing Committee of Analysts (SCA)
The American Association of Official Analytical Chemists
American Public Health Association

The demonstration that new methods are at least as accurate and precise as reference methods is, however, a complex procedure. This section describes a protocol for comparing the recoveries of confirmed target organisms by two or more methods. The protocol and procedures described have been developed from those derived for the Drinking Water Inspectorate[25].

9.2 Scope of procedures

This section describes the procedures for establishing the equivalency of microbiological cultural methods used in drinking water bacteriology, for example the replacement of a reference or standard method by an alternative new method. Instructions, including the preparation of spiked samples and the recommended number of measurements, are described to evaluate whether a new method as a replacement for a reference method could be adopted for routine use in the laboratory. The new method should, before evaluation, be thoroughly validated. The comparisons involve two stages. Stage 1 compares the results of 2 methods using samples containing about 20 - 50 target organisms per test volume, usually 100 ml. Stage 2 compares the results of the same methods using samples with lower levels of target organisms, usually in the range of about 1-10 target organisms per test volume. Only paired samples with at least one positive result are considered, as paired samples with zero counts do not provide additional information on the comparative recovery of target organisms.

9.2.1 Statistical approach and acceptance criteria

The comparison of a new method with a reference method should be undertaken with an appropriate diversity of target and competing non-target organisms (obtained from a variety of sources) relevant to the test methods. The preparation of suitable samples (see section 9.6.2) is very important and the waters used should be derived from several sources. Each source is referred to as a “category of origin” and samples of water are taken over different periods of time. In stage 1, samples should be used which produce enumerated counts within the optimum ranges of both methods. These counts should yield sufficient numbers of organisms to provide a meaningful statistical comparison. For example, with a membrane filtration method, a suitable range of 20 - 50 target organisms per unit volume (typically, 100 ml) is estimated to be sufficient. If the method enumerates more than one target group of organism (for example *E. coli* and coliform bacteria) then separate tests may be necessary to ensure that each target group is enumerated in the range. In addition, guidance is given on the number of samples needed to provide sufficient statistical information to reach a positive conclusion.

These criteria, described in section 9.6, require a clear presentation of the data, a statistical comparison within each category of origin of samples, and finally, an overall statistical comparison. The new method should be rejected if it is shown that significantly lower average counts are obtained than those obtained using the reference method. The new method may be accepted, subject to the conclusions reached in stage 2 of the trail, if it is shown to be better than the reference method or the following criteria are satisfied:

(i) it is demonstrated that there is no statistical, significant difference between the methods; and

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(ii) the 95 % confidence interval for the average difference lies above the value which would indicate that the new method was finding 10 % fewer organisms than that found by the reference method.

A new method, which is found to be acceptable after stage 1 has been completed, should then be tested against the reference method as described in stage 2. This should then demonstrate that there are no major differences between the two methods when much lower counts are compared.

9.3 Basic concepts and definitions

A laboratory considering an alternative new method to one currently in use should obtain sufficient comparative data to demonstrate the equivalence of the two methods before adopting the new method for routine use. If appropriate, other laboratories should then undertake the process of full data comparison and the data from all laboratories may then be pooled and reviewed to establish robustness.

9.3.1 Microbiological cultural methods

Methods are considered microbiological cultural methods when growth and multiplication of micro-organisms are essential features for their detection and/or quantification.

9.3.2 Definitions

Confirmed counts - The number of presumptive counts multiplied by the proportion confirmed, that conform to the definition of the target organism.

Error - The statistical variation including natural variation and imprecision of the method.

Over-dispersion - The variation in excess of that shown by the Poisson distribution.

Poisson distribution - Fully random distribution of particle numbers when sampling a perfectly mixed suspension, exhibiting no attraction or repulsion between micro-organisms.

Precision - The closeness of agreement between independent test results obtained under stipulated conditions.

Presumptive counts - The number of organisms that produce a response typical of the target organism in or on a primary detection medium.

Primary validation - Establishment of the specifications for the performance of a new method and/or experimental verification that a method meets theoretically derived quality criteria.

Repeatability - Closeness of the agreement between the results of successive measurements of the same measure carried out under repeatability conditions of measurement. For example, this can be calculated from replicate counts from sub-samples obtained from a well-mixed sample, analysed by one analyst using the same reagents and method.

Reproducibility - Closeness of the agreement between the results of measurements on the same measure carried out under reproducibility conditions of measurement. For example, this can be calculated from replicate counts from sub-samples obtained from a well-mixed sample,

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analysed by more than one analyst or laboratory using different reagents, but the same method.

Robustness - The insensitivity of an analytical method to small changes in procedure.

Validation - The procedures undertaken which establish the performance of a new method.

Verification or secondary validation - Demonstration by experiment that an established method functions according to its specifications in the user's hands.

9.4 Sources of variation and error

As already described in section 8.3, there are several sources of variation that may complicate the evaluation of comparing alternative methods for drinking water bacteriology. These include sample variation, natural variation and systematic imprecision inherent in the methods.

9.4.1 Sample variation

A water source, sampled for monitoring purposes, may exhibit enormous variation in its microbial content over time and between sampling sites[9]. Samples, used in comparative trials of alternative methods, should, therefore, not be collected or prepared separately. A paired or split sample approach (see also section 8.5.2) should be used. A suitable sample should be thoroughly mixed, and two aliquots of this sample taken for analysis. The analysis of each aliquot should then be carried out at the same time, the first aliquot being analysed by one method and the second aliquot being analysed by the other method. Over time and on average, the theoretically expected number of organisms in both aliquots should be the same.

9.4.2 Natural (random) variation

Figure 9.1 illustrates the volume of a thoroughly mixed sample of water containing 30 micro-organisms that are randomly distributed. For each of the ten identically marked aliquots, it is important to note that the number of organisms present in each aliquot may not be the same and that these numbers may differ purely by chance. Overall, the average number of organisms is 3 per unit aliquot. However, as depicted, the range is shown to be 0 – 7. This type of variation within a sample will always occur in water microbiology. In addition, over-dispersion may occur, as a result of the attraction or repulsion between organisms and suspended matter, laboratory equipment or other organisms.

To accommodate this natural variation, many samples need to be analysed to evaluate the systematic variations that may exist when different methods are compared. Sufficient data should be generated to average out the effects caused by the natural variation depicted in Figure 9.1. An example of this natural variation is illustrated in Figure 9.2 which shows the results of 50 paired water samples examined for the same organism using the same method[23]. As shown, the results are scattered and the correlation between the pairs of counts appears low. The correlation coefficient or product-moment statistic, r^2 is calculated as 0.39, even though it might be expected that a value of 1 should be generated under theoretical or ideal conditions. This illustrates that the use of this statistic, r^2, is not appropriate in these cases. Hence, the correlation between paired counts needs to be assessed and interpreted against this background of inevitable variability.

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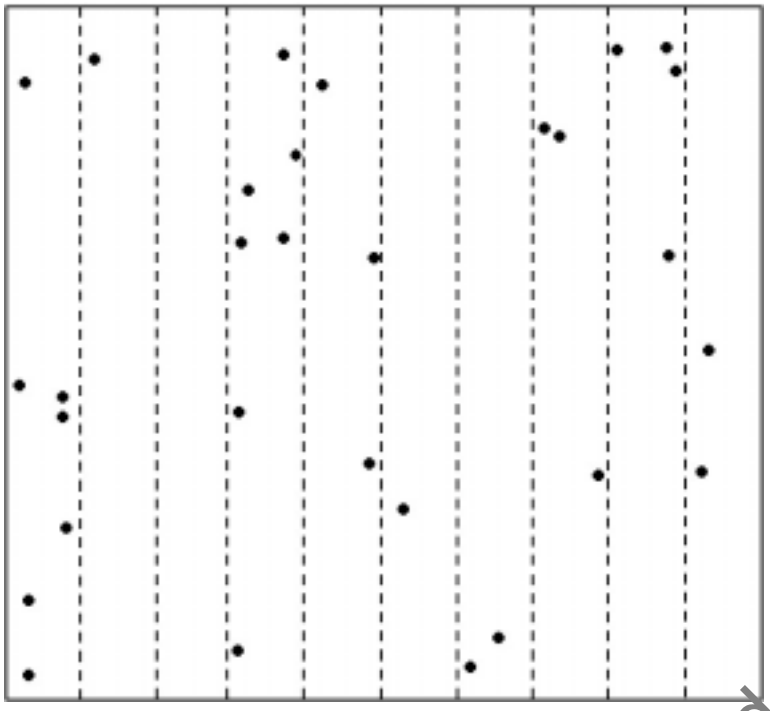
Figure 9.1 Random variation of organisms in aliquots from a sample containing 30 organisms



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9.4.3 Other sources of variation

Other factors can affect either the number of organisms present or the numbers detected and reported. These factors include inadequate mixing of samples and inaccurate measurement of aliquot volumes. Also, errors in the number of organisms reported can be introduced by equipment, analysts or laboratory procedures, as well as by the methods used. A small amount of random variability is expected from every procedure and this can be acceptable. However, excessive random variability might indicate an imprecise method and this should become apparent during the characterisation of a new method (see section 9.5). Non-random or systematic variation, for example due to the inadequacy of the method, should be highlighted during method validation when a new method is being evaluated. Any investigation, therefore, needs to separate or distinguish the variation caused by or inherent to the methods used and that resulting from natural or random variability.

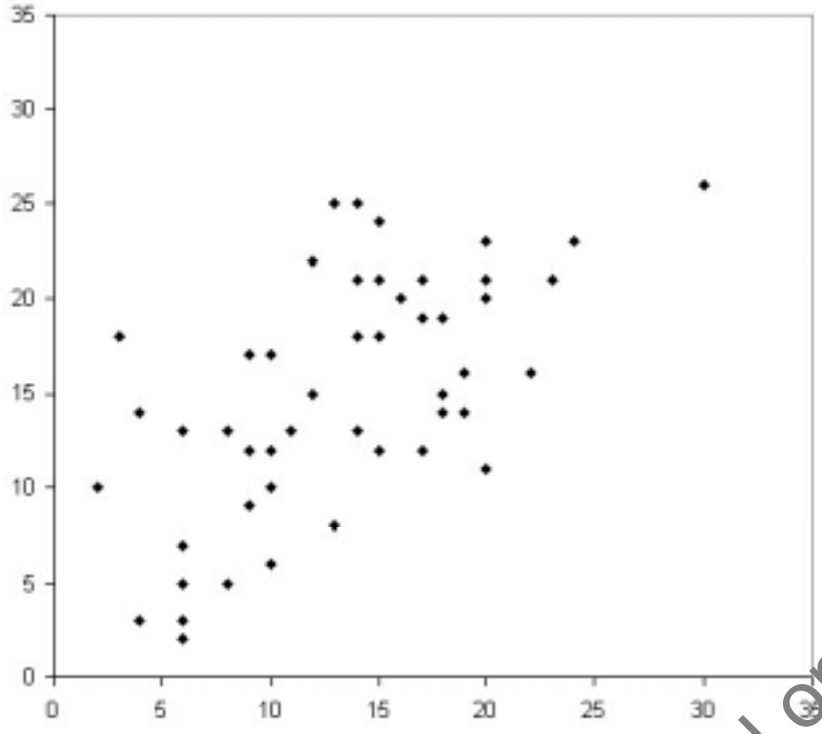
9.4.4 Statistical detection of other sources of variation

Method comparison studies have been designed and analysed to detect whether other sources of variation are present, and whether they are microbiologically and/or statistically significant[26]. The sources of variation in the enumeration of the relevant organism become apparent when the components of that count are studied. For example,

$$y_i = \mu + \varepsilon_i \qquad (1)$$

where: i is 1 or 2, representing the first or second aliquot in the paired sample;
y_i is the number of organisms enumerated;
μ is the mean value for the sample; and
ε_i is the random error.

Figure 9.2 Pairs of replicate counts of coliform organisms



The y-axis represents counts on the first sub-sample
The x-axis represents counts on the second sub-sample

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Equation (1) can be expanded to:

$$y_i = \mu_t + m_l + m_m + \varepsilon_{ti} + \varepsilon_{li}$$

where: μ_t is the true mean value of organisms present in the whole sample;
m_l is the laboratory effect (independent of the two methods);
m_m is the method effect (m_{ref} = reference method and m_{new} = new (trial) method);
ε_{ti} is the random or natural error between aliquots;
ε_{li} is the random measurement error in the laboratory.

The laboratory effect plus the method effect (ie $m_l + m_m$) is the systematic, average difference from the true mean when that method is used. It represents the bias and is inversely proportional to the "trueness" of the measurement.

Random variation reflects precision and hence, the difference between the paired counts is:

$$y_1 - y_2 = (\mu_t + m_l + m_{ref} + \varepsilon_{t1} + \varepsilon_{l1}) - (\mu_t + m_l + m_{new} + \varepsilon_{t2} + \varepsilon_{l2})$$

$$= (m_{ref} - m_{new}) + (\varepsilon_1 + \varepsilon_2)$$

If sufficient samples are examined then the random errors should average to zero. Thus, the expected value of $y_1 - y_2$ may be represented as:

$$E(y_1 - y_2) = m_{ref} - m_{new}$$

Any interaction between method and laboratory will be included in this expression but does not affect the conclusions about the effectiveness of the new (trial) method in a particular laboratory undertaking the trial. Because the absolute errors may be large (due to the natural random variation) the precision will be low and a large amount of data will be required for a powerful statistical estimate of ($m_{ref} - m_{new}$).

9.4.5 Limitation of errors

Errors should be minimised or eliminated by implementing a quality assurance programme that includes the use of internal quality control samples and participation in an appropriate external inter-laboratory quality assessment scheme.

9.4.6 A “confidence level” approach

When two methods are compared, it may not be statistically acceptable to analyse a pre-determined number of samples for each stage of the comparison tests. Whilst it may be possible to theorise about the number of samples that might give a 95 % probability of detecting a difference, the distributions of counts in the samples examined might be different from those predicted. A “confidence level” approach is, therefore, used to provide the statistical power necessary for the evaluation of a new validated method to be undertaken before it is accepted for routine use within a laboratory.

The approach for comparing method A (for example, a reference method) with method B (for example, a newly proposed trial method) is made on the basis of recording the difference in results obtained for paired sub-samples of a sample. This data set, when complete, is then progressively evaluated to ascertain whether the average results are comparable and the confidence intervals are acceptable.

9.5 Prerequisites for method comparisons

9.5.1 Initial characterisation of a new method

During method development of a new, modified or previously inadequately characterised method, it is paramount that certain optimum conditions should be established. Primary validation should establish the operational limits and performance characteristics. The validation should provide numerical and descriptive specifications for the performance and include a detailed and unambiguous description of the target organisms. An example of a validation study[11] is shown in Figure 9.3.

Primary validation of the method should establish the specificity, selectivity, relative recovery and other characteristics[11]. The method should be tested with samples containing low numbers of target organisms. Additionally, primary validation should establish that the repeatability and reproducibility of the method are acceptable.

Descriptions of methods for the bacteriological examination of water should provide details of performance characteristics and their specifications including the scope of the method, incubation robustness and time sensitivity, reliable working limits, and target definition and identification. Additionally, advice should be provided on, and requirements stated for, quality control of media and equipment. Protocols should provide laboratories with structured procedures to assist the application of the method and, therefore, the capability to generate valid results. For example, details should include statements on sensitivity, selectivity, counting uncertainty, replicate analysis, within-sample variation, and proportionality.

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9.5.2 Common identification of target and non-target organisms

Microbiological methods should be designed to detect and/or enumerate particular types of micro-organisms, ie target organisms. All other micro-organisms, ie non-target organisms, that may be present in the sample should be not detected, or if they are, should be readily differentiated, and therefore, should not interfere with the detection or enumeration of the target organisms. Non-target organisms are often described as competitive or background flora. The definition of target organisms should reflect current microbiological understanding, and be sufficient to ensure common differentiation between target and non-target organisms when two different methods are being compared.

9.5.3 False-positive and false-negative results

If a non-target organism is mistakenly identified as a target organism, a false-positive result is obtained. Alternatively, a false-negative result is obtained when a target organism is not correctly identified. Note that a false-positive result or a false-negative result may be reported for individual colonies, as well as for the overall final result of a sample. The nature and concentration of target organisms and non-target organisms often vary considerably between samples taken from a specific location, and especially from those taken from different locations. A consequence of this is that a method that has been evaluated for a particular type of sample may not necessarily have universal applicability. Methods have been described in international standards, or prescribed as legal requirements, as a means of achieving a standardised approach to analysis. If these methods are clearly and unambiguously described then inter-method differences are eliminated. This does not mean that an international standard or prescribed method is suitable for all situations and samples. The laboratory should be responsible for evaluating the performance of a method, especially when different types of samples are analysed by the same method. The temporal variation of the performance of a method, in relation to variable characteristics of the micro-flora, should be evaluated as part of any quality assurance programme.

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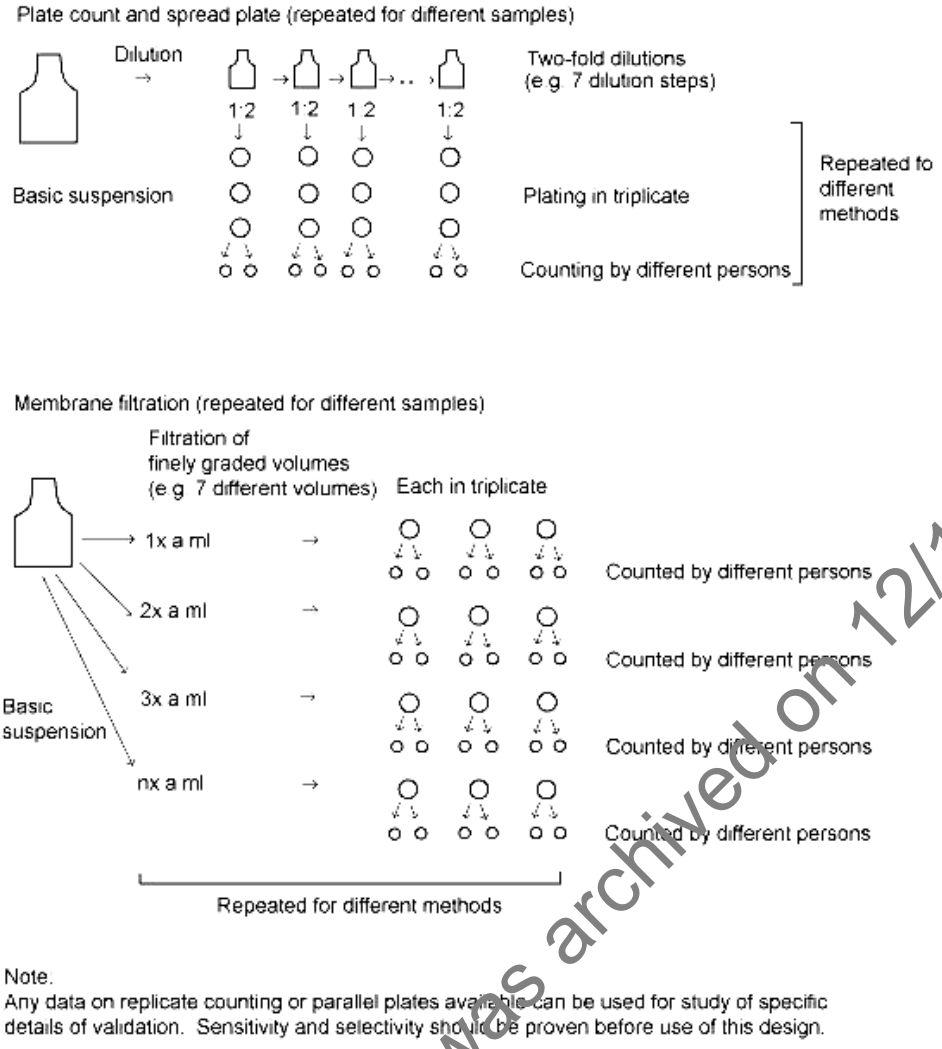
9.5.4 Quality assurance

It is essential that any laboratory that carries out a comparison of microbiological methods according to this guidance should adopt appropriate quality assurance systems. Accreditation, whilst beneficial to the laboratory in terms of documentation, does not guarantee a laboratory's performance. The sources of "error" or variability (as defined in 9.3.2) that affect microbiological methods include;

(i) sample error - the variation in enumerated counts as a result of taking of sub-samples;
(ii) natural error - the variation due to the Poisson distribution of organisms in a liquid matrix;
(iii) systematic error - the imprecision inherent in the method used; and
(iv) random laboratory error - the imprecision that is particular to a laboratory or analyst.

In an attempt to minimise the effects of systematic and random "errors" it is essential that laboratories make use of appropriate reference materials and take part in inter-laboratory, external quality assessment or proficiency testing schemes. In addition, attention should be paid to media, incubators and membrane filters.

Figure 9.3 Example of a validation experiment



Note.
Any data on replicate counting or parallel plates available can be used for study of specific details of validation. Sensitivity and selectivity should be proven before use of this design.

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9.5.4.1 Media

It has been shown that small changes in the composition of the medium used in a particular method can affect the method performance, in particular its ability to recover target organisms from certified reference materials[27]. There may also be batch to batch variation in the quality of the medium used. Ideally, the comparison of microbiological methods should use a single batch of medium that has been performance tested using reference materials. Media should be prepared correctly, paying attention to the conductivity of the water, the pH of the medium (before and after sterilisation) and ensuring that the time the medium is exposed to high temperatures is minimised.

9.5.4.2 Incubators

The temperatures of incubation used for water microbiology are, usually, critical. The temperature in an incubator can vary between individual shelves and be affected by loading patterns. Incubators should preferably be fan-assisted and temperatures should be controlled within stringent limits. During incubation, temperatures should be recorded regularly, preferably by continuous monitoring.

9.5.4.3 Membrane filters

The quality of membrane filters differs between different manufacturers and also, between different batches prepared by the same manufacturer. This can affect the recovery of target organisms. The batch number of the membrane filters used should, therefore, be recorded, and tests using reference materials should be carried out on each new batch of membrane filters to ensure they are satisfactory. Membrane filters from a single batch should be used when undertaking comparisons of microbiological methods.

9.6 Stage 1 of the comparison of method A versus method B

The following procedures apply to the comparison of enumeration methods. The comparison of two microbiological methods involves processing (at the same time) aliquots of the same sample by the two methods under study. A number of different samples from each source or category of origin and from different sources or categories of origin are similarly analysed and then a statistical analysis of all the results is carried out.

The methods to be compared should be tested with the types of samples, which it is anticipated, will be routinely analysed by the methods. These samples, generally, will comprise waters that have been subject to some form of treatment, usually including disinfection. Because of the high quality of most treated water supplies it will, generally, be necessary to prepare samples that mimic the effect of inadequate treatment. Protocols for the preparation of suitable samples, containing chlorine as a disinfectant, are given in section 9.6.2. For alternative disinfectants, it will be necessary to determine by experiment those conditions appropriate for the survival of suitable numbers of target organisms.

It is estimated that a minimum of 150 samples should be included in the comparison trial, which reflect the range of source waters. The methods used should be tested with the appropriate volume of sample relevant to the target organism and the prescribed limit. This is usually 100 ml and this volume is used in this document for illustrative purposes. The samples should not be diluted and should be tested over a period of several of days, generally, testing approximately 10 - 15 samples per day.

9.6.1 Preparation of samples

There are a several ways of preparing suitable samples (based on chlorinated waters) for carrying out comparisons of microbiological methods and these are listed in order of preference.

(i) Chlorinated tap water plus river water with the addition of extra quantities of chlorine to produce chlorine-stressed organisms, to a final concentration of chlorine of approximately 0.1 - 0.5 mg/l (see section 9.6.2.1).

(ii) Through treatment samples (for example, following granulated activated carbon or post rapid gravity filter treatment) if necessary, with a final concentration of chlorine of approximately 0.1 mg/l.

(iii) Chlorinated tap water plus sewage effluent with the addition of extra quantities of chlorine to produce chlorine-stressed organisms, to a final concentration of chlorine of approximately 1.2 - 2.5 mg/l (see section 9.6.2.2).

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(iv) Naturally contaminated un-chlorinated groundwater with the addition of extra quantities of chlorine to produce chlorine-stressed organisms, to a final concentration of chlorine of approximately 0.1 mg/l.

In certain situations, it may be necessary to use environmentally stressed organisms instead of chlorine-stressed organisms. In these cases, suitable samples may be prepared by prolonged storage of sewage effluent or river water samples.

9.6.2 Preparation of spiked samples

For the initial phase of stage 1, spiked samples are prepared which contain chlorine-stressed target organisms, non-target organisms and organisms closely related to target organisms. Ideally, samples should contain 20 - 50 target organisms per test aliquot (for example, 100 ml).

9.6.2.1 Generation of chlorine-stressed target organisms using river water

9.6.2.1.1 Collect approximately 10 litres of tap water from a supply that is representative of the water supplies to be tested, and cool to 2 - 8 °C (store overnight if necessary). Collect at least 1000 ml of river water. If the tap water being used is derived from surface water, then the water source from which the tap water is derived should be used.

9.6.2.1.2 Remove a small quantity of the cooled tap water and determine the concentration of free and total chlorine in a suitable aliquot. This determination is used to calculate the amount of chlorine that should be added to the remaining volume of tap water, to produce a free chlorine concentration of approximately 0.1 - 0.5 mg/l. The calculated amount of chlorine can be added using a solution prepared from sodium hypochlorite or chlorine-generating tablets. The chlorinated tap water should be stoppered or capped and thoroughly mixed. Store the chlorinated tap water at 2 - 8 °C.

9.6.2.1.3 Add 900 ml of the cooled chlorinated tap water to a suitable container, bottle or flask. To the container, add 100 ml of the river water, mix well, leave for 5 minutes, and then determine the free and total chlorine concentration. To a second container, add 900 ml of the cooled chlorinated tap water and 100 ml of deionised or distilled water, mix well, leave for 5 minutes and then determine the free and total chlorine concentration. These two containers are used as controls for assessing whether the chlorine demand is too high. For example, if the concentration of chlorine in the mixed tap and river water falls to non-detectable levels within the 5 minutes, then 10 litres of tap water containing a higher concentration of chlorine, ie greater than 0.1 - 0.5 mg/l, will be required. The concentration of chlorine in the tap water, required to achieve the desired concentration of free chlorine in the mixed tap and river water solution, will vary according to the pH and organic and inorganic contents of the river and tap water. It may be necessary to carry out preliminary trials to determine the optimum concentration of chlorine in the tap water. When satisfactorily resolved and 10 litres of tap water of the correct concentration of chlorine have been prepared, add 900 ml of cooled tap water containing the correct level of chlorine to each of seven suitable containers, bottles or flasks.

9.6.2.1.4 Add 100 ml of the river water to one of the containers and mix well. Allow the chlorination process to react for 1 minute ± 5 seconds, and then add 1 ml of 18 % m/v sodium thiosulphate pentahydrate solution to the container. Cap and mix well, and store at 2 - 8 °C. Repeat the procedure using each of the remaining six containers and chlorination times of 1.5, 2.0, 2,5, 3.0, 3.5, and 4.0 minutes (± 5 seconds) respectively.

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9.6.2.1.5 Remove 10 ml of the mixed tap and river water from each container and analyse each of the seven samples for the target organism. A method should be used that will yield a presumptive result, ideally, within 24 hours. Store the containers at 2 - 8 °C.

9.6.2.1.6 After incubation, determine the number of organisms in each 10 ml aliquot, and identify those containers, bottles or flasks found to contain 30 - 90 target organisms in the corresponding 10 ml aliquots. This number of organisms is higher than the target range of 20 - 50, in order to allow for some decay in the population of the organisms during overnight storage.

9.6.2.1.7 For the number of identified containers possessing 30 - 90 target organisms in 10 ml aliquots, add 900 ml of fresh tap water from the original source (9.6.2.1.1) to separate clean 1000 ml containers. To each of these containers, add sufficient sodium thiosulphate pentahydrate solution to neutralise any residual chlorine and mix well. To each separate container, add 100 ml of the corresponding mixed tap and river water samples from those identified containers possessing 30 - 90 target organisms in 10 ml aliquots. Cap and mix well. Each 1000 ml of diluted mixed tap and river water sample now enables up to 10 replicate 100 ml samples to be analysed by two or more methods used in parallel by one or more analysts. Alternatively, larger volumes of diluted mixed tap and river water samples can be prepared, by increasing proportionately the volumes of fresh tap water from the original source (9.6.2.1.1) and mixed tap and river water.

9.6.2.2 Generation of chlorine-stressed target organisms using sewage effluent

9.6.2.2.1 Collect 10 litres of tap water from a supply that is representative of the water supplies to be tested, and cool to 2 - 8 °C (store overnight if necessary). Collect at least 1000 ml of sewage effluent and store for one hour at 2 - 8 °C to ensure solid material settles.

9.6.2.2.2 Prepare a solution of chlorine, containing 12 - 25 mg/l by dissolving the appropriate amount of hypochlorite solution or chlorine-generating tablets in 1 litre of distilled or deionised water. Cap and mix well.

9.6.2.2.3 Taking care not to disturb any settled solid material, transfer 500 ml of the sewage effluent into a clean 10 litre container (one fitted with a tap will make the following procedures easier to carry out) containing a magnetic stirrer bar, or other stirring mechanism. Add 8.5 litres of the tap water previously stored at 2 - 8 °C. Cap the container, mix the contents thoroughly and stand the container on a magnetic stirrer and stir vigorously.

9.6.2.2.4 Whilst maintaining the stirring action, add to the container, sufficient volume, up to 1000 ml, of the solution of chlorine to produce a free chlorine concentration in the mixed tap water-sewage effluent solution of 1.2 - 2.5 mg/l. (The exact volume of chlorine solution may have to be adjusted accordingly). Mix the contents vigorously. After 3 minutes, transfer 500 ml of the chlorinated mixed tap water-sewage effluent solution into a suitable vessel, bottle or flask containing 1 ml of 18 % m/v sodium thiosulphate pentahydrate solution. Stopper and mix well by inverting several times to ensure the chlorine is rapidly neutralised. Repeat the procedure at one-minute intervals, by transferring 500 ml of the chlorinated mixed tap water-sewage effluent solution into other, separate vessels, bottles or flasks each containing 1 ml of 18 % m/v sodium thiosulphate pentahydrate solution, until 16 samples have been taken and prepared.

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9.6.2.2.5 Remove 10 ml of the mixed tap water-sewage effluent solution from each container and analyse each of the 16 solutions for the target organism. A method should be used that will yield a presumptive result, ideally, within 24 hours. Store the containers at 2 - 8 °C.

9.6.2.2.6 After incubation, determine the number of organisms in each 10 ml aliquot, and identify those containers, bottles or flasks found to contained 30 - 90 target organisms in the corresponding 10 ml aliquots. This number of organisms is higher than the target range of 20 - 50, in order to allow for some decay in the population of the organisms during overnight storage.

9.6.2.2.7 For the number of identified containers possessing 30 - 90 target organisms in 10 ml aliquots, add 900 ml of fresh tap water from the original source (9.6.2.2.1) to separate clean 1000 ml containers. To each of these containers, add sufficient sodium thiosulphate pentahydrate solution to neutralise any residual chlorine and mix well. To each separate container, add 100 ml of the corresponding mixed tap water-sewage effluent solution from those identified containers possessing 30 - 90 target organisms in 10 ml aliquots. Cap and mix well. Each 1000 ml diluted mixed tap water-sewage effluent solution now enables up to 10 replicate 100 ml samples to be analysed by two methods used in parallel by one or more analysts. Alternatively, larger volumes of diluted mixed tap water-sewage effluent solution can be prepared, by increasing proportionately the volumes of fresh tap water from the original source (9.6.2.2.1) and mixed tap water-sewage effluent solution.

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9.6.3 Confirmation tests

If confirmation of presumptive target colonies is required, then this should be carried out according to the requirements of the method. Generally, for drinking water, 10 presumptive colonies should be tested for confirmation if there are more than 10 presumptive colonies present, and all colonies should be tested if there are 10 presumptive colonies or less. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be examined. See also section 8.3.3.

9.6.4 Verification of identity of target and non-target organisms

Methods should already have undergone validation that should have included a determination of the proportion of false-positive and false-negative results. However, this determination may have been carried out on a limited range of samples or sources of organisms. It is possible that different sources or categories of origin of water may contain different spectra of organisms from those examined in the initial validation trial and this may affect the proportion of false-positive and false-negative results. It can be useful, therefore, to carry out a more extensive identification of a selection of target or presumptive target colonies and non-target or presumptive non-target colonies. This identification is distinct from any confirmation steps that may be an integral part of the methods under test.

A minimum of 100 target colonies and, where appropriate, 100 non-target colonies per method should be selected for full identification by a suitable method. For most purposes, commercial identification kits may be adequate, but other approaches to identification may be more appropriate for some organisms. If the method under test is used for more than one target or presumptive target organism then at least 100 representative colonies of each organism should be examined. For example, if a method detects *Salmonella* species and *E. coli* simultaneously, then examination of 100 presumptive colonies of *Salmonella* and

100 presumptive colonies of *E. coli* would be required. The colonies should be selected so that they are evenly distributed over the sources of water examined. When confirmation tests are conducted, the most appropriate procedure of selecting target colonies for further identification is to choose the first one identified for confirmation. The advantage of this is that it will be known whether the colony confirmed or not. Non-target colonies should be selected at random, preferably one colony per Petri dish or plate and selected so that there are similar numbers examined from each source of water.

The spectrum of target or non-target organisms detected should be compared with that expected from previous validation data. If a particular source or category of origin of water exhibits differences from other sources, examination of the identification data may facilitate an interpretation of the differences.

9.7 Interpretation of stage 1 data

Pilot work with the preparation of samples is essential. It is necessary to ensure that as many samples as possible give counts within the required range of 20 - 50 target organisms. Once the study has commenced, all enumerated counts should be recorded. If any result is higher than expected, for example, a result is too numerous to count (such as greater than 100 for membrane filtration, or in the multiple tube technique, all tubes exhibit growth in the medium) then the subsequent data analysis may be biased. If the paired results obtained by both methods are too numerous to count, then both results can be omitted from the data analysis. This is because both results contribute little or no information about whether the new (trial) method gives a higher or lower result than the reference method. However, if only one method produces a result that is too numerous to count, then both results must be included in the data analysis. The exclusion of such results could bias the conclusions. In some situations, it is possible to allocate arbitrary numbers to those results reported as being too numerous to count, for example, 181 for a result reported as greater than 180. However, if there are many such results, the use of non-parametric statistical techniques may be more appropriate because there will be no proper estimate of the difference in counts for these paired results. Clearly, the data analysis will be much easier to conduct and interpret, if the results fall within the target range.

When a zero count obtained by one method is reported but is associated with a non-zero count obtained by the other method, then both results must be recorded and included in the data analysis. If paired zero counts are reported by both methods then these results can be excluded from the data analysis because they contribute little or no information about whether the new (trial) method gives a higher or lower result than the reference method.

9.7.1 Statistical comparison of methods

When performing confirmation tests, representative colonies should be chosen at random and typically, 10 colonies per plate are chosen. Depending upon circumstances, it may be necessary to test more colonies. See also section 8.3.3.

9.7.1.1 Stage 1 - spiked samples (20 - 50 target organisms/aliquot)

The procedures described in sections 9.6.2.1 and 9.6.2.2 should enable aliquots of samples to be prepared that contain 20 - 50 target organisms. The samples may be stored and appropriate aliquots withdrawn and tested by both methods. This procedure should then be repeated on different days. However, on every occasion, the sample should be thoroughly mixed before the appropriate volumes are withdrawn for analysis by both methods. The results from both

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methods must be recorded as a “paired sample” result and examples of data analysis are given in Annex B.

It is preferable that the prepared samples described in sections 9.6.2.1 and 9.6.2.2 are derived from a selection of sources or categories of origin (see section 9.2.1). Each category of origin will involve material from a particular source (for example, a specific section of river, a treatment works, etc.). Material can be collected over a period of time. For convenience, these categories of origin are referred to as “sources”, although it is noted that the actual samples prepared are not taken directly from particular sources but have involved some manipulation according to the details within sections 9.6.2.1 and 9.6.2.2.

A sufficient number of samples (at least 15) from each source or category of origin (usually 5 - 10) should be analysed to give statistical information to enable the following questions to be answered satisfactory.

Question (I) Is the relative performance of the two methods similar for all the sources or categories of origin used in the study?

Question (II) On average, does the new (trial) method find similar numbers of target organisms, or significantly lower or significantly higher numbers of organisms?

Question (III) If the new method finds similar numbers of organisms, does the 95 % confidence interval for the estimated difference between the average counts exclude the value where the new (trial) method finds greater than, or equal to, 10 % lower numbers of organisms than the reference method?

The analysis of at least 15 samples per source or category of origin, together with a total of not less than 150 samples for all sources or categories should provide sufficient information to answer the above questions. However, the difficulty of being able to predict the numbers of target organisms in a sample makes it difficult, in turn, to predict the statistical information in a fixed size trial. The numbers of samples and sources suggested above are, therefore, to be used as a guide and the final numbers will be dependent on the outcome of the comparison. If the comparison appears inconclusive, then more samples should be analysed.

If the results of stage 1 of the comparison indicate that the new (trial) method appears acceptable, then stage 2 of the comparison should be undertaken to confirm the outcome.

9.7.1.2 Data collection and evaluation

Analyse 15, or more, samples per source or category of origin by the two methods under investigation, ie the reference method and the new (trial) method, ensuring that at least 150 samples are examined.

Plot the paired results, differentiating each source or category of origin. Also, plot the differences (actual or transformed data) on appropriate scales.

9.7.1.2.1 From the data, ascertain whether the data are suitable for parametric analysis, ie are the data distributed in an approximately Gaussian (or normal) manner? If the answer to this question is yes, perform a data analysis, for example using the *t*-test, analysis of variance (ANOVA) test etc. If the answer to the question is no, transform or convert the data to an appropriate scale, if there is one, and carry out a parametric data analysis. Alternatively,

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perform a non-parametric data analysis, using, for example Wilcoxon signed rank test. Whether a parametric or non-parametric data analysis is carried out, Question (I) should then be considered. Namely, is the relative performance of the two methods similar for all the sources or categories of origin used in the study? Depending on the data analysis, further questions need to be addressed.

For a parametric data analysis, do the *t*-tests or ANOVA tests show significant differences between the sources or categories of origin? If the answer to this question is no, Question (II) should be considered. If the answer is yes, then possible microbiological causes should be investigated and decisions taken whether or not the differences affect part of (ie, a particular source) or the whole of the data (ie, all sources). Depending upon these actions and decisions, the whole of the data may need to be rejected and the comparison terminated, ie, the new (trial) method is rejected. See example B2.

For a non-parametric data analysis, does the tabulation of data by source or category of origin of frequencies of paired results show differences between the sources or categories of origin? If the answer to this question is no, Question (II) should be considered. If the answer is yes, then possible microbiological causes should be investigated and decisions taken whether or not the differences affect part of, or the whole of, the data. Depending upon these actions and decisions, the whole of the data may need to be rejected and the comparison terminated, ie the new (trial) method is rejected. See example B2.

9.7.1.2.2 Question (II) concerns whether or not, on average, the new (trial) method finds similar numbers of target organisms, or significantly lower or significantly higher numbers of organisms. Depending on the data analysis, further questions need to be addressed.

For a parametric data analysis, does the mean difference between enumerated counts differ significantly from zero? If the answer to this question is no, Question (III) should be considered. If the answer is yes, then a further question needs to be addressed. Namely, does the new (trial) method show significantly lower or significantly higher numbers of organisms? If the counts are significantly lower then the comparison is terminated, ie the new (trial) method is rejected. If the counts are significantly higher, then the new (trial) method can be accepted.

For a non-parametric data analysis, does the median difference between enumerated counts differ significantly from zero? If the answer to this question is no, Question (III) should be considered. If the answer is yes, then a further question needs to be addressed. Namely does the new (trial) method show significantly lower or significantly higher numbers of organisms? If the counts are significantly lower then the comparison is terminated, ie the new (trial) method is rejected. If the counts are significantly higher, then the new (trial) method can be accepted.

9.7.1.2.3 Question (III) concerns whether or not, the 95 % confidence interval for the estimated difference between the average counts excludes the value where the new (trial) method finds greater than, or equal to, 10 % lower numbers of organisms than the reference method. Depending on the data analysis, further questions need to be addressed.

For a parametric data analysis, does the 95 % confidence interval of the mean difference include the value where the new (trial) method finds greater than, or equal to, 10 % lower numbers of organisms than the reference method? If the answer to this question is no, then the results of stage 1 of the comparison would indicate the data appear acceptable and stage 2 of

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the comparison should be undertaken. If the answer is yes, then stage 1 of the comparison should be expanded and more samples analysed, and the whole process repeated.

For a non-parametric data analysis, does the 95 % confidence interval of the median difference include the value where the new (trial) method finds greater than, or equal to, 10 % lower numbers of organisms than the reference method? If the answer to this question is no, then the results of stage 1 of the comparison would indicate the data appear acceptable and stage 2 of the comparison should be undertaken. If the answer is yes, then stage 1 of the comparison should be expanded and more samples analysed, and the whole process repeated.

9.8 Stage 2 evaluation

The stage 2 of the comparison should be undertaken only when satisfactory results are obtained from stage 1. Stage 2 comprises the comparison of results of paired analyses of samples containing less than 20 target organisms per unit test volume. This comparison is carried out to ensure that the results remain valid at lower levels of organisms, approaching those numbers closer to statutory limits, but not so low as to be based on presence/absence criteria.

The paired results of at least 30 samples are required, where enumerated counts in the range 1 - 10 are recorded by at least one of the methods used. The samples can be prepared in the same way as described in sections 9.6.2.1 and 9.6.2.2 but with extra dilution steps. Successive two-fold dilutions of the same sample can be prepared, but samples should be derived from more than one source or category of origin. In addition, samples should contain an appropriate diversity of organisms.

If, the paired results obtained in stage 1 contain at least 30 samples giving counts in this lower range for all sources or categories of origin, then the data from these samples can be used for the stage 2 evaluation.

9.8.1 Data analysis for stage 2

As for stage 1, all the results should be plotted. With low counts it may be more of a problem to use a parametric data analysis approach, and it becomes more efficient to use a non-parametric analysis, see the worked examples in annex B. The proportion of paired results where the count by the new (trial) method exceeds the count given by the reference method should not be significantly lower than 50 %. Thirty samples should give an estimate of the proportion, with a confidence interval that is not too large, for example ± 20 %. If the confidence interval is large and there is evidence to suggest the new (trial) method is not performing well, then more samples should be analysed to establish whether or not there is any significant difference within these bounds.

9.9 Evaluation of a method for use in a laboratory

Any laboratory considering the introduction of a microbiological method should be familiar with its use within the laboratory. For example, staff should analyse samples containing pure cultures in suspensions in waters in the range 20 - 50 target and non-target organisms per unit test volume.

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9.9.1 Evolution of method introduction

On a national or international scale, the adoption of a new method involves a sequential series of events. Namely,

(i) comparison of new (trial) method with reference method in one laboratory;
(ii) subsequent comparison of new (trial) method with reference method in four , or more laboratories;
(iii) assessment of robustness; and
(iv) adoption of new method.

A new method should undergo full comparative testing, using the procedure outlined in this document, in at least five laboratories before being regarded as potentially of general applicability. Where adequate comparative assessments have been undertaken in a single laboratory and these assessments indicate that the results obtained using a new (trial) method are comparable to the results obtained by a reference method, then the new method could be adopted for routine use by that laboratory. The adoption of the new method for routine use would not depend on whether other laboratories had carried out similar studies. When five, or more, laboratories have demonstrated that the performance of a new method is equivalent to, or better than the performance of a reference method, then wider adoption by other laboratories can be considered. In these cases, the comparison exercises undertaken by other laboratories may involve fewer numbers of samples. The comparison studies carried out in the initial five, or more, laboratories may require the replicate analysis of at least 180 samples (150 samples for stage 1 and 30 samples for stage 2) in each laboratory. Ideally, all procedures described in section 9.6.1 should be used, and samples should be representative of the sources of waters that the laboratory is likely to analyse by the new method. Data from the comparison studies undertaken in the different laboratories should be then be combined and reviewed following further statistical appraisal. By combining the data, it is possible to assess more confidently the robustness, repeatability and reproducibility of the new method.

Once the robustness, repeatability and reproducibility of the new method have been satisfactory established, the new method is generally acceptable for adoption for routine use. Therefore, the numbers of samples that subsequent laboratories need to analyse by the new method can be reviewed in light of the expanding database. However, a minimum of 30 samples, containing low numbers of a variety of organisms, should be analysed and results compared with those obtained using the reference method.

9.10 Comparison of an MPN method with an enumeration method

The design of the study and the same procedures described in sections 9.6 and 9.7 should be used for comparing results obtained using an MPN method and those obtained using an enumeration method. When an MPN method is the new method, the aim of the comparison exercise is to show that the MPN method does not find significantly lower numbers of organisms than found by the enumeration method, and if this is the case, the average difference in counts is accurately established. The required level of accuracy is such that the 95 % confidence interval for the average difference in counts should not include the situation where the MPN method finds 10 % lower numbers of organisms. In situations where the MPN method is the reference method, the investigation should establish that the enumeration method does not give significantly lower counts. If this is so, then the 95 % confidence interval for the average difference in counts should not include the situation where the enumeration method finds 10 % lower numbers of organisms.

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Water samples should be prepared which contain organisms from a variety of sources or categories of origin, and be typical of the samples analysed in the laboratory. Whilst the same procedures as described in sections 9.6 and 9.7 are used, the only difference will be those factors influencing the choice of statistical methods. Paired results are used but, in the situation where an enumerated method is compared with an MPN method, results are based on the comparison of enumerated counts and most probable numbers.

The range of values achievable with a multiple tube method is discontinuous within the range of the method. For example, in an 11-tube series (1 x 50 ml, 5 x 10 ml, 5 x 1 ml) if 9 tubes exhibit growth in the medium (say 1, 5, 3) then from appropriate tables, the MPN is 91 per 100 ml. If 10 tubes exhibit growth in the medium (say 1, 5, 4) then the MPN is 160 per 100 ml. It is impossible to obtain a count between 91 and 160. Appropriate tables[28] show the counts (MPNs) and ranges of counts (MPRs) corresponding to 11-tube series (1 x 50 ml, 5 x 10 ml, 5 x 1 ml) and 15-tube series (5 x10 ml, 5 x 1 ml, 5 x 0.1 ml). One approach[29], especially where a tube series exhibits large gaps in MPNs is to group the results from the counting methods and compare them with the corresponding MPN. The grouping is carried out by consideration of each count and determining the tube combination that would be the most appropriate from a sample containing this number of organisms. (This should not be confused with MPRs or confidence intervals published for MPNs[28] which are obtained from different conditional probabilities). Appropriate conditional probabilities have been published[29] and resulting ranges tabulated for tube combinations. For example, with the 11-tube series given above, it has been shown that counts of between 69-110 would probably give a tube result of 1, 5, 3 and an MPN of 91. Counts of between 111-175 would probably give a tube result of 1, 5, 4 and an MPN of 160. Enumerated counts 69-110 could be interpreted as “equivalent” to an MPN of 91. Alternatively, the MPN can be regarded as the end result and compared directly with the count from the paired result. Careful plots of the results should be made and consideration given to using non-parametric analyses along similar lines to those described in the worked examples with enumeration methods in the second example of annex B.

These problems can be reduced, by using suitable samples where less than half of the tubes in the series exhibit growth. If not, the MPN will be an approximate count, and the comparison with the enumeration method might become biased. Multiple tube methods that require large numbers of tubes (at several dilutions) are more reliable than multiple tube methods with fewer dilutions and tubes.

As with a comparison of two enumeration methods, it is important that when the study is underway, all results are used. The only results that should be discarded are those where both methods recorded zero counts, or both methods failed to give an estimate of the count because the enumeration method showed overgrowth and the MPN method resulted in all tubes within the series exhibiting growth, ie were positive.

9.11 Comparison of two MPN methods

The same procedures described in sections 9.6 - 9.8 should be used when two MPN methods are compared. The points raised in section 9.10 still apply to both MPN methods and the principles of the comparison remain the same. Again, factors may influence the choice of statistical methods, which should be decided after thorough scrutiny of the data summaries and plots. It is likely that non-parametric data analyses should be used. The preparation of samples should be such that the number of tubes in the series exhibiting growth in the medium for the reference MPN method should be less than half of those tubes inoculated.

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Annex A **95 % Confidence intervals for the (unobserved) count from the second sub-sample for the observed count from the first sub-sample (see section 8.5.2)**

Observed count in first sub-sample	95 % CI for unobserved count in second sub-sample	Observed count in first sub-sample	95 % CI for unobserved count in second sub-sample
0	0 – 5	51	33 – 73
1	0 – 7	52	33 – 75
2	0 – 9	53	34 – 76
3	0 – 11	54	35 – 77
4	0 – 12	55	36 – 78
5	0 – 14	56	37 – 79
6	1 – 16	57	38 – 80
7	1 – 17	58	38 – 82
8	2 – 19	59	39 – 83
9	2 – 20	60	40 – 84
10	3 – 22	61	41 – 85
11	3 – 23	62	42 – 86
12	4 – 24	63	42 – 88
13	5 – 26	64	43 – 89
14	5 – 27	65	44 – 90
15	6 – 28	66	45 – 91
16	6 – 30	67	46 – 92
17	7 – 31	68	47 – 93
18	8 – 32	69	47 – 95
19	8 – 34	70	48 – 96
20	9 – 35	71	49 – 97
21	10 – 36	72	50 – 98
22	10 – 38	73	51 – 99
23	11 – 39	74	52 – 100
24	12 – 40	75	52 – 102
25	13 – 41	76	53 – 103
26	13 – 43	77	54 – 104
27	14 – 44	78	55 – 105
28	15 – 45	79	56 – 106
29	16 – 47	80	57 – 107
30	16 – 48	81	58 – 108
31	17 – 49	82	58 – 110
32	18 – 50	83	59 – 111
33	19 – 52	84	60 – 112
34	19 – 53	85	61 – 113
35	20 – 54	86	62 – 114
36	21 – 55	87	63 – 115
37	22 – 56	88	63 – 117
38	22 – 58	89	64 – 118
39	23 – 59	90	65 – 119
40	24 – 60	91	66 – 120
41	25 – 61	92	67 – 121
42	26 – 63	93	68 – 122
43	26 – 64	94	69 – 123
44	27 – 65	95	69 – 125
45	28 – 66	96	70 – 126
46	29 – 67	97	71 – 127
47	29 – 69	98	71 – 128
48	30 – 70	99	73 – 129
49	31 – 71	100	74 – 130
50	32 – 72		

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Annex B Worked examples of comparison of methods for drinking water bacteriology

This annex describes worked examples of trials comparing two methods following the procedures detailed in sections 9.6 - 9.8. The first example is based on counts from membrane filtration methods and contains real data that have been selected to illustrate relevant situations. Both parametric and non-parametric data analyses have been applied to demonstrate both approaches. In practice, only one approach might be used.

Example B1

Stage 1 evaluation

A total of 150 samples were each split into 2 sub-samples. One sub-sample was analysed by a reference method and the second sub-sample was analysed by a new (trial) method. Counts were reported as confirmed coliform bacteria counts per 100 ml. Samples were derived from five geographically distinct areas, all relevant to the routine work of the laboratory where the trial was undertaken. The data are listed in Table B1. Whilst the aim was to produce counts in the range 20 - 50, in practice, the range was wider. Summary data analysis of all counts in stage 1 reveals

	Median value	Range of counts
Reference method	30	5 - 180
Trial method	30	3 - 170

The data are plotted as paired counts (shown in Figure B1) and on a logarithmic (base 10) scale as log paired counts (shown in Figure B2). Also, the difference in the paired counts (count for trial method - count for reference method) is plotted on a frequency distribution plot or histogram, shown in Figure B3. Similarly, the difference in the log paired counts, ie log (count for trial method) - log (count for reference method) is plotted and shown in Figure B4.

From a casual study of the data in Table B1, it appears that the trial method gives counts similar in magnitude to counts produced by the reference method. Also, the trial method appears to be comparable to the reference method in that there are similar numbers of observations, as shown in Figures B1 and B2, above and below the theoretical line of equivalence. The paired differences, both in counts and as the log scale, appear clustered around zero as shown in Figures B3 and B4, but some differences are large in magnitude. This is not unusual in “real life” microbiology where occasional observations display variation much greater than random (Poisson) variation. The median difference in paired counts is 1 and the range is between - 61 to + 90.

From the data, it now needs to be ascertained whether the data are suitable for parametric analysis, ie are the data distributed in an approximately Gaussian (or normal) manner, see section 9.7.1.2.1.

The histogram of the frequency distribution of the difference in paired counts, as shown in Figure B3, appears symmetric but with long tails. The histogram based on the logarithmically converted scale, as shown in Figure B4, shows that the tails are reduced, but are still affected by possible “outlying” values. If cumulative Gaussian frequency distribution data are plotted on “normal probability paper”, a straight line is produced.

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Table B1 Data for example B1 - Stage 1

Source 1		Source 2		Source 3		Source 4		Source 5	
Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method
96	106	13	7	35	41	8	10	25	25
22	24	15	21	27	24	11	3	60	52
22	30	21	28	20	23	76	70	19	20
40	40	23	34	40	108	15	17	11	12
40	20	14	35	20	13	19	33	10	8
15	23	5	29	40	16	27	28	9	10
6	11	8	74	70	84	21	21	7	11
80	80	16	12	37	81	21	30	19	26
10	15	84	52	50	15	50	81	58	75
60	40	71	10	80	32	50	60	43	39
63	43	9	69	57	95	34	19	142	140
50	50	80	78	80	60	30	25	8	10
80	170	60	60	14	10	41	47	20	30
14	14	33	16	40	15	22	24	180	170
40	38	24	14	60	126	100	98	10	30
60	40					17	13	10	20
21	32					75	77	13	12
31	45					11	7	32	24
86	72					14	17	10	10
14	23					33	33	70	76
						41	37	15	17
						6	20	27	28
						30	17	21	21
						27	23	21	30
						26	18	50	81
						15	8	34	19
						18	15	25	30
						20	28	24	22
						58	30	100	98
						17	21	13	17
						58	56	75	77
						21	19	14	17
						30	38	41	37
						64	64	27	23
						41	44	39	50
						25	25	17	21
						17	7	58	56
						52	36	21	16
						67	63	30	38
						12	32	52	36
						44	34	63	67
						32	19	34	44
						64	68	25	25
						39	36	64	68
						57	32	39	33
						39	42	39	42
						40	51	40	51
						21	29	12	16
						24	35	41	80
						41	80	32	27

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Figure B1 **Example B1 - Stage 1 - Plot of paired counts for trial and reference methods**

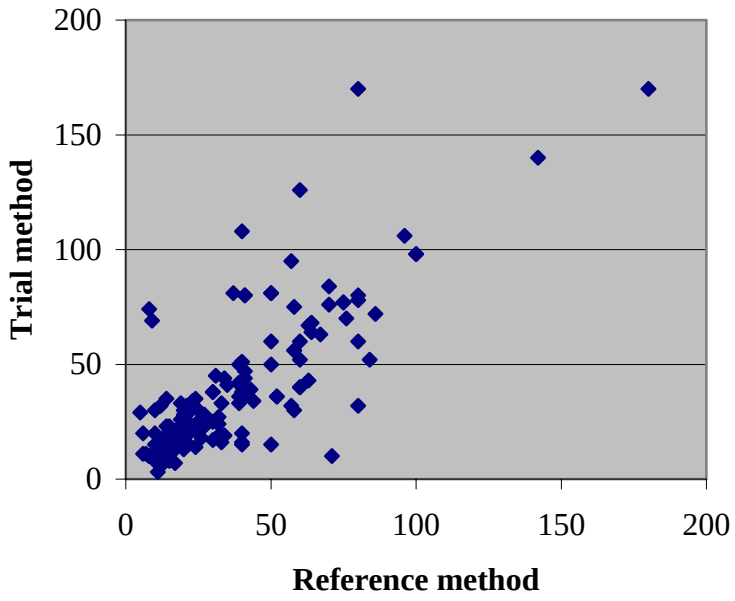

Figure B2 **Example B1 - Stage 1 - Plot of paired counts (converted to log_{10} scale) of trial and reference methods**

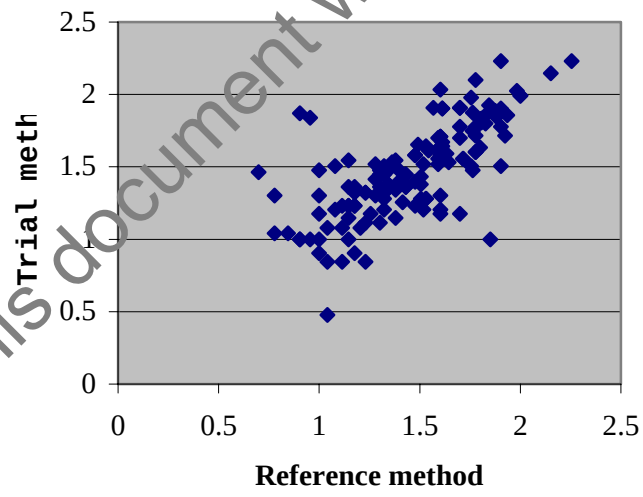

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Figure B3 Example B1 - Stage 1 - Difference in paired counts between trial and reference methods plotted as a histogram

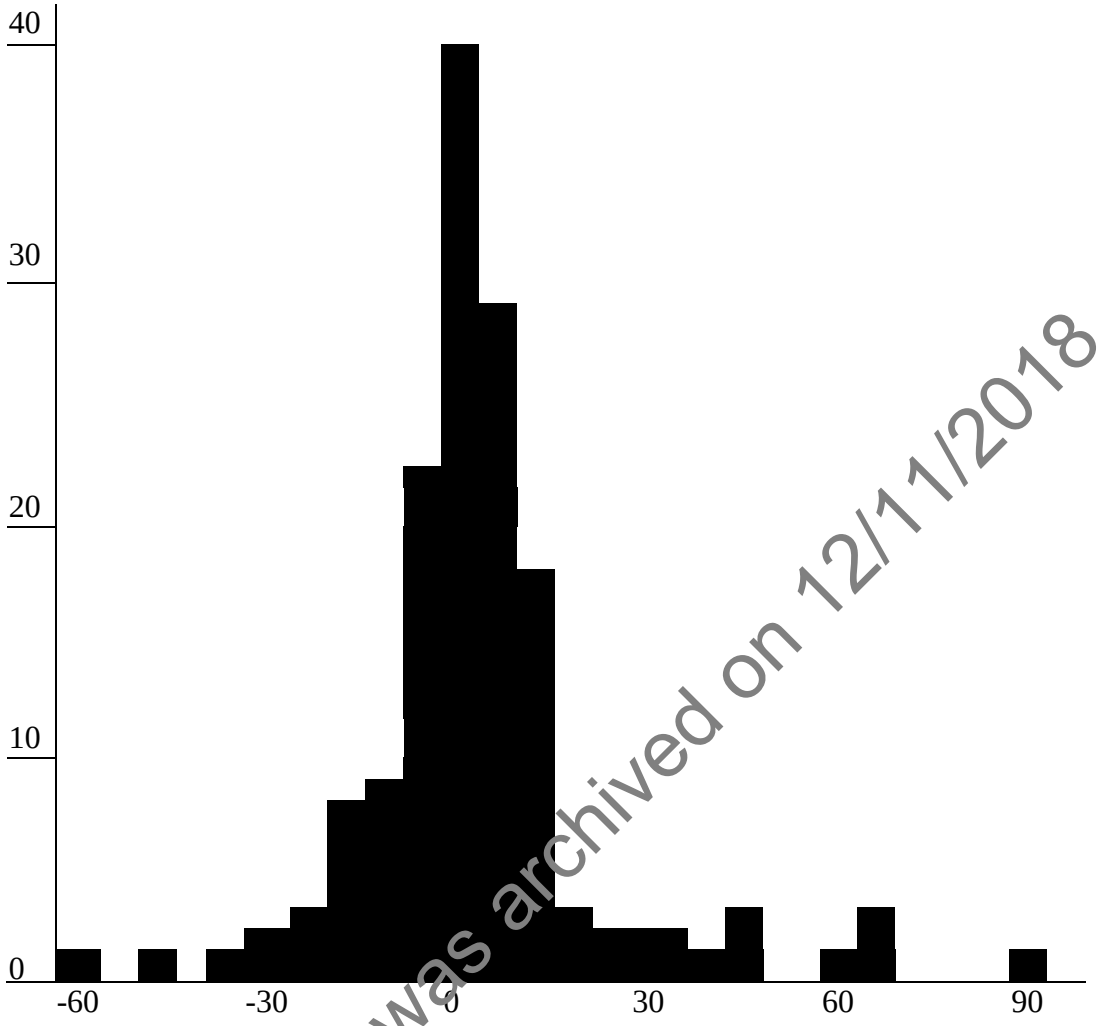

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Figure B5 Example B1 - Stage 1 - Normalised probability plot for differences in paired counts between trial and reference methods

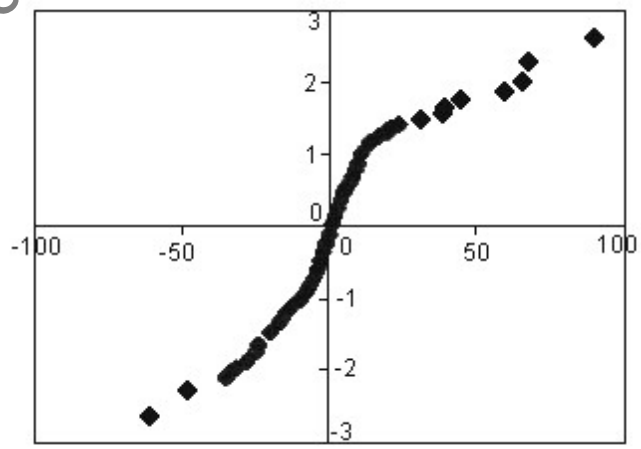


Figure B4 Example B1 - Stage 1 - Difference in log paired counts between trial and reference methods plotted as a histogram

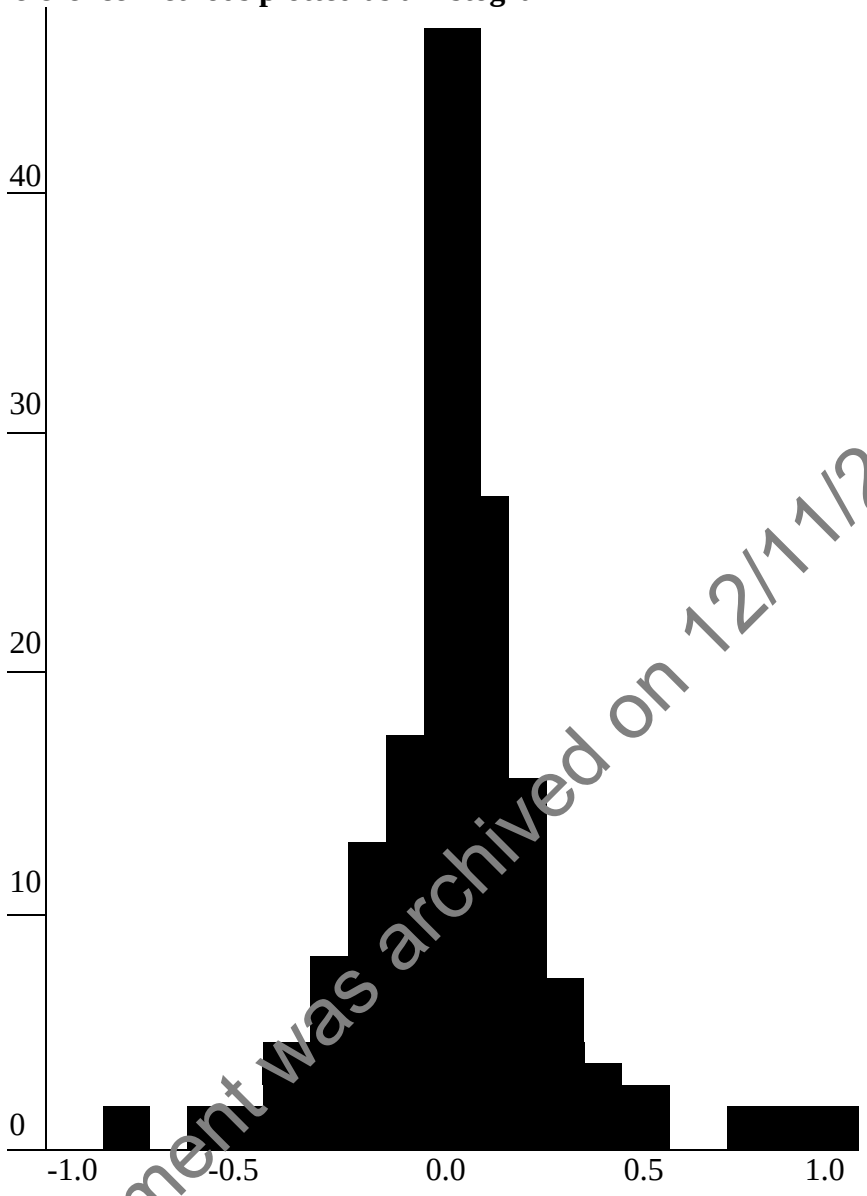
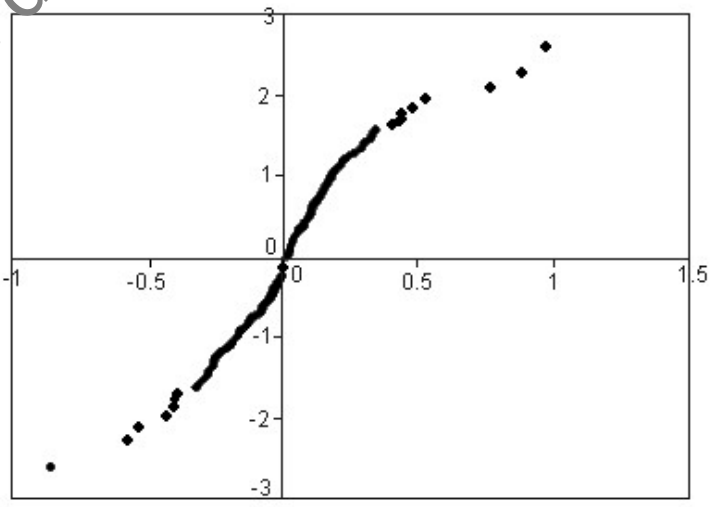


Figure B6 Example B1 - Stage 1 - Normalised probability plot for differences in log paired counts between trial and reference methods



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When this procedure is carried out (as shown in Figures B5 and B6 for both histograms shown in Figures B3 and B4 respectively) it can be seen that the probability plots produce lines that are approximately linear. This is good evidence that the data are distributed in an approximately Gaussian or normal manner. Normal probability values for the differences, using both scales, were computed and plotted against the differences as normal plots as shown in Figures B5 and B6. This was performed using the NSCORES function of the Minitab statistical software package (Minitab Inc.,1989, release 7, State College, PA, USA) but other packages are also available. As already stated a plot for perfectly Gaussian or normal data is linear. The data based on original paired counts, as shown in Figure B5, produce more of an S-shape plot, but approach linearity when logarithmically converted data, as shown in Figure B6, are used. The affects of the "outlying" data can, however, still be observed.

Whilst a parametric analysis based on logarithmically converted data might be acceptable, a cautious approach might also be to use a non-parametric analysis.

Parametric analysis of stage 1 of example B1

Question (I) should now be considered. Namely, is the relative performance of the two methods similar for all the sources or categories of origin used in the study? The differences were compared between the sources or categories of origin using a one way ANOVA test. The output from a Minitab software package is shown in Table B2. As indicated, there is no significant difference between the sources. A significant difference (due to one or more sources showing higher counts from the trial method) would not affect this outcome, but a significant difference (due to one or more sources showing significantly lower counts from the trial method) would lead to significant differences. As no significant differences between the sources are shown, Question (II) should now be considered. (See section 9.7.1.2.2). The ANOVA test of differences between log counts using data from Table B1 is shown in Table B2.

Table B2 Example B1 - Stage 1 - Parametric analysis

	Degrees of freedom	Sum of squares	Mean square	F ratio	p-value
Source	4	0.2393	0.0598	1.12	0.349
Error	145	7.7427	0.0534		
Total	149	7.9821			

The 2-sided tabulated F-test value at the 95% confidence level with the associated degrees of freedom is approximately 2.8, hence, since this value is greater than the F ratio calculated value of 1.12, there is probably no statistical difference between the data from all sources.

In a similar manner, it can be shown that using a pooled or combined standard deviation of 0.23109, the range of the individual 95 % confidence intervals for each source includes zero, ie, the value expected for methods giving similar results. The pooled or combined standard deviation, s_c, is calculated from

$$s_c = \sqrt{\frac{{s_1}^2(n_1-1)\ldots+\ldots{s_2}^2(n_2-1)\ldots{s_3}^2(n_3-1)\ldots+\ldots{s_4}^2(n_4-1)\ldots+\ldots{s_5}^2(n_5-1)}{(n_1+n_2+n_3+n_4+n_5-N)}}$$

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Where s_i is the standard deviation for source i (where i = 1 to 5) and n_i is the number of samples for source i. N is the number of sources. As expected, it is noted that the combined standard deviation is almost identical to the overall standard deviation shown below. Thus,

Source	Number of samples	Mean difference	Standard deviation	Range of 95% confidence interval
1	20	0.040485	0.16596	- 0.067 to + 0.148
2	15	0.09601	0.492393	- 0.031 to + 0.223
3	15	-0.04865	0.302399	- 0.176 to + 0.079
4	50	-0.0109	0.191284	- 0.077 to + 0.055
5	50	0.042763	0.123882	- 0.023 to + 0.108

Addressing Question (II) now, namely, on average, does the new (trial) method find similar numbers of target organisms, or significantly lower or significantly higher numbers of organisms? The overall mean of the 150 differences in the log paired results, ie log (count for trial method - log (count for reference method), is calculated as 0.02075 with a standard deviation, s, of 0.230681, and a standard error, se, of 0.0188. The standard error being calculated from

$$se = s / \sqrt{n}$$

where n is the number of paired results, ie in this case, 150.

If a new (trial) method were to find similar numbers of target organisms compared to a reference method, then it would be expected that over a period of time, the average difference of the methods would be zero. Using a 2-sided t-test, and comparing the overall mean of 0.02075 with a null hypothesis of zero, then t_{cal} = 0.02075 / 0.0188, ie 1.10. The 2-sided tabulated t-test value for 149 degrees of freedom at the 95 % confidence level is 1.97. Hence, since this value is greater than the corresponding calculated value of 1.10, there is probably no statistical difference between the mean of the differences and zero (p-value = 0.27). Thus, both methods find similar numbers of target organisms and there is no significant difference, on average, between the counts from the two methods. Question (III) should now be considered, see section 9.7.1.2.3, namely, does the 95 % confidence interval for the estimated difference between the average counts exclude the value where the new (trial) method finds greater than, or equal to, 10 % lower numbers of organisms than the reference method?

To address this question, the 95 % confidence interval, 95 CI, needs to be calculated from

$$95\ CI = mean \pm (t_{149,\ 0.05} \times se)$$

where $t_{149,\ 0.05}$ from statistic tables is the 2-sided t-test value at the 95 % confidence level for 149 degrees of freedom, namely 1.97. Hence

$$95\ CI = 0.02075 \pm (1.97 \times 0.0188)$$
$$= 0.02075 \pm 0.037036$$

ie, the overall 95 % confidence interval range is - 0.016 to + 0.058.

Question (III) seeks to determine whether the lower end of the overall 95 % confidence interval for the average difference includes the situation where the trial method finds 10 % fewer organisms than the reference method. In other words, whether an unacceptable situation occurs when, on average, trial count, TC, is 90 % of the reference count, RC, ie

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$$TC = 0.9 \times RC$$

Using the logarithmically converted data, the equation becomes

$$\log(TC) = \log(0.9) + \log(RC)$$

Thus, the difference in the log paired results becomes

$$\log(TC) - \log(RC) = \log(0.9) + \log(RC) - \log(RC)$$
$$= \log(0.9)$$
$$= - 0.04576$$

The overall 95 % confidence interval for the average difference, - 0.016 to + 0.058, lies entirely above the value of - 0.04576, hence, the data for the trial method appear acceptable and stage 2 of the comparison should be undertaken.

Non-parametric analysis of stage 1 of example B1

Question (I) should be considered. Namely, is the relative performance of the two methods similar for all the sources or categories of origin used in the study? The differences were compared between the sources or categories of origin as shown in Table B3 where the count from the trial method is compared with the count from the reference method using two-tailed, binomial probability.

Table B3 Example B1 - Stage 1 - non-parametric analysis

Source or category of origin	Number of results where trial method finds lower counts than reference method	Number of results where trial method finds an equal number of counts as the reference method	Number of results where trial method finds higher counts than reference method	Number of samples	p-value
1	6	4	10	20	0.4
2	7	1	7	15	0.6
3	8	0	7	15	0.5
4	23	4	23	50	0.6
5	17	4	29	50	0.052
All data	61	13	76	150	

The probability values test the null hypothesis that any discrepant paired result, where the counts were not identical, was as likely to give a higher count by the trial method as by the reference method. The values were calculated using exact binomial probabilities, parameter p = 0.5, two-tailed. Alternatively, McNemar's test can be used, but this becomes approximate when the number of discrepant pairs is less than about 15. The results show that there was no significant difference between methods in any of the sources or categories of origin and also, there was no significant difference between the sources or categories of origin.

Addressing Questions (II) and (III) the median of the 150 differences in the paired counts was compared with the null hypothesis of zero average difference and the 95 % confidence interval estimated. The Wilcoxon confidence interval was calculated using a Minitab software package.

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The median difference (count for trial method - count for reference method) is 1, whilst the 95 % confidence interval range is - 0.5 to + 3. As already stated, the median count by the reference method was 30. The lower value of the confidence interval would represent an average of 1.7 % deficiency in counts by the trial method (ie 100 - [{(30 - 0.5) / 30} x 100]). Hence, the data for the trial method appear acceptable and stage 2 of the comparison should be undertaken.

Stage 2 evaluation

Stage 2 of the comparison is undertaken to ascertain if results complement the conclusions of stage 1. Six additional samples from each source or category of origin were obtained using the same five geographically distinct areas as used in stage 1 of the comparison. The 30 paired results are listed in Table B4 and plotted as shown in Figures B7 and B8. As can be seen there is a scatter of results above and below the theoretical line of equivalence for all sources. These data appear to agree with the findings from stage 1.

Table B4 Data for example B1 - Stage 2

Source 1		Source 2		Source 3		Source 4		Source 5	
Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method
4	0	0	3	5	9	9	6	1	1
2	1	4	1	3	3	1	0	3	3
8	7	0	6	1	0	6	11	13	3
1	4	14	10	1	1	2	2	0	2
0	2	13	7	3	2	0	1	2	3
2	4	4	7	7	14	6	7	0	6

Figure B7 Example B1 - Stage 2 - Plot of paired counts for trial and reference methods

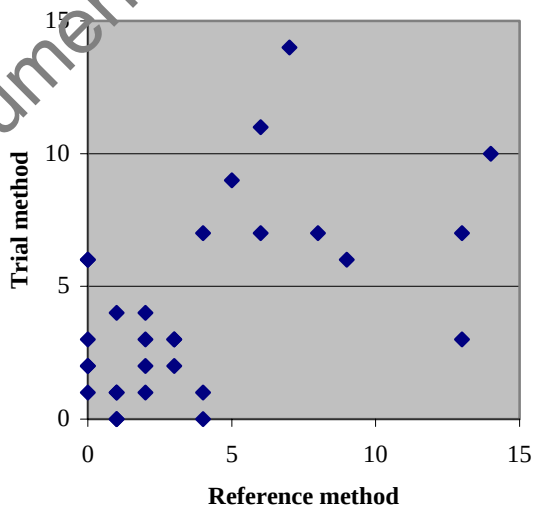

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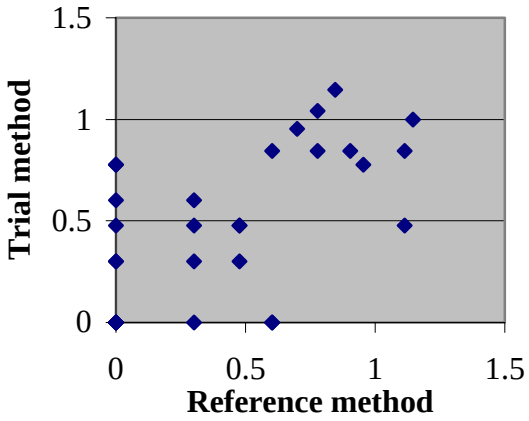
Figure B8 Example B1 - Stage 2 - Plot of paired counts (converted to $\log_{10}$ scale) of trial and reference methods



Parametric analysis of stage 2 of example B1

Summary data analysis of all counts in stage 2 reveals

	Mean	Median value	Range of counts
Reference method	3.8	2.5	0 - 14
Trial method	4.2	3	0 - 14

In addition, the median difference in paired counts is 0, and the range is - 10 to + 7. The mean difference in paired counts is calculated to be 0.3667 with a standard error of 0.6704. Using a 2-sided t-test, and comparing the mean difference of 0.3667 with a null hypothesis of zero, then t_{calc} = 0.3667 / 0.6704, ie 0.547. The 2-sided tabulated t-test value for 29 degrees of freedom at the 95 % confidence level is 2.045. Hence, since this value is greater than the corresponding calculated value of 0.547, there is probably no statistical difference between the mean of the differences and zero. Thus, both methods find similar numbers of target organisms and there is no significant difference, on average, between the counts from the two methods.

Addressing Question (ii), the overall 95 % confidence interval, 95 CI, needs to be calculated from

$$95\ CI = \text{mean} \pm (t_{29,\,0.05} \times se)$$

where $t_{29,\,0.05}$ from statistic tables is the 2-sided t-test value at the 95% confidence level for 29 degrees of freedom, namely 2.045. Hence

$$95\ CI = 0.3667 \pm (2.045 \times 0.6704)$$
$$= 0.3667 \pm 1.3710$$

ie, the 95 % confidence interval range is - 1.00 to + 1.74.

Since the range includes the value zero, and is within the 20 % criterion (see 9.8.1), ie in this case 16 % lower (ie 100 - [{4.2 - 1.0} / 3.8} x 100}), the trial method, therefore, appears comparable to the reference method at stage 2.

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Non-parametric analysis of stage 2 of example B1

The count from the trial method is compared with the count from the reference method using two-tailed, binomial probability.

Table B5 Example B1 - Stage 2 - non-parametric analysis

Source or category of origin	Number of results where trial method finds lower counts than reference method	Number of results where trial method finds an equal number of counts as the reference method	Number of results where trial method finds higher counts than reference method	Number of samples	p-value
All data	11	5	14	30	0.5

From the information available, it would seem reasonable to accept the trial method as being comparable to the reference method in this laboratory.

Example B2

Stage 1 evaluation

The results presented for this example represent a smaller study where the available evidence casts doubt on the suitability of a trial method when compared to a reference method. This illustrates how a large study is not always necessary to demonstrate whether a new trial method is satisfactory

Forty-five samples were each split into 2 sub-samples, and the first sub-sample analysed by the trial method and the second sub-sample analysed by the reference method. Counts were recorded as confirmed *E. coli* counts per 100 ml. The samples were derived from three geographically distinct areas, all relevant to the routine work of the laboratory. The data are listed in Table B6. Whilst the aim was to produce counts in the range 20 - 50, in practice, the range was wider. Summary data analysis of all counts in stage 1 reveals the median and range of counts were:

	Median	Range
Reference method	30	10 - 84
Trial method	28	3 - 78

The data are plotted as paired counts (shown in Figure B9) and on a logarithmic (base 10) scale as log paired counts (shown in Figure B10). The median difference in paired counts is 1 and the range is - 41 to + 41. As in example B1, the difference in paired log counts was computed. The results of the parametric analysis of differences in paired log counts from Table B6 using the ANOVA test is shown in Table B7. The 2-sided tabulated F-test value at the 95% confidence level with the associated degrees of freedom is approximately 4, hence, since this value is less than the F ratio calculated value of 5.50, there is probably a statistical difference between the data from all sources. The results of the non-parametric approach are shown in Table B8, where the count for the trial method was compared with the count for the reference method using two-tailed, binomial probability. This approach shows that the counts for the second source displayed significant differences by the trial method compared to the

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reference method. As indicated in Table B6, of the 15 paired results, 11 results were higher by the reference method, two paired results were the same and two results were lower (p-value = 0.02). In addition, the individual 95 % confidence interval for the results from source 2 indicate that the mean difference in paired log counts is entirely below zero, while the individual 95 % confidence intervals for the sources 1 and 3 includes zero.

Thus,

Source	Number of samples	Mean difference	Standard deviation	Range of 95% confidence interval
1	15	0.0503	0.2495	- 0.067 to + 0.148
2	15	- 0.2399	0.2407	- 0.031 to - 0.223
3	15	-0.0245	0.2564	- 0.176 to + 0.079

The results from sources 1 and 3 show no significant differences between the 2 methods. For source 2, the performance of the trial method compared to the reference method would suggest that it would not be advantageous to investigate further comparisons until, at least, further study resolved the indicative poor performance for source 2 and that this could be corrected. From the information available, it would seem reasonable to reject the trial method as being comparable to the reference method in this laboratory and that the comparison be terminated and a different trial method be investigated.

Table B6 Data for example B2 - Stage 1

Source 1		Source 2		Source 3	
Ref. method	Trial method	Ref. method	Trial method	Ref. method	Trial method
32	24	76	51	35	15
18	21	27	18	23	40
21	28	21	12	40	40
28	27	18	18	40	16
10	32	33	37	30	44
19	60	30	11	37	18
16	9	30	10	50	18
84	52	22	3	57	34
71	30	15	10	14	28
80	78	32	15	40	51
60	60	20	23	44	60
33	46	23	10	15	15
24	44	20	12	33	60
55	50	32	18	26	29
20	30	24	24	30	40

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Figure B9 Example B2 - Stage 1 - Plot of paired counts for trial and reference methods

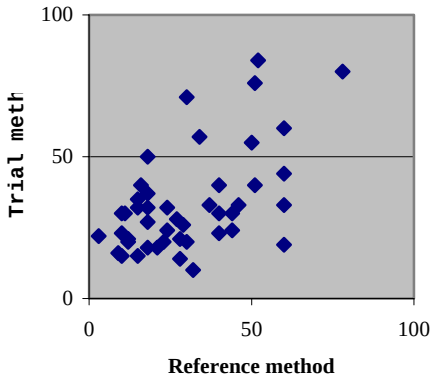

Figure B10 Example B2 - Stage 1 - Plot of paired counts (converted to $\log_{10}$ scale) of trial and reference method

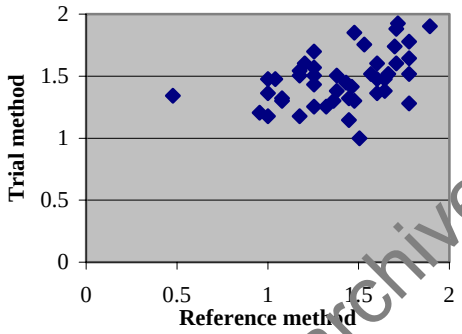

Table B7 Example B2 - Stage 1 - Parametric analysis

	Degrees of freedom	Sum of squares	Mean square	F ratio	p-value
Source	2	0.6812	0.3406	5.50	0.008
Error	42	2.6027	0.0620		
Total	44	3.2839			

Table B8 Example B2 - Stage 1 - non-parametric analysis

Source or category of origin	Number of results where trial method finds lower counts than reference method	Number of results where trial method finds an equal number of counts as the reference method	Number of results where trial method finds higher counts than reference method	Number of samples	p-value
1	7	1	7	15	0.5
2	11	2	2	15	0.02
3	5	2	8	15	0.3
All data	23	5	17	45	

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RESEARCH

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Emergence of antimicrobial resistance to *Pseudomonas aeruginosa* in the intensive care unit: association with the duration of antibiotic exposure and mode of administration

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Abstract

Background: Antibiotics are frequently used in intensive care units (ICUs), and their use is associated with the emergence of bacterial resistance to antibiotics. The aim of this study was to investigate the association between the emergence of *Pseudomonas aeruginosa* resistance and the duration of antibiotic exposure or mode of administration in an ICU unit.

Methods: A 4-year cohort study of intensive care unit was performed in patients with *P. aeruginosa* isolates from clinical specimens, initially susceptible to the investigated antibiotics (piperacillin/tazobactam, ceftazidime, ciprofloxacin, meropenem and amikacin). Odds ratios (ORs) with 95% confidence interval (95% CI) of emergence of resistance were calculated using logistic regression analysis for various exposure periods to antibiotics (1–3, 4–7, 8–15 and >15 days) relative to no exposure with adjustment for age, sex, Simplified Acute Physiology Score 3 (SAPS 3) and length of stay. ORs on the emergence of *P. aeruginosa* resistance were also calculated for the various modes of administration.

Results: Included were 187 patients [mean age 61 years, 69% male, mean SAPS 3 score (SD): 59 (12.3)]. None of the antibiotics investigated showed the emergence of resistance within 1–3 days. Significant meropenem resistance emerged within 8–15 days [OR 79.1 (14.9–421.0)] after antibiotic exposure unlike other antibiotics (>15 days). No difference was observed between intermittent and extended administration of meropenem and between beta-lactam mono- or combined therapy.

Conclusions: Use of meropenem was associated with the emergence of resistance as soon as 8 days after exposure to the antibiotic.

Keywords: *Pseudomonas aeruginosa*, Antibiotic resistance, Extended infusion

Background

Pseudomonas aeruginosa is responsible for 9% of all healthcare-associated infections [1], and the resistance to *P. aeruginosa* is increasing [2]. Data from the European Centre for Disease Prevention and Control show that around 18% of *P. aeruginosa* strains are resistant to carbapenems, 15% resistant to at least three out of five antimicrobial classes with an antipseudomonal spectrum (piperacillin/tazobactam, ceftazidime, fluoroquinolones, carbapenems and aminoglycosides) and even 5% resistant to all five [3]. Antimicrobial resistance is of particular importance in the intensive care unit (ICU) because antibiotic use in this setting is extremely common [4]. Several studies have explored the risk factors for *P. aeruginosa* antimicrobial resistance in non-critically ill patients [5, 6]. These studies showed that among antipseudomonas antibiotics, meropenem was associated with the highest

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risk of resistance emergence [4, 7]. The risk factors for emerging antimicrobial-resistant *Pseudomonas aeruginosa* in the ICU are also worth investigating, especially to answer the question how quick the antibiotic resistance emerges after the exposure to antibiotics. Also, worthy of investigation is whether the use of extended infusion of meropenem and the combination of beta-lactam antibiotics with aminoglycosides are associated with less risk of the emergence of resistance. Extended infusion of meropenem (over 3–4 h) has been shown to improve microbiologic and clinical cure since it shows a time-dependent effect on bacterial eradication [8] but it is not clear whether extended infusion also reduces the emergence of resistance. Combination of beta-lactam antibiotics with aminoglycosides is often used in daily practice [9], for example in critically ill patients, because it increases the chance of treating the patients with one active antibiotic. Arguably, the risk of inappropriate antibiotic therapy (no active molecule) will increase in case of use of a beta-lactam alone, and inappropriate therapy is usually associated with a poor prognosis. Yet, the use of combination has not shown to have an added benefit, for example in neutropenic patients [10].

Therefore, the aim of this study was twofold: first to quantify the duration of antibiotic exposure and other risk factors for emergence of resistance and second to investigate whether the mode of administration of meropenem and combination beta-lactams and aminoglycosides were associated with lower emergence of resistance of previously susceptible *P. aeruginosa*.

Methods

Study setting and population

The cohort study was performed between June 2007 and June 2011 in adult patients admitted to the ICU of the Antwerp University Hospital (UZA) for at least 48 h. This ICU consists of 45 beds with approximately 2600 adult admissions yearly; it serves as tertiary care hospital in a city with the population of around 500,000 inhabitants. Included in the study were patients with >1 clinical (i.e., not screening) culture positive with *P. aeruginosa* from any site of the body (blood, upper (sputum) and lower respiratory tract (bronchoalveolar lavage), and wound, that were taken from a patient at least 2 days apart. Patients who were re-admitted after an initial stay in the ICU during the observed period as well as those on antibiotics prior to admission were excluded. On the included patients, we collected demographic data (age and sex), clinical characteristics (reason of ICU admission and Simplified Acute Physiology Score 3 ((SAPS 3), a validated marker of disease severity [11]), use of mechanical ventilation) and data on all antimicrobial drugs the patients received during the ICU stay (duration and mode of administration). The patients received standard dose of antibiotics or adjusted dose according to renal clearance. The standard intravenous doses were 4 g/2 g q.i.d. for piperacillin/tazobactam, 6 g o.d. for ceftazidime, 400 mg b.d. for ciprofloxacin, 1 g t.d. for meropenem and 15 mg/kg divided into three doses for amikacin. Amikacin was, apart from treatment of endocarditis with gentamicin, the only aminoglycoside used in our ICU.

Specimens

All cultures were analyzed according to standard hospital and laboratory protocol. Antibiotic susceptibility testing was performed using disk diffusion technique on Mueller–Hinton agar for piperacillin/tazobactam, ceftazidime, ciprofloxacin, meropenem and amikacin [12]. Zone diameters were interpreted according to CLSI (Clinical & Laboratory Standards Institute) guidelines (CLSI M100) as susceptible, intermediate or resistant. Intermediate isolates were considered in this present study as susceptible since the intermediate category also implies that a higher-than-normal dosage of a drug can be used [13].

Statistical analysis

All variables were assessed and described according to their distribution. Normally distributed variables were presented as mean with standard deviation (SD), and not normally distributed variables as median with range.

The outcome of the investigation was the emergence of resistance in *P. aeruginosa*, defined as phenotype switch of the relevant antibiotic from susceptible/intermediate at the initial culture to resistant at any follow-up culture. The determinant variable was the duration of the relevant antibiotic used by the patients. The patients were divided into groups according to the number of days of antibiotic exposure according to the literature [6, 14] as follows: not exposed, 1–3, 4–7, 8–15 and >15 days.

Goodman and Kruskal's gamma tests were first used to investigate an association between higher categories of exposure and higher numbers of the emergence of resistance. Associations were considered significant if p-values were <0.05. To further quantify the effect of antibiotic exposure on emergence of resistance per antibiotic, logistic regression analysis was performed to calculate odds ratios (ORs) with 95% confidence interval (95% CI) of emerging resistance to an antibiotic for patients in one of the exposed groups (1–3, 4–7, 8–15 and >15 days) compared to the group without exposure with adjustment for possible confounders age, sex and SAPS 3 scores. Further adjustment was made for the length of stay. The analyses were repeated using the emergence of resistance antibiotic on the phenotype switch of any antipseudomonal

antibiotic as the outcome. Re-analyses were also performed after excluding possible cross-transmission. Arbitrarily, we defined possible cross-transmission as cases with phenotype switch to identical antibiogram with a potential source patient with overlapping time periods of 30 days. Due to the small number of the included study participants, the effect of interaction between antipseudomonal antibiotics was not investigated.

To investigate the effect of meropenem administration through extended or intermittent infusion and the effect of combination therapy versus monotherapy, the ORs (95% CI) were calculated using logistic regression analysis comparing the risk of emerging resistance in one regimen versus the risk in the comparator regimen. All analyses were performed using IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, NY).

Results

Demographic of study participants

We identified 405 patients with at least one positive culture with *P. aeruginosa* during the study period. After exclusion of patients with only one isolate ($n = 168$), the length of stay <48 h ($n = 18$) and patients re-admitted to the ICU ($n = 32$), 187 patients (mean age of 61 years, 69% male) were included. The flowchart of included patients is presented in Fig. 1. The included patients had mean SAPS 3 score of 59 (12.3). Most of the patients had mechanical ventilation (84.0%) and vasopressors (88.8%) (Table 1).

During the ICU admission, 96% of the patients received at least one of the antipseudomonal antibiotics. The most common antibiotics used were piperacillin/tazobactam (61.0%) and meropenem (54.8%). The median length of stay was 29 days (range 2–145 days).

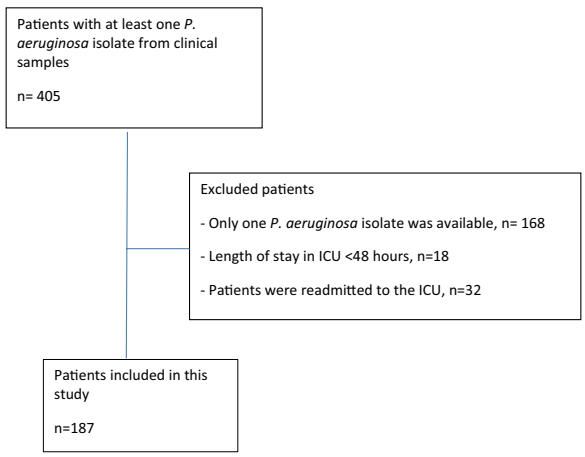

Fig. 1 Flowchart of included patients

Table 1 Demographic and clinical characteristics of the study cohort ($n = 187$)

Characteristics	Values
Mean age (SD), years	61 (14)
Male gender, *n* (%)	129 (69.0)
Mean Simplified Acute Physiology Score 3 score (SD)	59 (12)
Length of stay in intensive care unit, days, mean (range)	29 (2–145)
Previous non-antipseudomonal antibiotics, *n* (%)	79 (42.2)
Antibiotics during ICU stay	
Piperacillin/tazobactam, *n* (%)	114 (61.0)
Meropenem, *n* (%)	102 (54.8)
Ceftazidime, *n* (%)	73 (39.0)
Amikacin, *n* (%)	72 (38.5)
Ciprofloxacin, *n* (%)	69 (36.9)
Types of antibiotics received	
None	13 (7.4)
1	35 (19.9)
2–3	94 (53.5)
4–5	34 (19.2)
Reason for ICU admission, n (%)	
Cardiovascular/vascular	55 (29.4)
Respiratory	46 (24.6)
Neurological	32 (17.1)
Gastrointestinal	25 (13.4)
Sepsis/septic shock	15 (7.5)
Trauma	10 (5.3)
Others	5 (2.7)
Mechanical ventilation, *n* (%)	160 (83.8)
Therapy with one or more vasopressors, *n* (%)	169 (88.5)

SD standard deviation

From these patients, 1158 cultures were available (mean number cultures per patient 6, range 2–42). At baseline, the number of patients with an isolate susceptible to the antibiotic was 176 (94.1%) for piperacillin/tazobactam, 171 (91.4%) for ceftazidime, 133 for ciprofloxacin (71.1%), 124 (66.3%) for meropenem and 155 (82.9%) for amikacin. During the follow-up, phenotype switch from susceptible/intermediate to resistant for at least one antibiotic occurred in isolates from 38.1% of the patients during the ICU stay. During the ICU stay, 31.6% of *Pseudomonas* isolates became resistant to piperacillin/tazobactam, 27.5% to ceftazidime, 22.0% to meropenem, 13.3% to ciprofloxacin and 12.9% to amikacin.

Duration of antibiotics use and the emergence of resistance

Exposure to the relevant antibiotics was significantly associated with the emergence of resistance of *P. aeruginosa* for meropenem ($p < 0.001$), piperacillin/tazobactam ($p = 0.006$) and ceftazidime ($p = 0.006$). For

ciprofloxacin, there was an association but did not reach statistic significant level ($p = 0.06$). There was no association with amikacin ($p = 0.33$). Other variables such as age, gender, SAPS 3 scores, previous antibiotic use, necessity use of mechanical ventilation or vasopressors and ICU length of stay were not associated with the emergence of *P. aeruginosa* resistance to any of the antipseudomonal antibiotics during ICU stay (data not shown).

Using antibiotics for less than 4 days was not significantly associated with emergence of resistance (Table 2). When the antibiotic was administered for longer than 15 days, we observed the emergence of resistance of *P. aeruginosa* to all antipseudomonal antibiotics, except to amikacin. The OR (95% CI) after adjusting for age, gender and SAPS 3 scores for the emergence of resistance for piperacillin–tazobactam was 4.7 (1.8–12.4) and for ciprofloxacin was 14.5 (2.8–75.0). Giving ceftazidime or meropenem between 8 and 15 days was associated with phenotypic switch of *P. aeruginosa* to these antibiotics. The ORs (95% CI) were 2.6 (1.1–6.0) and 10.0 (19.8–551.0), respectively. Further adjustment for length of stay changed the significant associations to not significant, except for meropenem (Table 2). The association between meropenem use and emergence of resistance remained even after adjustment for length of stay.

Among all antipseudomonal antibiotics, only meropenem use of 8–15 days and >15 days was associated with the emerging of resistance of any of antipseudomonal antibiotics. The ORs were 6.3 (2.4–16.8, $p = 0.0001$) and 2.6 (1.1–6.3, $p = 0.04$), respectively.

Eight patients (4.2%) were excluded due to possible cross-contamination. Re-analysis after excluding these patients did not significantly alter the results (data not shown).

Association between the mode of administration of meropenem and combination beta-lactams and aminoglycosides with emergence of resistance

Extended administration of meropenem was not associated with a lower risk of emerging *P. aeruginosa* resistance in comparison with intermittent administration (OR 1.1 (0.2–4.0). The proportion of patients who acquired resistance was 45.5% for extended administration ($n = 46$) and 43.5% for intermittent administration ($n = 11$).

The combination of piperacillin/tazobactam with amikacin was also not associated with a lower risk of acquiring *P. aeruginosa* resistance [OR 1.0 (0.4–2.5)]. The proportion of patients who acquired resistance was 37.5% for combination therapy ($n = 24$) and 37.2% for patients with monotherapy ($n = 86$).

Discussion

We observed in this study that exposure to meropenem, as short as 8 days, was already associated with emergence of resistance in *Pseudomonas aeruginosa*, even after adjustment for length of stay. We also found that extended infusion of meropenem and combination of beta-lactam antibiotics with amikacin did not reduce emergence of resistance. The present study used patient-based instead of surveillance-based approach. Instead of detecting the emergence of resistance in patients who endured regular screening cultures, the emergence of resistance was studied in clinical cultures. This approach is convenient in a clinical situation where surveillance cultures are not always routinely performed. The results of the present study are in line with those from surveillance-based approach studies that showed carbapenem as antibiotic class with the strongest potential for selection of resistance, for example from Carmeli et al. [15]. Unlike Carmeli and co-workers who used imipenem in their study, we used meropenem. Whether one type of carbapenem is more associated with emergence of resistance should be further explored. Earlier studies also showed that antipseudomonal antibiotics piperacillin/tazobactam, ceftazidime and ciprofloxacin were associated with the emergence of *P. aeruginosa* resistance in critically ill patients [7, 16, 17]. However, in our study, the significant effect disappeared after further adjustment with the length of stay, except for meropenem, which highlights meropenem as an antibiotic that selected for *P. aeruginosa* resistance.

Several studies have been published recently that investigated the molecular mechanisms of the resistance of *P. aeruginosa* to antipseudomonal antibiotics showing that mutated genes during the emergence of beta-lactam resistance differed for each antibiotic [18, 19]. Since we do not perform molecular analysis, our study does not allow the analysis of mechanisms involved in the emergence of resistance, either expression of intrinsic resistance mechanisms in response to antimicrobial exposure such as reduction in expression of the outer membrane proteins (porins) or increase production of an efflux pump to a variety of antibiotics.

To the best of our knowledge, the present study is the first to show that the emergence of resistance in initially susceptible *P. aeruginosa* isolates does not occur when the antibiotic was given for less than 4 days, even for meropenem. This is in line with results from Lodise et al. [6] that showed patients infected with a carbapenem-resistant *P. aeruginosa* were more likely to have been exposed to carbapenems for 3 days or more compared to patients infected with a carbapenem susceptible strain. The combination therapy beta-lactam and amikacin failed to decrease the emergence of resistance unlike in

Table 2 Odds ratios for the emergence of antipseudomonal antibiotic resistance by *Pseudomonas aeruginosa* in relation to antibiotic duration

Antibiotic duration	Number of patients	Events (%)	Odds ratio (95% confidence interval) adjusted for age, gender and SAPS 3 scores	Odds ratio (95% confidence interval) adjusted for age, gender, SAPS 3 scores and length of stay
Piperacillin–tazobactam ($n = 176$)				
Reference (i.e., use of other antibiotic than piperacillin–tazobactam or no antibiotic)	65	13 (21.2)	1	1
1–3 days	17	6 (35.3)	2.5 (0.8–8.0)	2.3 (0.7–8.2)
4–7 days	29	11 (37.9)	2.3 (0.8–6.1)	2.2 (0.7–6.4)
8–15 days	34	9 (26.5)	1.5 (0.6–4.1)	1.5 (0.5–4.3)
>15 days	29	16 (55.2)	4.7 (1.8–12.4)‡	1.7 (0.6–5.3)
Ceftazidime ($n = 171$)				
Reference (i.e., use of other antibiotic than ceftazidime or no antibiotic)	99	22 (22.2)	1	1
1–3 days	13	0	NA	NA
4–7 days	12	8 (66.7)	1.7 (0.4–6.6)	1.7 (0.4–7.4)
8–15 days	34	14 (41.2)	2.6 (1.1–6.0)‡	1.7 (0.7–4.3)
>15 days	13	7 (53.8)	3.4 (1.0–12.4)‡	0.7 (0.2–3.4)
Ciprofloxacin ($n = 128$)				
Reference (i.e., use of other antibiotic than ciprofloxacin or no antibiotic)	78	7 (9)	1	1
1–3 days	18	2 (5.6)	1.5 (0.3–7.9)	2.0 (0.3–11.7)
4–7 days	10	0	0	0
8–15 days	13	3 (23.1)	2.7 (0.5–13.3)	1.4 (0.2–850)
>15 days	9	5 (55.6)	14.5 (2.8–75.0)‡	1.9 (0.2–15.6)
Meropenem ($n = 123$)				
Reference (i.e., use of other antibiotic than meropenem or no antibiotic)	64	3 (4.7)	1	1
1–3 days	13	0 (0)	0.0	0.0
4–7 days	9	2 (5.6)	4.9 (0.7–37.2)	3.4 (0.4–30.2)
8–15 days	21	17 (81.0)	104.5 (19.8–551.0)‡	79.1 (14.9–421.0)‡
>15 days	16	5 (31.2)	10.0 (2.0–49.5)‡	4.6 (1.7–32.2)‡
Amikacin ($n = 148$)				
Reference (i.e., use of other antibiotic than amikacin or no antibiotic)	87	10 (11.5)	1	1
1–3 days	41	7 (17.1)	1.3 (0.4–4.2)	0.7 (0.2–2.6)
4–7 days	13	1 (7.7)	0.3 (0.03–3.0)	0.1 (0.01–2.2)
8–15 days	7	1 (14.3)	0.5 (0.05–5.1)	0.1 (0.01–2.9)
>15 days	0	0	0.0	0.0

‡ Statistically significant at $p < 0.05$

an in vitro study [20]. One of the possible explanations is that amikacin drug concentration may be suboptimal for *P. aeruginosa* in ICU patients. We did not always perform peak measurement of amikacin, and therefore, we do not know whether the pharmacokinetic/pharmacodynamic (PK/PD) target was achieved. In this study, the dose of 15 mg/kg divided into three doses was mostly used, yet some authors advised the use of 25 mg/kg/day [21].

Extended meropenem infusion was also not associated with less emergence of resistance compared with intermittent infusion. This effect may be true but alternative explanations such as optimal dosing are also possible and further study on this issue is needed.

This study has further limitations. Firstly, although using clinical specimens approach is convenient and mimic daily practice in many ICUs, this study cannot determine the exact date of phenotype switch because cultures were not regularly performed. Since the study was based on clinical specimens, the follow-up duration of the patients varied, i.e., the follow-up duration

was the time frame between the first and the last positive culture for *P. aeruginosa*. A prospective study design with cultures taken at regular time frame will be a better study design despite in the routine practice *P. aeruginosa* could not always be re-isolated. Secondly, this study does not use genotyping to investigate the relatedness of the strains and to confirm the cross-transmission of resistant *P. aeruginosa*. However, cross-transmission estimated in this study (4.2%) is comparable with cross-transmission confirmed by genotyping (2.8%) as shown in a previous study [7], and the results of the analysis remain the same after excluding possible cross-transmission. It is also possible that several strains could be responsible for infection and this selection pressure cannot be differentiated from the emergence of resistance since no genotyping was performed. Thirdly, the results of this study might underestimate the effect since no minimum inhibitory concentration (MIC) values were available. Antipseudomonal antibiotics use might be associated with increasing MIC values that does not cause phenotypic switch on disk diffusion. Fourthly, the number of patients was relatively small and the study may be underpowered for several antibiotics that were used in low number, and lastly this study was performed in one center only.

Conclusions

The results of this study suggested that meropenem is associated with resistance of *P. aeruginosa* as soon after 8 days of use. This finding should be confirmed in other studies. All antipseudomonal antibiotics except amikacin are associated with the emergence of *P. aeruginosa* resistance. Physicians should weigh up the risk of resistance and the therapeutic benefit when meropenem or other antipseudomonal antibiotics are going to be used for a long period. Moreover, when physicians start empiric therapy with a carbapenem antibiotic, they should consider to de-escalate it within 48–72 h, once the culture results are known since the switch might be clinically beneficial for the patients.

Abbreviations

ICU: intensive care unit; SAPS 3: Simplified Acute Physiology Score 3; CLSI: Clinical & Laboratory Standards Institute; SD: standard deviation; OR: odds ratio; 95% CI: 95% confidence interval; MIC: minimum inhibitory concentration.

Authors' contributions

All authors contributed to the conception and design of the work. All authors performed the acquisition, analysis and interpretation of data. EY and BVH drafted the work. EY, BVH, WV, KW, PGJ and HG revised the work critically for important intellectual content. All authors gave final approval of the version to be published. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Data can be requested from the corresponding author in SPSS format.

Ethics approval and consent to participate

The study was reviewed and approved by the hospital's institutional review board (reference number EC110219), and informed consent was waived as the data were anonymized and gathered in a retrospective manner.

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NHS Greater Glasgow & Clyde Acute Division
RHC HAEMATOLOGY AND ONCOLOGY ADMISSIONS
(DC=DAY CASE, IP EL=INPATIENT ELECTIVE,IP NON EL=IP NON ELECTIVE)

	2015 (FROM JUNE)				2016				2017				2018				2019				2020				2021				2022				
ADMISSION WARD	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	DC	IP EI	IP Non EI	Tot	TOT
RHC Ward 2B	732	218	99	1049	1191	423	158	1772	1638	421	233	2292	1123	314	258	1695																	6808
QEUH 6A DCH Day Unit													387	87	93	567	1506	278	256	2040	1024	227	149	1400	1384	317	97	1798	262	49	13	324	6129
RHC Area 1B Day surgery	191	12		203	373	26		399	329	19	1	349	388	23		411	455	25	1	481	120	5		125	169	11		180	224	10	3	237	2385
RHC Schiehallion Day Unit 2B																													1106	221	141	1468	1468
RHC Clinical Decision Unit			3	3			5	5		2	119	121		1	143	144	1	8	108	117		2	165	167		5	405	410	2	1	408	411	1378
RHC Ward 2A	6	83	165	254	3	160	331	494	1	124	151	276		80	101	181																	1205
QEUH 6ACH Inpatients														23	49	72	5	112	184	301		52	78	130	8	49	58	115	1	12	14	27	645
RHC Ward 1A 23 Hour Ward											1	1		1		1		1		1	152	8		160	210	17		227	189	20		209	599
RHC Ward 2A Schiehallion																													2	54	74	130	130
RHC Area 1C Day Care Unit									5			5						1		1	66	4		70	17	2		19					95
RHC Paediatric Haematology/Oncology																	70	10	9	89			2	2									91
Yorkhill Schiehallion Day Care Unit - Haematology	40	9	3	52																													52
RHC Ward 2C Beds 1-20			3	3			3	3		1	3	4			1	1			2	2		2	7	9	1	2	10	13	3	2	7	12	47
RHC Ward 3B Multiple Spec		1	4	5			1	1		2	3	5		1	2	3					13	4	1	18	1		2	3		1	2	3	38
RHC Ward 3C	1	2	2	5		2	4	6	1	4	7	12			2	2																	25
RHC Ward 3A Multiple Spec		2	4	6			3	3		1	3	4			3	3		1		1						3	2	5					22
RHC Ward 2C Beds 21-40			2	2			5	5		2	3	5			2	2		1	1	2			1	1			4	4		1		1	22
Yorkhill Day Surgery Unit - XSU	17			17																													17
RHC Ward 1D Paediatric Critical Care			4	4			1	1			4	4			2	2			1	1			1	1			2	2			1	1	16
QEUH 4BCH Bone Marrow Transplant														2		2		8	1	9		2		2		1		1			1	1	15
RHC Ward 3C Orthopaedics															1	1			2	2	1	1	8	10			2	2					15
RHC Ward 1E Cardio/CS							2	2		2	2	4		1	2	3			2	2							2	2					13
Yorkhill Schiehallion Ward - Haematology		3	7	10																													10
RHC Ward 3C Renal															2	2			1	1	2			2			1	1					6
RHC Ward 2A Clinical Decisions Unit																			6	6													6
Yorkhill Ward 4A - Medical Paediatrics			1	1																													1
Grand Total	987	330	297	1614	1567	611	513	2691	1974	578	530	3082	1898	533	661	3092	2037	445	574	3056	1378	307	412	2097	1790	407	585	2782	1789	371	664	2824	21238

SCOTTISH STATUTORY INSTRUMENTS

2001 No. 207

WATER SUPPLY

The Water Supply (Water Quality) (Scotland) Regulations 2001

Made - - - - 4th June 2001
Laid before the Scottish Parliament - - - - 5th June 2001
Coming into force in accordance with regulation 1(2) to (4)

The Scottish Ministers, in exercise of the powers conferred by sections 76B, 76F(5) and (6), 76J, 101(1) and (1A), and 109(1) of the Water (Scotland) Act 1980(**1**) and section 2(2) of the European Communities Act 1972(**2**), and of all other powers enabling them in that behalf, hereby make the following Regulations:

PART I

GENERAL

Citation, commencement and extent

1.—(1) These Regulations may be cited as the Water Supply (Water Quality) (Scotland) Regulations 2001.

(2) This regulation and regulations 2, 36 and 37 shall come into force on 26th June 2001.

(3) Regulations 3 and 38 shall come into force on 1st June 2003.

(4) All other provisions of these Regulations shall come into force on 25th December 2003.

(5) These Regulations extend to Scotland only.

Interpretation

2.—(1) In these Regulations–

(**1**) 1980 c. 45. Sections 76B, 76F and 76J were inserted by the Water Act 1989 (c. 15), Schedule 22. Section 101(1A) was inserted by the Natural Heritage (Scotland) Act 1991 (c. 28), Schedule 10, paragraph 9(5). The functions of the Secretary of State were transferred to the Scottish Ministers by virtue of section 53 of the Scotland Act 1998 (c. 46).

(**2**) 1972 c. 68. Section 2(2) was amended by the Scotland Act 1998 (c. 46), Schedule 8, paragraph 15(3). The functions conferred on the Minister of the Crown under section 2(2) of the European Communities Act 1972, insofar as within devolved competence, were transferred to the Scottish Ministers by virtue of section 53 of the Scotland Act 1998.

"the the Act" means the Water (Scotland) Act 1980;

"the 1990 Regulations" means the Water Supply (Water Quality) (Scotland) Regulations 1990(**3**);

"appropriate local authority" and "appropriate health board" in relation to a departure authorised under regulation 20 or 21 or an application for any such authorisation, means the local authority and the health board, respectively, whose area contains any part of the water supply zone to which the authorisation relates or, in the case of an application, would apply if a departure were authorised in the terms sought;

"blending point" means a point at which waters originating from two or more sources and treated for the purposes of their supply for regulation 4(1) purposes are combined under conditions that are designed to secure that, after such combination, the requirements of paragraph (2) of regulation 4 are met;

"consumer" means a person to whom water is supplied for regulation 4(1) purposes by a water authority in the discharge of its duties under Part II of the Act;

"disinfection" means a process which removes or renders inactive pathogenic micro-organisms so as to satisfy the requirements of Part III of these Regulations in respect of micro-organisms (other than parameters), parasites and the parameters listed in Table A in Schedule 1 to these Regulations; and "disinfected" shall be construed accordingly;

"ground waters" has the same meaning as in section 30A(1)(d) of the Control of Pollution Act 1974(**4**), but does not include waters contained in underground strata in a sewer, pipe or service reservoir within the meaning of regulation 2(4);

"health board" means a board constituted by order made under section 2(1) of the National Health Service (Scotland) Act 1978(**5**);

"indicator parameter" means a parameter listed in Schedule 2;

"local authority" means a council constituted under section 2 of the Local Government etc. (Scotland) Act 1994(**6**);

"parameter" means a property, element, organism or substance listed in the second column of Table A or Table B in Schedule 1 to these Regulations, or in Schedule 2, as read, where appropriate, with the notes to Schedule 2 and those Tables;

"pesticides and related products" means–

(a) any organic insecticide;

(b) any organic herbicide;

(c) any organic fungicide;

(d) any organic nematocide;

(e) any organic acaricide;

(f) any organic algicide;

(g) any organic rodenticide;

(h) any organic slimicide; and

(i) any product related to any of (a) to (h) (including any growth regulator),

and includes their relevant metabolites, degradation and reaction products;

(**3**) S.I.1990/119, amended by S.I. 1991/1333.
(**4**) 1974 c. 40.
(**5**) 1978 c. 29.
(**6**) 1994 c. 39.

"prescribed concentration or value", in relation to any parameter, means the maximum or minimum concentration or value specified in relation to that parameter in Table A or Table B in Schedule 1 as measured by reference to the unit of measurement so specified, and as read, where appropriate, with the notes to those Tables;

"regulation 4(1) purposes", in relation to the supply of water, means a supply–

(a) for such domestic purposes as consist in or include, cooking, drinking, food preparation or washing; or

(b) for any of these domestic purposes to premises in which food is produced;

"sampling point"–

(a) in relation to water supplied from a distribution network, means a point, being a consumer's tap, that is selected for the purposes of Part IV of these Regulations; and

(b) in relation to water supplied from a tanker, means the point at which the water emerges from the tanker;

"specification", in relation to an indicator parameter, means the concentration, value or state, shown as applicable to that parameter in Schedule 2 as measured by reference to the unit of measurement so shown;

"state", in relation to an indicator parameter, means the state specified in relation to that parameter in Schedule 2 as measured by reference to the unit of measurement so specified;

"supply point" means a blending point, service reservoir, treatment works or other point, not being a sampling point, which the Scottish Ministers may, in accordance with regulation 8, authorise for the purposes of regulation 6;

"water authority" means a new water and sewerage authority constituted under section 62 of the Local Government etc. (Scotland) Act 1994;

"water supply zone", in relation to a water authority and a year, means an area designated for that year by the water authority in accordance with regulation 3; and

"year" means a calendar year.

(2) Other expressions used both in these Regulations and in Council Directive 98/83/EC (on the quality of water intended for human consumption)(**7**) have the same meaning in these Regulations as they have in that Directive.

(3) Any reference in these Regulations to a numbered regulation shall be treated as the regulation bearing that number in these Regulations; any reference to a numbered paragraph shall be treated as the paragraph bearing that number in the regulation in which it appears; and any reference to a Schedule shall be treated as a reference to a Schedule to these Regulations;

(4) Subject to paragraph (5), references in these Regulations to a service reservoir are references to any structure, other than a structure at a treatment works, in which water that has been treated with a view to complying with the requirements of regulation 4 is contained and stored for the purpose of meeting a variable demand for the supply of water.

(5) Where references in these Regulations to a service reservoir would, but for this paragraph, include references to a structure comprising more than one compartment–

(a) each compartment which has its own water inlet and water outlet and is not connected hydraulically to any other compartment shall be treated as a single service reservoir;

(b) the compartments which are connected hydraulically shall be treated as a single service reservoir; and

(c) unless all of the compartments are connected hydraulically, the structure as a whole shall not be treated as a service reservoir.

(**7**) O.J. No. L 330, 5.12.98, p.32.

PART II

WATER SUPPLY ZONES

Water supply zones

3.—(1) Before the beginning of each year in which it intends to supply water for regulation 4(1) purposes, a water authority shall designate the names and areas within its area of supply that are to be its water supply zones for that year.

(2) A water supply zone may not comprise an area whose population immediately before the beginning of the year in question is estimated by the water authority to exceed 100,000.

(3) A water authority may not vary a designation under paragraph (1) after the beginning of the year in relation to which the designation has effect.

PART III

WHOLESOMENESS

Wholesomeness

4.—(1) Water supplied–

(a) for such domestic purposes as consist in or include, cooking, drinking, food preparation or washing; or

(b) for any of those domestic purposes to premises in which food is produced,

shall, subject to paragraphs (4) and (5) below, be regarded as wholesome for the purposes of Part VI A of the Act, as it applies to the supply of water for those domestic purposes, if the requirements of paragraph (2) are satisfied.

(2) The requirements of this paragraph are–

(a) that the water does not contain–

(i) any micro-organism (other than a parameter) or parasite; or

(ii) any substance (other than a parameter),

at a concentration or value which would constitute a potential danger to human health;

(b) that the water does not contain any substance (whether or not a parameter) at a concentration or value which, in conjunction with any other substance it contains (whether or not a parameter) would constitute a potential danger to human health;

(c) that the water does not contain concentrations or values of the parameters listed in Tables A and B in Schedule 1 in excess of or, as the case may be, less than the prescribed concentrations or values; and

(d) that the water satisfies the formula [nitrate]/50 + [nitrite]/3 • 1, where the square brackets signify the concentrations in mg/1 for nitrate (NO_3) and nitrite (NO_2).

(3) The point at which the requirements of paragraph (2), in so far as they relate to the parameters set out in Part I of Table A and in Table B in Schedule 1 are to be complied with is–

(a) in the case of water supplied from a tanker, the point at which the water emerges from the tanker;

(b) in any other case, the consumer's tap.

(4) Water supplied for regulation 4(1) purposes shall be regarded as unwholesome for the purposes of Part VI A of the Act if, on transfer from a treatment works for supply for those purposes–

(a) it contains a concentration of the coliform bacteria or E. coli. parameter (items 1 and 2 in Part II of Table A in Schedule 1) in excess of the prescribed concentrations; or

(b) it contains a concentration of nitrite in excess of 0.1 $mgNO_2/l$.

(5) Subject to paragraph (6), water supplied for regulation 4(1) purposes shall be regarded as unwholesome for the purposes of Part VI A of the Act if, on transfer from a service reservoir for supply for those purposes, it contains a concentration of the coliform bacteria or E. coli parameter in excess of the prescribed concentrations.

(6) Water transferred from a service reservoir for supply for regulation 4(1) purposes shall not be regarded as unwholesome for the purposes of Part VI A of the Act because the maximum concentration for the coliform bacteria parameter listed in Part II of Table A in Schedule 1 is exceeded if, as regards the samples taken in any year in which the reservoir in question is in use, the results of analysis for that parameter establish that in at least 95% of those samples coliforms were absent.

PART IV

MONITORING OF WATER SUPPLIES

Interpretation and application

5.—(1) In this Part, "audit monitoring" means monitoring for the purpose of obtaining information from which it may be established–

(a) as regards the parameters listed in Tables A and B in Schedule 1, whether water supplied for regulation 4(1) purposes satisfies the provisions of Part III of these Regulations or, if a departure has been authorised under Part VI in relation to that supply, those provisions as read with the terms of that departure; and

(b) as regards indicator parameters in Schedule 2, whether water supplied for regulation 4(1) purposes meets the specifications for those parameters.

(2) In this Part, "check monitoring" means monitoring for the purpose of obtaining information at regular intervals–

(a) as to the organoleptic and microbiological quality of water; and

(b) where relevant, as to the effectiveness of drinking-water treatment (particularly of disinfection),

for the purpose of determining–

(i) as regards the parameters listed in Tables A and B in Schedule 1, whether water supplied for regulation 4(1) purposes satisfies the provisions of Part III of these Regulations or, if a departure has been authorised under Part VI in relation to that supply, those provisions as read with the terms of that departure; and

(ii) as regards indicator parameters listed in Schedule 2, whether water supplied for regulation 4(1) purposes meets the specifications for those parameters.

(3) This Part applies to water supplied for regulation 4(1) purposes by a water authority in the performance of its duties under Part VI A of the Act.

Monitoring: general provisions

6.—(1) For the purpose of determining whether water to which this Part applies satisfies the provisions of Part III or, if a departure has been authorised under Part VI in relation to that supply,

those provisions as read with the terms of that authorisation, a water authority shall take, or cause to be taken, and analyse, or cause to be analysed, not less than the number of samples of the water within each of its water supply zones specified in, or in accordance with the provisions of, this Part.

(2) Except in a case to which paragraph (3) applies, the parameters listed in Tables A and B in Schedule 1 and the indicator parameters in Schedule 2 shall be subject–

(a) as regards a parameter listed in column (2) of Table 1 in Schedule 3, in relation to which there is no entry in column (3) of that Table, to check monitoring;

(b) as regards a parameter so listed in relation to which there is an entry in column (3), check monitoring in the circumstances specified in that column;

(c) in any other case, audit monitoring.

(3) Where–

(a) the distribution of water in any part of a water supply zone is by tanker; and

(b) is or is likely to be an intermittent short-term supply,

samples of water from each tanker from which water is distributed shall be taken 48 hours after the commencement of the distribution from that tanker and every 48 hours thereafter until the distribution is discontinued.

(4) Of the samples taken in accordance with paragraph (3) in relation to each distribution, the first shall be analysed for compliance with the parameters *E. Coli*, hydrogen ion and conductivity (item 2 in Part I of Table A in Schedule 1, item 3 in Part II of Table B in Schedule 1 and item 6 in Schedule 2, respectively), and the second and any subsequent samples shall be analysed for compliance with those and every other parameter.

(5) For the purposes of the application of paragraph (2)(b) to the aluminium, *clostridium perfringens*, iron and manganese parameters (items 1, 3, 10 and 11 in Table 1 in Schedule 3), a supply which consists of both ground waters and surface water shall be deemed to be a supply which consists only of surface water.

(6) The copper, lead and nickel parameters and, subject to paragraph (7), the parameters relevant to radioactivity (total indicative dose and tritium), shall be monitored in such manner as the Scottish Ministers shall specify from time to time by notice in writing (or in electronic form) given to each water authority.

(7) If, in relation to any water supply zone, the Scottish Ministers are satisfied that water supplied to that zone for regulation 4(1) purposes–

(a) gives rise to a calculated total indicative dose in respect of radioactivity that is well below the specification; or

(b) contains levels of tritium that are well below the specification,

they shall notify the water authority who supplies water to that zone that the total indicative dose parameter (item 8 in Schedule 2) or, as the case may be, the tritium parameter (item 10 in Schedule 2) need not be monitored.

(8) The Scottish Ministers shall, by notice in writing (or in electronic form)–

(a) withdraw a notice under paragraph (7) given in relation to the total indicative dose parameter if they believe that water supplied to the zone in question for regulation 4(1) purposes gives rise to a calculated total indicative dose in respect of radioactivity that is not well below the specification;

(b) withdraw a notice under paragraph (7) given in relation to the tritium parameter if they believe that water supplied to the zone in question for regulation 4(1) purposes contains levels of tritium that are not well below the specification.

(9) A water authority which receives a notice under paragraph (8) shall then monitor the total indicative dose parameter or, as the case may be, the tritium parameter in accordance with the notice having effect for the time being under paragraph (6).

Determination of sampling points

7. Except in relation to water supplied from a tanker, sampling points in respect of every parameter, other than a parameter for which samples are taken from a supply point authorised by or under regulation 8, shall be selected at random unless, by notice in writing (or in electronic form acceptable to them) to a water authority (whether or not on the application of that authority), the Scottish Ministers otherwise direct.

Authorisation of supply points

8.—(1) The Scottish Ministers, being satisfied that analysis of samples taken from–

(a) any blending point;

(b) the water leaving any service reservoir which receives water from a treatment works before its supply to any consumer; and

(c) the water leaving any treatment works,

will produce data in respect of the parameters specified in Table 3 of Schedule 3 which are unlikely to differ in any material respect from the data that would be produced in respect of those parameters from analysis of samples obtained from sampling points, authorise the use for the purposes of regulation 6 of samples in relation to those parameters taken for a water supply zone from a blending point, a service reservoir of that description or a treatment works.

(2) Subject to paragraph (3), the Scottish Ministers may, in relation to any parameter other than a parameter specified in Table 3 in Schedule 3, on the application in writing or in electronic form of a water authority, authorise the use for the purposes of regulation 6 of samples taken for a water supply zone otherwise than from a sampling point, and any such authorisation may extend to all samples in relation to that parameter or to such number or proportion of those samples as is specified in the authorisation.

(3) The Scottish Ministers shall not grant an authorisation under paragraph (2) unless they are satisfied that analysis of samples taken from a point other than a sampling point will produce data in respect of the parameter in question which are unlikely to differ in any material respect from the data that would be produced in respect of that parameter from analysis of samples obtained from sampling points.

(4) Subject to paragraph (5), the Scottish Ministers may at any time modify or revoke an authorisation under paragraph (2).

(5) Unless it appears to the Scottish Ministers that the immediate modification or revocation of an authorisation under paragraph (2) is required in the interests of public health, they shall not modify or revoke such an authorisation without giving to the water authority to which the authorisation relates at least six weeks' notice of their intention to modify or revoke.

(6) A water authority shall notify the Scottish Ministers as soon as it has reasonable grounds for believing that an analysis of samples taken for a water supply zone from a point other than a sampling point would produce data in respect of the parameter in question which would differ in a material respect from the data produced by an analysis of samples taken from any of the sampling points within that zone; and the Scottish Ministers shall thereupon, and without the need for prior notice to the water authority, revoke the authorisation.

Numbers of samples

9.—(1) Subject to paragraph (2), in each year a water authority shall take, or cause to be taken–

(a) from its sampling points; or

(b) to the extent authorised by or under regulation 8, from its supply points,

the standard number of samples for analysis as regards residual disinfectant and each parameter listed in column (1) of Table 2 in Schedule 3 or, as the case may be, Tables 3 or 4 in that Schedule.

(2) Where, in respect of a parameter subject to check monitoring–

(a) a water authority is of the opinion that the quality of water supplied by it to a water supply zone is unlikely to deteriorate; and

(b) in each of two successive years the results of samples taken, subject to paragraph (3), in accordance with these Regulations show no significant variation and–

(i) if the parameter is colony counts, have shown no abnormal change;

(ii) if the parameter in question is hydrogen ion concentration (item 9 in Table 1 in Schedule 3), have established a pH value that is not less than 6.5 and not more than 9.5;

(iii) in any other case, have established a concentration or value for that parameter that is significantly lower than the prescribed concentration or value, or specification,

the number of samples to be taken in the following year for that parameter may be the reduced number.

(3) Where the following year is 2004, paragraph (2)(b) shall apply as if for "these Regulations" there were substituted "the 1990 Regulations"; and where the following year is 2005, paragraph (2)(b) shall apply as if before "these Regulations" there were inserted "the 1990 Regulations and".

(4) Samples required to be taken by this regulation shall be taken at regular intervals.

(5) In this regulation–

(a) in relation to water supply zones, residual disinfectant or a parameter and the supply of water to an estimated population within one of the ranges shown in column (2) of Table 2 in Schedule 3, "the reduced number" and the "standard number" mean the number shown in column (3) and column (4), respectively, of that Table as applicable to that substance or parameter by reference to a population within that range; and

(b) in relation to supply points, each of the parameters specified in Table 3 of Schedule 3, and the supply of a volume of water within one of the ranges shown in column (3) of that Table, "the reduced number" and "the standard number" mean the number shown in column (4) and column (5), respectively, of that Table as applicable to that parameter by reference to a volume supplied within that range.

Sampling: further provisions

10. As soon as a water authority has reasonable grounds for believing that any element, organism or substance, other than residual disinfectant or a parameter, whether alone or in combination with a parameter or any other element, organism or substance, may cause the supply within any of its water supply zones to be a supply which does not satisfy–

(a) the provisions of Part III of these Regulations or;

(b) if a departure has been authorised under Part VI, those provisions as read with the terms of that authorisation,

it shall take, or cause to be taken, sufficient samples from water within that zone (whether from a service reservoir, a treatment works or otherwise) in respect of that element, organism or substance, in order to establish whether that water is wholesome.

PART V

MONITORING – ADDITIONAL PROVISIONS

Interpretation

11. In this Part, in relation to residual disinfectant or a parameter specified in Table 4 of Schedule 3, and the supply of a volume of water within one of the ranges shown in column (3) of that Table, "the reduced number" and "the standard number" mean the number shown in column (4) and column (5), respectively, of that Table as applicable to residual disinfectant or the parameter in question by reference to a volume of water within that range.

Sampling for particular substances and parameters

12. For the purposes of establishing the quality of water to be supplied to any of its water supply zones, a water authority shall take, or cause to be taken, and analyse, or cause to be analysed, not less than the number of samples specified in this Part.

Sampling at treatment works

13.—(1) Subject to paragraphs (2) and (4), in each year a water authority shall take, or cause to be taken, from the point at which water leaves each treatment works which serves its water supply zones, the standard number of samples for analysis–

(a) for determining the concentration of residual disinfectant;

(b) for determining whether, in relation to the colony counts and turbidity parameters, water leaving treatment works meets the specifications for those parameters set out in Schedule 2; and

(c) for testing for compliance with the prescribed concentrations or values set out in Schedule 1 in respect of the coliform bacteria, *E. coli* and nitrite parameters for water leaving treatment works.

(2) Where in each of two successive years the results of the analysis of samples taken, subject to paragraph (3), in accordance with these Regulations have established–

(a) in respect of the coliform bacteria, *E. coli* or nitrite parameter, that the maximum concentration has not been exceeded;

(b) in respect of the turbidity parameter that the specification has been met;

(c) in respect of the colony counts parameter, that there has been no significant increase,

the number of samples to be taken in respect of that parameter in the next following year from the point at which water leaves that treatment works may, subject to paragraph (4), be the reduced number.

(3) Paragraph (2) shall apply–

(a) where the following year is 2004, as if for "these Regulations" there were substituted "the 1990 Regulations"; and

(b) where the following year is 2005, as if before "these Regulations" there were inserted "the 1990 Regulations and".

(4) In respect of the coliform bacteria parameter and the *E. coli* parameter, the reduced number of samples may be taken in accordance with paragraph (2) only if the water authority is of the opinion–

(a) that there is no foreseeable risk that the supply will exceed the maximum concentration for that parameter; or

(b) that the treatment works is designed to secure that, in the event of a failure of the disinfection process, water that has not been disinfected cannot enter the supply.

(5) Samples required to be taken by this regulation shall be taken at regular intervals.

Sampling at service reservoirs

14. A water authority shall take, or cause to be taken, from each of its service reservoirs in each week in which the reservoir is in use, one sample for analysis–

(a) for testing for compliance with the prescribed concentrations or values in respect of the E. coli and coliform bacteria parameters set out in Part II of Table A to Schedule 1;

(b) for determining the concentration of residual disinfectant; and

(c) for determining whether the specification in relation to the colony counts parameter set out in Schedule 2 is met.

Sampling: new sources

15.—(1) This regulation applies as respects–

(a) any source which has not been used for the supply of water by a water authority at any time since 25th December 2003; and

(b) any source which has been so used but not so used for a period of six months preceding the date on which the water authority proposes to supply water from it.

(2) A water authority shall–

(a) before it supplies water from a source mentioned in paragraph (1)(a); and

(b) as soon as is reasonably practicable after it has begun to supply water from a source mentioned in paragraph (1)(b),

take, or cause to be taken, in accordance with paragraph (3), such samples of that water as will enable it to establish–

(i) whether water can be supplied from that source without contravening section 76A(1) of the Act; and

(ii) the treatment necessary to ensure that section 76A(2) of the Act is complied with in relation to the supply of that water.

(3) Samples shall be taken–

(a) in the case of a source mentioned in paragraph (1)(a), in respect of–

(i) the parameters listed in Schedules 1 and 2; and

(ii) any other element, organism or substance which, in the opinion of the water authority, may cause the supply to contravene section 76A(1) of the Act;

(b) in the case of a source mentioned in paragraph (1)(b), in respect of–

(i) the parameters listed in Table A in Schedule 1;

(ii) the conductivity, hydrogen ion and turbidity parameters; and

(iii) any other parameter as regards which the water authority is of the opinion that its concentration or value is likely to have altered since the last occasion on which water from that source was analysed.

Collection and analysis of samples

16.—(1) A water authority shall secure, so far as is reasonably practicable, that in taking, handling, transporting, storing and analysing any sample required to be taken for the purposes of Part IV or this Part of these Regulations, or causing any such sample to be taken, handled, transported, stored and analysed, the appropriate requirements are satisfied.

(2) In paragraph (1) "the appropriate requirements" means such of the following requirements as are applicable–

(a) the sample is representative of the quality of the water at the time of sampling;

(b) the sample is not contaminated when being taken;

(c) the sample is kept at such temperature and in such conditions as will secure that there is no material alteration of the concentration or value or specification for the measurement or observation for which the sample is intended;

(d) the sample is analysed as soon as may be after it has been taken–

(i) by or under the supervision of a person who is competent to perform that task; and

(ii) with the use of such equipment as is suitable for the purpose;

(e) any laboratory at which samples are analysed has a system of analytical quality control that is subjected from time to time to checking by a person who is–

(i) not under the control of either the laboratory or the water authority; and

(ii) approved by the Scottish Ministers for that purpose.

(3) For the purposes of paragraph (2)(e), "laboratory" includes a person who undertakes the analysis of samples for this Part, whether at the time and place where the samples are taken or otherwise.

(4) A water authority shall maintain such records as are sufficient to enable it to establish, in relation to each sample taken for the purposes of Part IV or this Part of these Regulations, that such of the appropriate requirements as are applicable to that sample have been satisfied.

(5) Subject to paragraph (7), for the purpose of establishing, within acceptable limits of deviation and detection, whether the sample contains concentrations or values which contravene the prescribed concentrations or values, or exceed the specifications for indicator parameters–

(a) the method of analysis specified in column (2) of Table A1 in Schedule 4 shall be used for determining compliance with the parameter specified in relation to that method in column (1);

(b) the method of analysis used for determining compliance with a parameter specified in column (1) of Table A2 in Schedule 4 must be capable, at the time of use–

(i) of measuring concentrations and values equal to the parametric value with the trueness and precision specified in relation to that parameter in columns (2) and (3) of that Table; and

(ii) of detecting the parameter at the limit of detection specified in relation to that parameter in column (4) of that Table;

(c) the method of analysis used for determining compliance with the hydrogen ion parameter must be capable, at the time of use, of measuring concentrations equal to the parametric value with a trueness of 0.2 pH unit and a precision of 0.2 pH unit; and

(d) the method of analysis used for the odour and taste parameters must be capable, at the time of use, of measuring values equal to the parametric value with a precision of 1 dilution number at 25°C.

(6) For the purposes of paragraph (5)–

"limit of detection" is to be calculated as:

(a) three times the relative within batch standard deviation of a natural sample containing a low concentration of the parameter; or

(b) five times the relative within batch standard deviation of a blank sample;

"precision" (the random error) is to be calculated as twice the standard deviation (within a batch and between batches) of the spread of result about the mean; and

"trueness" (the systematic error) is to be calculated as the difference between the mean value of the large number of repeated measurements and the true value.

(7) Subject to paragraph (9), the Scottish Ministers may, on the application of any person, authorise a method of analysis other than that specified in paragraph (5)(a) ("the prescribed method").

(8) An application for the purpose of paragraph (7) shall be made in writing (or in electronic form acceptable to the Scottish Ministers) and shall be accompanied by–

(a) a description of the method of analysis; and

(b) the results of the tests carried out to demonstrate the reliability of that method and its equivalence to the prescribed method.

(9) The Scottish Ministers shall not authorise the use of the method proposed in the application unless they are satisfied that the results obtained by the use of that method are at least as reliable as those produced by the use of the prescribed method.

(10) An authorisation under paragraph (7) may be subject to such conditions as the Scottish Ministers think fit.

(11) The Scottish Ministers may at any time by notice in writing (or in electronic form) served on the water authority to whom an authorisation under paragraph (7) has been given revoke the authorisation, but no such notice shall be served later than 3 months before the date on which the revocation is stated to take effect.

PART VI

INVESTIGATIONS, AUTHORISATION OF DEPARTURES AND REMEDIAL ACTION

Investigations: Schedule 1 parameters

17.—(1) Subject to paragraph (3), where a water authority has reason to believe that water supplied by it for regulation 4(1) purposes–

(a) fails, or is likely to fail, to satisfy a requirement of paragraph (2) of regulation 4; or

(b) is to be regarded as unwholesome by virtue of paragraph (4) of that regulation; or

(c) if paragraph (6) of that regulation were ignored, would be regarded as unwholesome by virtue of paragraph (5) of that regulation,

the water authority shall immediately take such steps as are necessary to identify the matters specified in paragraph (2) below.

(2) The matters referred to in paragraph (1) are–

(a) the cause and extent of the failure, or as the case may be, the apprehended failure;

(b) the Schedule 1 parameters in respect of which the prescribed concentration or value has not been, or is unlikely to be, achieved; and

(c) in relation to each parameter so identified, whether the failure, or apprehended failure, to achieve the prescribed concentration or value is attributable–

(i) to the domestic distribution system;

(ii) to the maintenance of that system; or

(iii) to neither of those matters.

(3) Where a departure has been authorised under Part VI–

(a) paragraph (1) shall apply only in respect of the Schedule 1 parameters (if any) that are not specified in the authorisation; and

(b) a water authority which has reason to believe that water supplied by it for regulation 4(1) purposes fails, or is likely to fail, to satisfy the concentration or value required by the authorisation in relation to any Schedule 1 parameter, shall immediately take such steps as are necessary to identify the matters specified in paragraph (4).

(4) The matters referred to in paragraph (3) are–

(a) the cause and extent of the failure or, as the case may be, the apprehended failure;

(b) the Schedule 1 parameters in respect of which the required concentration or value has not been, or is unlikely to be, achieved; and

(c) in relation to each parameter so identified, whether the failure, or apprehended failure, to achieve that concentration or value is attributable–

(i) to the domestic distribution system;

(ii) to the maintenance of that system; or

(iii) to neither of those matters.

(5) As soon as may be after the matters specified in paragraph (2) or (4), as the case may be, have been identified, the water authority shall notify the Scottish Ministers–

(a) of those matters;

(b) in relation to each parameter identified in accordance with paragraph (2)(b) or (4)(b), whether it is the opinion of the water authority that a failure in respect of that parameter is likely to recur; and

(c) of the action (if any) taken by the water authority in relation to a failure which is attributable to the domestic distribution system or the maintenance of that system.

(6) Where the water authority has identified a failure attributable to the domestic distribution system or to the maintenance of that system, it shall, at the same time as notice is given under paragraph (5)–

(a) by notice in writing (or in electronic form) to those of its consumers–

(i) to whom it supplies water for regulation 4(1) purposes; and

(ii) who are likely to be affected by the failure,

inform them of the nature of the failure and provide details of the steps (if any) that, in the opinion of the water authority, it is necessary or desirable for those consumers to take in the interests of their health; and

(b) send a copy of that notice to the Scottish Ministers and to each appropriate local authority.

(7) A water authority which has complied with the requirements of paragraph (5) and (6) need not, in respect of the same failure or apprehended failure, comply with the requirements of regulation 31(9).

(8) Where such a failure as is mentioned in paragraph (6) affects the supply of water to the public in premises in which water is so supplied, the water authority shall, as soon as may be, notify such

persons as the Scottish Ministers may from time to time determine for the purposes of this paragraph of the matters of which notice is given to consumers in accordance with paragraph (6)(a).

(9) Where such a failure as is mentioned in paragraph (1) relates to the copper or lead parameter, the water authority shall, as soon as reasonably practicable after the occurrence, modify or replace such of its pipes and their associated fittings as it knows or has reason to believe have the potential for contributing to copper or lead in the water supplied to the premises, so as to eliminate that potential (whether or not the presence of copper or lead in those pipes contributed to the failure).

Investigations: Schedule 2 indicator parameters

18.—(1) Where a water authority has reason to believe that water supplied by it for regulation 4(1) purposes does not meet the specifications for indicator parameters set out in Schedule 2, it shall take such steps as are necessary to identify–

(a) the reason why the specifications are not met;

(b) the indicator parameters in respect of which the specifications are not met; and

(c) if the specification for the coliform bacteria or colony count parameter (items 4 and 5 in Schedule 2) is not met, whether the inability to meet that specification is attributable–

(i) to the domestic distribution system;

(ii) to the maintenance of that system; or

(iii) to neither of those matters.

(2) As soon as may be after the matters specified in paragraph (1) have been identified, the water authority shall notify the Scottish Ministers–

(a) of those matters; and

(b) in relation to each parameter identified in accordance with paragraph (1)(b), whether it is the opinion of the water authority that a recurrence of the inability to meet the specification in respect of that parameter is likely.

(3) Where, in a case to which paragraph (1)(c) applies, the inability to meet the specification has been identified as attributable to the domestic distribution system or to the maintenance of that system, the water authority shall, at the same time as notice is given under paragraph (2)–

(a) notify in writing (or in electronic form) those of its consumers–

(i) to whom it supplies water for regulation 4(1) purposes; and

(ii) who are likely to be affected,

of the nature of the problem and provide details of the steps (if any) that, in the opinion of the water authority, it is necessary or desirable for those consumers to take in the interests of their health; and

(b) send a copy of the notice to the Scottish Ministers and to each appropriate local authority.

(4) Where such an inability as is mentioned in paragraph (3) is, in the opinion of the water authority, likely to affect the supply of water to the public in premises in which water is so supplied, it shall, at the same time as notice is given under paragraph (2), notify such persons as the Scottish Ministers may from time to time determine for the purposes of this paragraph of the matters of which notice is given to consumers in accordance with paragraph (3)(a).

Action by the Scottish Ministers

19.—(1) Where–

(a) a notification given in accordance with paragraph (5) of regulation 17 in the circumstances mentioned in paragraph (1) of that regulation (including that paragraph as read with paragraph (3)(a) of that regulation)–

(i) discloses a failure in respect of a parameter specified in Part II of Table A or in Table B in Schedule 1; and

(ii) that the failure is not attributable to the domestic distribution system or the maintenance of that system; and

(b) it appears to the Scottish Ministers that the failure in respect of that parameter is not trivial and is likely to recur,

the Scottish Ministers may, by notice in writing (or in electronic form) to the water authority, require that authority to seek a departure in accordance with regulation 20.

(2) The exercise by the Scottish Ministers of the power conferred by paragraph (1) shall not preclude the exercise by them, in relation to the same circumstances, of the power conferred by section 76E of the Act.

(3) Where–

(a) a notification given in accordance with paragraph (5) of regulation 17 in the circumstances mentioned in paragraph (3)(b) of that regulation discloses–

(i) a failure in relation to any parameter specified in Part II of Table A or in Table B in Schedule 1; and

(ii) that the failure is not attributable to the domestic distribution system or the maintenance of that system; and

(b) it appears to the Scottish Ministers that the failure in respect of that parameter is not trivial and is likely to recur,

the Scottish Ministers shall consider whether the terms of the authorisation under regulation 20 should be modified.

(4) Where–

(a) a notification given in accordance with regulation 18(2) discloses an inability to meet the specification applicable to an indicator parameter; and

(b) the Scottish Ministers consider that the inability is likely to pose a risk to human health,

they may, by notice in writing (or in electronic form) to the water authority, require the water authority to take such steps as may be determined by them and specified in the notice.

(5) It shall be the duty of a water authority to which a notice under paragraph (4) has been given to take the steps specified in the notice.

Authorisation of temporary supply of water that is not wholesome

20.—(1) Subject to paragraph (2), the Scottish Ministers may, upon the written application of a water authority (or in electronic form acceptable to them), authorise in accordance with regulation 21 a departure from the provisions of Part III of these Regulations in so far as they relate to–

(a) a parameter specified in Part II of Table A in Schedule 1 or in Table B in Schedule 1; and

(b) the supply of water by that authority in any of its water supply zones.

(2) The Scottish Ministers shall not authorise a departure under paragraph (1) unless they are satisfied–

(a) that the authorisation is necessary to maintain in that zone a supply of water for regulation 4(1) purposes;

(b) that a supply of water for those purposes cannot be maintained in that zone by any other reasonable means; and

(c) that the supply of water in accordance with the authorisation does not constitute a potential danger to human health.

(3) A water authority shall provide with its application–

(a) a statement–

(i) of the grounds on which the authorisation is sought;

(ii) of the water supply zone in respect of which the authorisation is sought;

(iii) of the parameters in respect of which the prescribed concentration or value cannot be met;

(iv) in respect of each parameter to which paragraph (iii) applies, of the results of the analysis of the samples taken in the water supply zone in question during the 12 months immediately preceding the first day on which the prescribed concentration or value could not be met;

(v) in respect of each parameter to which paragraph (iii) applies, of the results of the analysis of the samples (if any) taken in the water supply zone in question between the first day on which the prescribed concentration or value could not be met and the date of the application;

(vi) of the average daily quantity of water supplied to that zone or, if that quantity cannot readily be ascertained, of the average daily quantity of water supplied from the treatment works that supplies water to that zone;

(vii) of the estimated population of that zone;

(viii) as to whether, if a departure were authorised in the terms sought, any relevant food-production undertaking would be affected;

(ix) of the period for which the authorisation is sought; and

(x) of the reasons why the supply cannot be maintained by other reasonable means;

(b) a scheme for monitoring the quality of water supplied in the zone during the period for which the authorisation is sought; and

(c) a summary of the steps that it proposes to take in order to secure that the supply fully satisfies the requirements of Part III, including–

(i) a timetable for the work;

(ii) an estimate of the cost of the work; and

(iii) provisions for reviewing the progress of the work and for reporting the result of the review to the Scottish Ministers.

(4) At the same time as it makes an application for an authorisation under paragraph (1), the water authority shall serve on–

(a) every appropriate local authority;

(b) every appropriate health board; and

(c) the Water Industry Commissioner for Scotland(**8**),

a copy of the application and of the statement, scheme and summary referred to in paragraph (3).

(5) A body on whom documents have been served in accordance with paragraph (4) may make representations to the Scottish Ministers in connection with the application; and any such

(**8**) The Water Industry Commissioner was established by virtue of section 67A of the Local Government etc. (Scotland) Act 1994 c. 39.

representations shall be made not later than the end of the period of 30 days beginning with the date on which the application for the authorisation is made.

Authorisations: terms and conditions

21.—(1) Subject to paragraph (2), a departure may be authorised under regulation 20 for such period as is in the Scottish Ministers' opinion reasonably required for securing a supply of water for regulation 4(1) purposes that fully satisfies the requirements of Part III ("the departure period").

(2) No departure period shall exceed three years.

(3) Subject to paragraph (4), an authorisation under regulation 20–

(a) shall specify–

(i) the grounds on which it is granted;

(ii) every water supply zone in respect of which it is granted;

(iii) the extent to which a departure from the prescribed concentration or value of any parameter is authorised;

(iv) in respect of each parameter to which paragraph (iii) applies, the results of the analysis of the samples taken in the water supply zone in question during the 12 months immediately preceding the first day on which the prescribed concentration or value could not be met;

(v) in respect of each parameter to which paragraph (iii) applies, the results of the analysis of the samples (if any) taken in each water supply zone in question between the first day on which the prescribed concentration or value could not be met and the date of the application;

(vi) the average daily quantity of water supplied from each of those zones or, if that quantity cannot readily be ascertained, the average daily quantity of water supplied from the treatment works that supplies water to that zone;

(vii) the estimated population of each of those zones;

(viii) whether or not any relevant food-production undertaking would be affected; and

(ix) the departure period; and

(b) shall require the implementation of a scheme for monitoring the quality of water supplied in each of those zones during the departure period (which may be, but need not be, the scheme submitted in accordance with regulation 20(3)(b)); and

(c) shall require the carrying out of the steps which, in their opinion, are reasonably required in order to secure that the supply fully satisfies the requirements of Part III (whether or not the steps are those proposed in the summary submitted in accordance with regulation 20(3)(c)); and

(d) shall specify, in relation to those steps–

(i) the timetable for the work;

(ii) an estimate of the cost of the work; and

(iii) provisions for reviewing the progress of the work and for reporting to them the result of the review; and

(e) require the taking of such steps as may be specified to give to the population within the water supply zones to which the authorisation applies and, in particular, to those groups of that population for which the supply of water in accordance with the authorisation could present a special risk, advice as to the measures (if any) that it would be advisable in the interests of their health for persons within that population or those groups to take for the whole or any part of the departure period.

(4) Where the Scottish Ministers are of the opinion–

(a) that the extent of the contravention of the requirements of Part III as respects any parameter is trivial; and

(b) that the prescribed concentration or value as respects that parameter is likely to be achieved within the period of 30 days beginning with the day on which the prescribed concentration or value in respect of that parameter was contravened,

the particulars to be specified in the authorisation shall be those required by paragraph (3)(a) (iii) and (ix), and sub-paragraphs (b) to (e) of that paragraph shall not apply.

(5) Where it appears to the Scottish Ministers that a supply of water that fully satisfies the requirements of Part III cannot be restored by the end of the departure period, they may authorise a further departure.

(6) Paragraphs (1) to (4) shall apply to a further departure as they apply to a departure authorised under regulation 20.

(7) Where it appears to the Scottish Ministers that a supply of water that fully satisfies the requirements of Part III cannot be restored by the end of the departure period relevant to an authorisation under paragraph (5), they may, in accordance with Article 9(2) of Council Directive 98/83/EC(**9**), authorise a third departure.

(8) Paragraph (3) shall apply to any departure authorised under paragraph (7) as it applies to a departure authorised under regulation 20, but with the substitution for the words "Subject to paragraph (4)" of the words "Subject to any decision of the Commission".

Authorisations: other limitations

22. An authorisation under regulation 20 or regulation 21 may be limited to water supplied–

(a) from particular sources or classes of source;

(b) to particular water supply zones or to zones of particular descriptions.

Publicity for authorisations

23. As soon as reasonably practicable after a departure has been authorised, the water authority shall–

(a) inform the population affected of the departure and of the conditions governing it–

(i) except in a case to which paragraph (4) of regulation 21 applies, a notice containing a statement of the matters specified in paragraph (3)(a)(ii), (iii), (viii) and (ix) of that regulation;

(ii) in a case to which paragraph (4) of regulation 21 applies, and if Scottish Ministers so require, a notice containing a statement of the matters specified in paragraph (3)(a)(ii), (iii) and (ix) of that regulation;

(b) give such other public notice of the authorisation and of its terms and conditions as the Scottish Ministers may, by notice in writing (or in electronic form) to the water authority, reasonably require.

Revocation and modification of authorisations

24.—(1) Subject to paragraphs (2) and (3), the Scottish Ministers may at any time modify or revoke an authorisation under regulation 20; and regulations 21 and 22 shall apply as respects a modification as they apply as respects an authorisation.

(**9**) O.J. No. L 330, 5.12.98.

(2) The Scottish Ministers shall not revoke or modify an authorisation under regulation 20 without giving at least six months' notice in writing (or in electronic form) of their intention to do so to–

(a) the water authority to which the authorisation relates;

(b) every appropriate local authority;

(c) every appropriate health board; and

(d) the Water Industry Commissioner for Scotland,

but they may revoke or modify an authorisation without notice if it appears to them that immediate revocation or modification is required in the interests of public health.

(3) A water authority on whose application a departure has been authorised under this Part shall notify the Scottish Ministers as soon as the circumstances which gave rise to the application cease to exist; and the Scottish Ministers shall thereupon revoke the authorisation without the need for prior notice.

PART VII

WATER TREATMENT

Treatment of raw water

25.—(1) In carrying out such of its functions under Part VI A of the Act as comprise the supply of water for regulation 4(1) purposes, a water authority shall not, subject to paragraph (2), supply water from any source which consists of or includes raw water unless the water has been disinfected and, in the case of surface water, subjected to at least such further treatment as is specified in paragraph (3).

(2) This paragraph shall not require a water authority to disinfect such ground waters as are specified in an authorisation given by the Scottish Ministers for the purposes of this paragraph.

(3) The further treatment mentioned in paragraph (1) is such treatment as is required to secure compliance with Council Directive 75/440/EC (quality required of surface water intended for the abstraction of drinking water)(**10**); and for the purposes of this regulation, surface water shall be treated as falling within category A1 or A2 or A3 set out in Annex I to that Directive if it is abstracted from waters for which the classification to be currently satisfied in accordance with section 30B of the Control of Pollution Act 1974(**11**) is, as appropriate, DW1 or DW2 or DW3, as set out in the Surface Waters (Abstraction for Drinking Water) (Classification) (Scotland) Regulations 1996(**12**).

(4) Except with the consent of the Scottish Ministers, water shall not be abstracted for supply for domestic purposes which include drinking except for waters to be treated as falling within category A1 or A2 or A3 in the Annex to Directive 75/440/EC.

Contamination from pipes

26.—(1) Where there is a risk ("the prescribed risk") that water supplied by a water authority would, for the reason mentioned in paragraph (2), after leaving the authority's pipes–

(a) contain a concentration of copper in excess of 2mg/litre; or

(b) contain a concentration of lead in excess of 10µg/litre,

the water authority shall, subject to paragraph (3), treat the water in such a way as will, in its opinion, eliminate the prescribed risk or reduce it to a minimum.

(**10**) O.J. No. L 194, 25.7.77, p.26.
(**11**) 1974 c. 40; section 30B was inserted by the Water Act 1989, Scehdule 23, paragraph 4.
(**12**) S.I. 1996/3047.

(2) The reason referred to in paragraph (1) is the presence in the water of a concentration of copper or lead which is attributable to the fact that copper or lead is the major component of such a pipe as is mentioned in section 76A(3)(a) of the Act, or its associated fittings.

(3) Paragraph (1) shall not require an authority to treat water–

(a) if the treatment is unlikely to achieve a significant reduction in the concentration of copper or lead; or

(b) in any water supply zone where the prescribed risk relates only to water supplied to an insignificant part of the zone; or

(c) if treatment is not reasonably practicable.

(4) Where at any time in the period beginning with 25th December 2003 and ending immediately before 25th December 2013, a water authority–

(a) has reason to believe that water supplied by it for regulation 4(1) purposes from a pipe to which paragraph (5) applies contains, at the consumer's tap, a concentration of lead which exceeds 10•g/1 but does not exceed 25•g/1; and

(b) has received from the owner of premises to which water is so supplied notice in writing (or in electronic form)–

(i) of the owner's intention to replace so much of the pipe as belongs to him; and

(ii) of his desire that the water authority replaces the remainder of the pipe,

the water authority shall modify or replace its part of the pipe.

(5) This paragraph applies to a pipe–

(a) of which the major component is lead;

(b) which is subject to water pressure from a water main or would be so subject but for the closing of some valve; and

(c) which belongs, as to part, to a water authority and, as to the remainder, to the owner of any premises to which the authority supplies water for regulation 4(1) purposes.

Application and introduction of substances and products

27.—(1) In this regulation–

"the Directive" means Council Directive 89/106/EEC on the approximation of laws, regulations and administrative provisions of the Member States relating to construction products(**13**);

"EEA State" means a State which is a contracting party to the agreement on the European Economic Area signed at Oporto on 2nd May 1992(**14**) as adjusted by the Protocol signed at Brussels on 17th March 1993(**15**);

"European technical approval" means a favourable technical assessment of the fitness for use of a construction product for an intended use, issued for the purposes of the Directive by a body authorised by an EEA State to issue European technical approvals for those purposes and notified by that body to the European Commission; and

"harmonized standard" means a standard established as mentioned in the Directive by the European standards organisation on the basis of a mandate given by the European Commission and published by the Commission in the Official Journal of the European Communities.

(**13**) O.J. No. L 40, 11.2.89, p.12.
(**14**) Cmnd 2073.
(**15**) Cmnd 2183.

(2) Subject to paragraph (3), a water authority shall not apply any substance or product to, or introduce any substance or product into, water which is to be supplied for regulation 4(1) purposes unless one of the requirements of paragraph (4) is satisfied.

(3) A substance or product which, at the time of its application or introduction, bears an appropriate CE marking in accordance with the Directive, or conforms to–

(a) an appropriate harmonised standard or European technical approval; or

(b) an appropriate British Standard or some other national standard of an EEA State which provides an equivalent level of protection and performance,

may be applied or introduced, notwithstanding that none of the requirements of paragraph (4) is satisfied; but any such application or introduction shall be subject to–

(i) such national conditions of use restricting the dosing concentration as are for the time being in force in relation to such substances and products pursuant to a determination of the Scottish Ministers by an instrument in writing; and

(ii) such other requirements, within the meaning of Council Directive 98/34/EC, as amended(**16**) (which lays down a procedure for the provision of information in the field of technical standards and regulations and of rules on information society services), in relation to such substances and products, as have been communicated to the Commission in the form of a draft technical regulation in accordance with Article 8 of that Directive, and whose adoption by a Member State has also been communciated to the Commission.

(4) The requirements of this paragraph are–

(a) that the Scottish Ministers have for the time being approved the application or introduction of that substance or product and it is applied or introduced in accordance with any conditions attaching to that approval;

(b) that the Scottish Ministers are satisfied that the substance or product either alone or in combination with any other substance or product in the water is unlikely to affect adversely the quality of the water supplied;

(c) that the substance or product is to be applied or introduced solely for the purposes of testing or research, and the water authority has given to the Scottish Ministers not less than 3 months' notice of their intention so to apply or introduce the substance or product.

(5) An application for such an approval as is mentioned in paragraph (4)(a) may be made by any person.

(6) If the Scottish Ministers decide to issue an approval under paragraph (4)(a), they may include in the approval such conditions as they consider appropriate and, subject to paragraph (10), may at any time revoke or vary any approval they have previously given.

(7) Where substances or products are applied or introduced in any case in which the requirement mentioned in paragraph (4)(c) is satisfied, their application or introduction shall be discontinued within 12 months of the date on which they were first applied or introduced or, if the Scottish Ministers by notice given in writing (or in electronic form) to the water authority so directs, within such other period (whether longer or shorter) as may be specified in the notice.

(8) The Scottish Ministers may, by notice given in writing (or in electronic form) to any water authority, prohibit it from applying to, or introducing into, water intended to be supplied for regulation 4(1) purposes any substance or product which the authority would otherwise be authorised to apply or introduce by virtue of–

(a) paragraph (2) and any of sub-paragraphs (b) or (c) of paragraph (4); or

(b) paragraph (3).

(**16**) O.J. No. L 204, 21.7.98, p.37 amended by Council Directive 98/48/EC (O.J. No. L 217, 5.8.98, p.18).

(9) A prohibition under paragraph (8) may be without limitation as to time or for such period as is specified in the notice.

(10) The Scottish Ministers may–

(a) revoke by an instrument in writing (or in electronic form) any approval given by them under paragraph (4)(a);

(b) modify any such approval by an instrument in writing (or in electronic form) by including conditions or varying existing conditions;

(c) issue any such notice as is mentioned in paragraph (8):

but, unless they are satisfied that it is necessary to do so in the interests of public health without notice, shall not do any of those things without giving all such persons as are, in their opinion, likely to be affected by the revocation or modification of the approval or by the issue of the notice at least six months' notice in writing (or in electronic form) of their intention.

(11) Notice shall be given forthwith by the Scottish Ministers to all persons likely to be affected by the making of such an instrument as is mentioned in paragraph (10)(a) or (b).

(12) At least once in each year beginning with the year 2004, the Scottish Ministers shall issue a list of all the substances and products in relation to which–

(a) an approval under paragraph (4)(a) has been granted or refused;

(b) such an approval has been revoked or modified;

(c) a notice has been issued under paragraph (8),

with particulars of the action taken.

Use of processes

28.—(1) The Scottish Ministers may at any time by notice in writing (or in electronic form) given to a water authority require it to make an application to them for approval of the use of any process; and may prohibit it for such period as may be specified in the notice from using any such process in connection with the supply by it of water for regulation 4(1) purposes.

(2) The Scottish Ministers may refuse the application or impose on any approval given for the purposes of this regulation such conditions as they think fit and, subject to paragraph (3), may at any time by notice in writing (or in electronic form) to the water authority revoke an approval so given or modify or revoke any condition imposed by virtue of this paragraph.

(3) Subject to paragraph (4), the Scottish Ministers shall not–

(a) revoke any approval given for the purposes of this regulation;

(b) modify any condition imposed by virtue of paragraph (2); or

(c) prohibit a water authority from using any process,

unless they have given to the water authority at least six months' notice in writing (or in electronic form) of their intention to revoke, modify or prohibit, as the case may be.

(4) Paragraph (3) shall not apply in any case in which the Scottish Ministers are of the opinion that the immediate revocation, modification or prohibition is necessary in the interests of public health.

(5) Paragraph (12) of regulation 27 shall apply for the purposes of this regulation as if–

(a) for the reference to a substance or product there were substituted a reference to a process; and

(b) for the reference to paragraph (4)(a) and paragraph (8) there were substituted a reference to this regulation and paragraph (1) of this regulation respectively.

Offences

29.—(1) A water authority which applies or introduces any substance or product in contravention of regulation 27(2) or a notice given under regulation 27(8), or uses any process in contravention of a prohibition imposed under regulation 28(1) or without complying with a condition imposed by virtue of regulation 28(2) shall be guilty of an offence and liable–

(a) on summary conviction, to a fine not exceeding the statutory maximum;

(b) on conviction on indictment, to a fine.

(2) If any person furnishing any information or making any application under regulation 27 or 28, makes any statement which that person knows to be false in a material particular, or recklessly makes any statement which is false in a material particular, that person shall be guilty of an offence and liable–

(a) on summary conviction, to a fine not exceeding the statutory maximum;

(b) on conviction on indictment, to a fine.

PART VIII

RECORDS AND INFORMATION

Maintenance of records

30.—(1) A water authority shall prepare and maintain, in respect of each of its water supply zones, a record containing–

(a) the name of the zone;

(b) the name of every water treatment works, service reservoir and other supply point from which water is supplied to premises within the zone;

(c) an estimate of the population of the zone;

(d) particulars of any departure authorised under Part VI of these Regulations which applies to water supplied in the zone;

(e) particulars of the action taken or required to be taken by the authority to comply with–

(i) any default order made under section 76E of the Act;

(ii) any departure authorised under Part VI; and

(iii) any notice under regulation 19(4).

(f) particulars of the result of any analysis of samples taken in accordance with Part IV of these Regulations or any of regulations 12 to 14; and

(g) such other particulars as the authority may determine.

(2) An authority shall make–

(a) initial entries in the record in respect of the matters mentioned in paragraph (1) (a) to (d) and (e)(ii) before 1st March 2004;

(b) entries in respect of the matters mentioned in paragraph (1)(e)(i) and (iii) within 28 days of the date of the order and notice respectively; and

(c) entries relating to the results of the analysis of samples within 28 days of the day on which the result is first known to the authority.

(3) Without prejudice to paragraph (2), the authority shall at least once in each year review and bring up to date the record required to be kept by paragraph (1).

(4) Nothing in this regulation shall require an authority to retain a record of any information mentioned in paragraph (1) at any time more than fifteen years after the date on which the information was first entered in the record.

Provision of information

31.—(1) A water authority shall make available for inspection by the public, in such places as it reasonably considers appropriate to ensure adequate accessibility to the public, at all reasonable hours and free of charge any record maintained by it in accordance with regulation 30.

(2) A water authority shall afford to any person facilities to take or obtain a copy of any part of a record maintained in accordance with regulation 30–

(a) in the case of information relating to that zone, free of charge if the person receives a supply of water in the zone;

(b) in any other case, on payment of such reasonable charge as the authority may determine.

(3) A water authority shall include in or append to at least one of the accounts sent to each customer in any year a statement informing them–

(a) that records of water quality may be inspected by the public free of charge; and

(b) of the address, telephone number and hours of opening of the office at which an inspection can be made.

(4) A water authority shall, not later than 31st March 2005 and not later than 31st March in each year thereafter, supply to each local authority to any part of whose area the water authority supplied water in the preceding year, information concerning the general quality of water supplied during that year to premises in the area of such local authority, and

(a) in respect of each treatment works from which water was so supplied, the particulars referred to in paragraph (5);

(b) in respect of each service reservoir, and every other supply point (other than a treatment works), from which water was so supplied, the particulars referred to in paragraph (6);

(c) in respect of–

(i) each water supply zone of which any part is within the area of such local authority; and

(ii) each parameter and residual disinfectant,

the particulars referred to in paragraph (7); and

(d) information as to the action taken by the authority in that year to comply with–

(i) any default order made under section 76E to the Act;

(ii) any departure authorised under Part VI; and

(iii) any notice under regulation 19(4).

(5) The particulars referred to in this paragraph are–

(a) the names of the water supply zones supplied from the works during the preceding year;

(b) the results of any analysis of samples taken in the preceding year in accordance with Part IV of these Regulations, or any of regulations 12 or 13;

(c) in relation to those samples and each parameter other than an indicator parameter–

(i) the number and percentage of samples which contravened the prescribed concentration or value; and

(ii) if at the time that samples were taken a departure had been authorised in respect of that parameter, the number and percentage of samples which exceeded the concentration or value specified in the authorisation;

(d) in respect of each indicator parameter, the number and percentage of the samples taken in the preceding year which did not meet the specification for that parameter;

(e) in respect of each parameter other than an indicator parameter, the minimum, mean and maximum concentrations; and

(f) in respect of residual disinfectant and each indicator parameter, the minimum, mean and maximum concentrations or values or, where a state is specified in relation to any such parameter, a commentary on its state.

(6) The particulars referred to in this paragraph are–

(a) the names of the water supply zones supplied from the service reservoir or, as the case may be, the supply point, during the preceding year;

(b) the results of any analysis of samples taken in the preceding year in accordance with Part IV of these Regulations, regulation 12 or regulation 14;

(c) in relation to those samples and each parameter other than an indicator parameter–

(i) the number and percentage of samples which contravened the prescribed concentration or value; and

(ii) if at the time that samples were taken a departure had been authorised in respect of that parameter, the number and percentage of samples which exceeded the concentration or value specified in the authorisation;

(d) in respect of each indicator parameter, the number and percentage of the samples taken in the preceding year which did not meet the specification for that parameter;

(e) in respect of each parameter other than an indicator parameter, the minimum, mean and maximum concentrations; and

(f) in respect of residual disinfectant and each indicator parameter, the minimum, mean and maximum concentrations or, values or, where a state is specified in relation to any such parameter, a commentary on the state.

(7) The particulars referred to in this paragraph are–

(a) the number of samples taken in the preceding year;

(b) the result of any analysis of samples taken in the preceding year in accordance with Part IV of these Regulations;

(c) in relation to those samples and each parameter other than an indicator parameter–

(i) the number and percentage of samples which contravened the prescribed concentration or value; and

(ii) if at the time that samples were taken a departure had been authorised in respect of that parameter, the number and percentage of samples which exceeded the concentration or value specified in the authorisation;

(d) in respect of each indicator parameter, the number and percentage of the samples taken in the preceding year which did not meet the specification for that parameter;

(e) in respect of each parameter other than an indicator parameter, the minimum, mean and maximum concentrations; and

(f) in respect of residual disinfectant and each indicator parameter, the minimum, mean and maximum concentrations or values or, where a state is specified in relation to any such parameter, a commentary on the state.

(8) As soon as may be after the occurrence of any event which, by reason of its effect or likely effect on the water supplied by a water authority, gives rise or is likely to give rise to a significant risk to health of persons residing in the area of a local authority or health board, the water authority shall notify each such local authority, each such health board and the Water Industry Commissioner for Scotland of the occurrence of the event.

(9) A water authority shall send to the Scottish Ministers a copy of every notification given under paragraph (8).

(10) A local authority or health board who has received a notification under paragraph (8) may require the water authority to provide them with such information relating to the event and its consequences as they may reasonably require.

Publication of information

32.—(1) A water authority shall, not later than 31st March 2005 and not later than 31st March in each year thereafter, publish a report relating to the preceding year containing–

(a) a statement of the number of treatment works, service reservoirs and other supply points from which it supplied water during any part of the year;

(b) a statement of the number of its water supply zones for the year;

(c) in respect of water supplied from its treatment works, the particulars referred to in paragraph (4);

(d) in respect of its service reservoirs and other supply points (other than treatment works), the particulars referred to in paragraph (5);

(e) in respect of its water supply zones, the particulars referred to in paragraph (6);

(f) a statement of the action taken by the water authority during the year to comply with–

(i) any default order made under section 76E of the Act;

(ii) any departure authorised under Part VI; and

(iii) any notice under regulation 19(4);

(g) a statement that any person may, free of charge, inspect the records of water quality kept by the water authority in accordance with regulation 31; and

(h) particulars of the times and places at which such inspection may be made.

(2) A report under paragraph (1) may include such other information as the water authority thinks fit.

(3) At the same time as it publishes a report in accordance with paragraph (1) the water authority shall send a copy of it to every local authority within whose area the water authority supplied water in the preceding year.

(4) The particulars referred to in this paragraph are–

(a) the total number of samples taken from all of the water authority's treatment works in the preceding year in respect of residual disinfectant and each parameter;

(b) in relation to those samples and each parameter other than an indicator parameter–

(i) the number and percentage of samples which contravened the prescribed concentration or value; and

(ii) if at the time that samples were taken a departure had been authorised in respect of that parameter, the number and percentage of samples which exceeded the concentration or value specified in the authorisation;

(c) the number and percentage of treatment works at which samples of the descriptions mentioned in paragraphs (i) and (ii) of sub-paragraph (b) were produced;

(d) in relation to the samples mentioned in sub-paragraph (a) and each indicator parameter, the number and percentage of the samples which did not meet the specification for that parameter; and

(e) the number and percentage of treatment works at which samples of the description mentioned in sub-paragraph (d) were produced.

(5) The particulars referred to in this paragraph are–

(a) the total number of samples taken from all of the water authority's service reservoirs and other supply points (other than treatment works), in the preceding year in respect of residual disinfectant and each parameter;

(b) in relation to those samples–

(i) the number and percentage which contravened the prescribed concentration or value; and

(ii) if at the time that they were taken a departure had been authorised, the number and percentage which exceeded the concentration or value specified in the authorisation;

(c) the number and percentage of service reservoirs, and other supply points (other than treatment works), at which samples of the descriptions mentioned in paragraphs (i) and (ii) of sub-paragraph (b) were produced;

(d) in relation to the samples mentioned in sub-paragraph (a) and each indicator parameter, the number and percentage of the samples which did not meet the specification for that parameter; and

(e) the number and percentage of service reservoirs, and other supply points (other than treatment works), at which samples of the description mentioned in sub-paragraph (d) were produced.

(6) The particulars referred to in this paragraph are–

(a) the total number of samples taken from all of the water authority's water supply zones in the preceding year in respect of residual disinfectant and each parameter;

(b) in relation to those samples and each parameter other than an indicator parameter–

(i) the number and percentage of samples which contravened the prescribed concentration or value; and

(ii) if at the time that samples were taken a departure had been authorised in respect of that parameter, the number and percentage of samples which exceeded the concentration or value specified in the authorisation;

(c) the number and percentage of water supply zones at which samples of the descriptions mentioned in paragraphs (i) and (ii) of sub-paragraph (b) were produced;

(d) in relation to the samples mentioned in sub-paragraph (a) and each indicator parameter, the number and percentage of the samples which did not meet the specification for that parameter; and

(e) the number and percentage of water supply zones at which samples of the description mentioned in sub-paragraph (d) were produced.

PART IX

FUNCTIONS OF LOCAL AUTHORITIES IN RELATION TO WATER QUALITY

Application and interpretation

33.—(1) This Part applies to the performance by a local authority of their duty under section 76F(1) of the Act insofar as that duty relates to water supplies which are not private supplies.

(2) In this Part "relevant water authority", in relation to a local authority, means a water authority any of whose supply zones include an area which is situated within the area of that local authority.

Duties of local authorities: supplementary provision

34.—(1) In performing their duty under section 76F(1) of the Act (general functions of local authorities in relation to water quality), a local authority–

(a) shall make such arrangements with the relevant water authority as will secure that the local authority is notified as mentioned in regulation 31(8); and

(b) may take, or cause to be taken, and analyse, or cause to be analysed, by a person designated by them in writing (or in electronic form), such samples of the water supplied to premises in their area as they may reasonably require.

(2) Regulation 16 shall apply to samples taken by virtue of paragraph (1) as it applies to samples taken for the purposes of Parts IV and V of these Regulations; but with the following modifications–

(a) in paragraph (1), for the words "A water authority" there shall be substituted the words "A local authority"; and

(b) in paragraph (2)(e), after the words "the water authority" there shall be inserted the words "or the local authority".

PART X

ENFORCEMENT

Contraventions by water authorities

35. Any duty or requirement imposed by Parts IV to VIII of these Regulations on a water authority shall be enforceable under section 76E of the Act by the Scottish Ministers, whether or not a failure to carry out the duty or requirement constitutes an offence.

PART XI

AMENDMENT AND REVOCATION OF REGULATIONS AND SAVING AND TRANSITIONAL PROVISION

Amendment of the Water Supply (Water Quality) (Scotland) Regulations 1990

36. The 1990 Regulations shall be amended, with effect from 31st January 2001, in accordance with Schedule 5.

Transitional provision: programmes of work

37.—(1) Every water authority which intends to supply water, on and after 25th December 2003, for regulation 4(1) purposes shall, not later than 25th September 2001 submit to the Scottish Ministers for their approval a programme of work designed to secure, so far as reasonably practicable–

(a) that on the coming into force of Part III of these Regulations, the supply of water for those purposes fully satisfies the requirements of that Part; and

(b) that on and after 25th December 2013, the supply of water for those purposes will fully satisfy the revised requirements of that Part in relation to the lead parameter (item 15 in Table B in Schedule 1),

and that, accordingly, section 76A (duties of water authorities with respect to water quality) of the Act is complied with.

(2) Programmes of work shall be drawn up in accordance with the provisions of Schedule 6.

(3) The Scottish Ministers may approve a programme of work with or without modification.

(4) If a water authority–

(a) fails to draw up a programme of work;

(b) draws up such a programme otherwise than in accordance with Schedule 6; or

(c) draws up such a programme that is unsatisfactory in any other respect,

the Scottish Ministers may draw up a programme of work and any such programme shall be treated for the purposes of paragraphs (5) and (6) as a programme of work drawn up by the water authority and approved by the Scottish Ministers.

(5) Whenever it comes to the attention of a water authority that the steps to be taken in accordance with a programme of work submitted to, or approved by, the Scottish Ministers, or treated as so approved, are insufficient to secure that the requirements of paragraph (1)(a) and (b) are met, it shall notify the Scottish Ministers of the additional steps to be taken to secure that those requirements are met.

(6) The Scottish Ministers may at any time, and whether or not on the application of a water authority, modify any programme of work where they are of the opinion that such modification is required to secure that the requirements of paragraph (1)(a) and (b) are met.

(7) It shall be the duty of each water authority–

(a) to take the steps for the time being specified in the programme of work approved or treated as approved in relation to its area of supply;

(b) to take those steps in accordance with the timetable so specified; and

(c) to submit reports to the Scottish Ministers at the times or within the periods specified.

(8) The duties imposed by paragraph (7) shall be enforceable under section 76E of the Act by the Scottish Ministers.

Transitional provision: authorisations

38.—(1) A water authority which–

(a) intends to supply water, on and after 25th December 2003, for regulation 4(1) purposes; and

(b) has reason to believe that water so supplied will not satisfy the requirements of Part III of these Regulations,

may apply to the Scottish Ministers for an authorisation under this regulation.

(2) An application under paragraph (1) shall be made not later than 25th September 2003.

(3) For the purpose of making and determining applications under paragraph (1) and publicising authorisations under this regulation, it shall be assumed–

(a) that regulations 4 and 20 to 23 of these Regulations, and so much of any other provision of these Regulations as relates to those regulations (in so far as not already in force), were in force at the material time; and

(b) that references in any provision specified or referred to in sub-paragraph (a) to an authorisation under regulation 20 (in whatever terms) were references to an authorisation under this regulation.

(4) On and after 25th December 2003, an authorisation under this regulation shall have effect for the purposes of these Regulations as an authorisation under regulation 20.

Revocation of Regulations and savings

39.—(1) On 25th December 2003–

(a) the 1990 Regulations;

(b) the Water Supply (Water Quality) (Scotland) Amendment Regulations 1991(**17**);

(c) regulation 23(b) of the Private Water Supplies (Scotland) Regulations 1992(**18**);

(d) regulation 16(1) of the Surface Waters (Abstraction for Drinking Water) (Classification) (Scotland) Regulations 1996(**19**); and

(e) regulations 36 to 38 of, and Schedules 5 and 6 to, these Regulations,

shall be revoked.

(2) Nothing in paragraph (1) shall affect any obligation of a water authority under the 1990 Regulations to compile and retain records, make information available and produce reports in respect of years ending on or before 31st December 2003.

St Andrew's House,
Edinburgh
4th June 2001

RHONA BRANKIN
Authorised to sign by the Scottish Ministers

(**17**) S.I. 1991/1333.
(**18**) S.I. 1992/575, amended by S.I. 1998/1856.
(**19**) S.I. 1996/3047.

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SCHEDULE 1 Regulations 2 and 4

PRESCRIBED CONCENTRATIONS AND VALUES

TABLE A

Microbiological parameters

Part I: Directive requirements

Item	*Parameters*	*Concentration or Value (maximum)*	*Units of Measurement*	*Point of compliance*
1.	Enterococci	0	number/100ml	consumers' taps
2.	*Escherichia coli (E. coli)*	0	number/100ml	consumers' taps

Part II: National requirements

Item	*Parameters*	*Concentration or Value (maximum)*	*Units of Measurement*	*Point of compliance*
1.	Coliform bacteria	0	number/100ml	service reservoirs* and water treatment works
2.	*Escherichia coli (E coli)*	0	number/100ml	service reservoirs and water treatment works

Note:

* compliance required as to 95% of samples from each service reservoir (regulation 4(6))

TABLE B

Chemical parameters

Part I: Directive requirements

Item	*Parameters*	*Concentration or Value (maximum)*	*Units of Measurement*	*Point of compliance*
1.	Acrylamide	0.10	µg/l	(i)
2.	Antimony	5.0	µgSb/l	consumers' taps
3.	Arsenic	10	µgAs/l	consumers' taps
4.	Benzene	1.0	µg/l	consumers' taps
5.	Benzo(a)pyrene	0.010	µg/l	consumers' taps

Item	*Parameters*	*Concentration or Value (maximum)*	*Units of Measurement*	*Point of compliance*
6.	Boron	1.0	mgB/l	consumers' taps
7.	Bromate	10	µgBrO_3/l	consumers' taps
8.	Cadmium	5.0	µgCd/l	consumers' taps
9.	Chromium	50	µgCr/l	consumers' taps
10.	Copper[(ii)]	2.0	mg Cu/l	consumers' taps
11.	Cyanide	50	µgCN/l	consumers' taps
12.	1,2 dichloroethane	3.0	µg/l	consumers' taps
13.	Epichlorohydrin	0.10	µg/l	[(i)]
14.	Fluoride	1.5	mg F/l	consumers' taps
15.	Lead[(ii)]	(a) 25, from 25/12/03 until 24/12/13	µgPb/l	consumers' taps
		(b) 10, from 25/12/13	µgPb/l	consumers' taps
16.	Mercury	1.0	µgHg/l	consumers' taps
17.	Nickel[(ii)]	20	µgNi/l	consumers' taps
18.	Nitrate[(iii)]	50	mgNO_3/l	consumers' taps
19.	Nitrite[(iii)]	0.50	mgNO_2/l	consumers' taps
		0.10	mgNO_2/l	treatment works
20.	Pesticides[(iv)(v)]–			
	Aldrin	0.030	µg/l	consumers' taps
	Dieldrin	0.030	µg/l	consumers' taps
	Heptachlor	0.030	µg/l	consumers' taps
	Heptachlor epoxide	0.030	µg/l	consumers' taps
	other pesticides	0.10	µg/l	consumers' taps
21.	Pesticides: Total[(vi)]	0.50	µg/l	consumers' taps
22.	PAH[(vii)]	0.10	µg/l	consumers' taps
23.	Selenium	10	µgSe/l	consumers' taps
24.	Tetrachloroethene & Trichloroethene[(viii)]	10	µg/l	consumers' taps
25.	THM: Total[(ix)]	100	µg/l	consumers' taps

Item	*Parameters*	*Concentration or Value (maximum)*	*Units of Measurement*	*Point of compliance*
26.	Vinyl chloride	0.50	µg/l	**(i)**

Notes:

(i) The parametric value refers to the residual monomer concentration in the water as calculated according to specifications of the maximum release from the corresponding polymer in contact with the water. This is controlled by product specification.

(ii) See also regulation 6(6).

(iii) See also regulation 4(2)(d).

(iv) See the definition of "pesticides and related products" in regulation 2.

(v) The parametric value applies to each individual pesticide.

(vi) "Pesticides: Total" means the sum of the concentrations of the individual pesticides detected and quantified in the monitoring procedure.

(vii) "PAH" means Polycyclic Aromatic Hydrocarbons, the specified compounds are:
- benzo(b)fluoranthene
- benzo(k)fluoranthene
- benzo(ghi)perylene
- indeno(1,2,3-cd)pyrene.

The parametric value applies to the sum of the concentrations of the individual compounds detected and quantified in the monitoring process.

(viii) The parametric value applies to the sum of the concentrations of the individual compounds detected and quantified in the monitoring process.

(ix) "THM: Total" means total Trihalomethanes, the specified compounds are:
- chloroform
- bromoform
- dibromochloromethane
- bromodichloromethane.

The parametric value applies to the sum of the concentrations of the individual compounds detected and quantified in the monitoring process.

Part II: National requirements

Item	*Parameters*	*Concentration or Value (maximum unless otherwise stated)*	*Units of Measurement*	*Point of compliance*
1.	Aluminium	200	µg Al/l	consumers' taps
2.	Colour	20	mg/l Pt/Co	consumers' taps
3.	Hydrogen ion	9.5	pH value	consumers' taps
		6.5 (minimum)		
4.	Iron	200	µgFe/l	consumers' taps
5.	Manganese	50	µgMn/l	consumers' taps
6.	Odour	3 at 25°C	Dilution number	consumers' taps
7.	Sodium	200	mgNa/l	consumers' taps
8.	Taste	3 at 25°C	Dilution number	consumers' taps

Item	*Parameters*	*Concentration or Value (maximum unless otherwise stated)*	*Units of Measurement*	*Point of compliance*
9.	Tetrachloromethane	3	µg/l	consumers' taps
10.	Turbidity	4	NTU	consumers' taps

SCHEDULE 2

Regulations 2 and 4

INDICATOR PARAMETERS

Item	*Parameters*	*Concentration or Value (maximum) or State*	*Units of Measurement*	*Point of monitoring*
1.	Ammonium	0.50	mgNH4/l	consumers' taps
2.	Chloride[(i)]	250	mgCl/l	supply point*
3.	*Clostridium perfringens* (including spores)	0	Number/100 ml	supply point*
4.	Coliform bacteria	0	Number/100ml	consumers' taps
5.	Colony count	No abnormal change	Number/1 ml at 22°C	consumers' taps
			Number/1 ml at 37°C	service reservoirs and treatment works
6.	Conductivity[(i)]	2500	µS/cm at 20°C	supply point*
7.	Sulphate[(i)]	250	mgSO4/l	supply point*
8.	Total indicative dose (for radioactivity)[(ii)]	0.10	mSv/year	supply point*
9.	Total organic carbon (TOC)	No abnormal change	mgC/l	supply point*
10.	Tritium (for radioactivity)	100	Bq/l	supply point*
11.	Turbidity	1	NTU	treatment works

Notes:

(i) The water should not be aggressive.

(ii) Excluding tritium, potassium – 40, radon and radon decay products.

* May be monitored from samples of water leaving treatment works or other supply point, as no significant change during distribution.

SCHEDULE 3 Part IV

MONITORING

TABLE 1

PARAMETERS AND CIRCUMSTANCES FOR CHECK MONITORING

(1) *Item*	*(2)* *Parameter*	*(3)* *Circumstances*
1.	Aluminium	– When used as flocculant or where the water originates from, or is influenced by, surface waters
2.	Ammonium	
3.	*Clostridium perfringens* (including spores)	– Where the water originates from, or is influenced by, surface waters
4.	Coliform bacteria	
5.	Colony counts	
6.	Colour	
7.	Conductivity	
8.	*Escherichia coli (E. Coli)*	
9.	Hydrogen ion	
10.	Iron	– When used as flocculant or where the water originates from, or is influenced by, surface waters
11.	Manganese	– Where the water originates from, or is influenced by, surface waters
12.	Nitrate	– When chloramination is practised
13.	Nitrite	– When chloramination is practised
14.	Odour	
15.	Taste	
16.	Turbidity	

TABLE 2

ANNUAL SAMPLING FREQUENCIES: WATER SUPPLY ZONES

(1) *Substances and parameters subject to check monitoring*	*(2)* *Estimated population of water supply zone*	*(3)* *Reduced*	*(4)* *Standard*
E.Coli	<100		4
Coliform bacteria Residual disinfectant	≥100		12 per 5000 population[(i)]
Aluminium*			
Ammonium			
Clostridium perfringes (including spores)*[(i)]			
Colony counts	<100	1	2
Colour	100 – 4,999	2	4
Conductivity*	5,000 – 9,999	6	12
Hydrogen ion	10,000 – 29,999	12	24
Iron+	30,000 – 49,999	18	36
Manganese+	50,000 – 79,999	26	52
Nitrate+	80,000 – 100,000	38	76
Nitrite+			
Odour			
Taste			
Turbidity			
Parameters subject to audit monitoring			
Antimony			
Arsenic			
Benzene*			

Notes:

\+ See regulation 6(2) and Table 1 in Schedule 3.

* Sampling for these parameters may be within water supply zones or at supply points as specified in Table 3, subject to notes (ii) and (iii) below.

(i) Where the population is not an exact multiple of 5000, the population figure should be rounded up to the nearest multiple of 5000.

(ii) Audit monitoring in water supply zones is required only where sodium hypochlorite is added after water has left the treatment works. In other circumstances, audit monitoring is required at supply points.

(iii) To monitor for total indicative dose (for radioactivity).

(1) *Substances and parameters subject to check monitoring*	*(2)* *Estimated population of water supply zone*	*(3)* *Reduced*	*(4)* *Standard*
Benzo(a)pyrene			
Boron*			
Bromate(ii)*			
Cadmium			
Chromium	<100		1
Copper	100 – 4,999		4
Cyanide*	5,000 – 100,000		8
1, 2 dichloroethane*			
Enterococci			
Flouride*			
Lead			
Mercury*			
Nickel			
Pesticides*			
PAH			
Selenium			
Sodium			
Trichloroethene/ Tetrachloroethene*			
Tetrachloromethane*	<100		1
THM	100 – 4,999		4
Chloride*	5,000 – 100,000		8
Sulphate*			
Total Organic Carbon*			
Tritium*			

Notes:

\+ See regulation 6(2) and Table 1 in Schedule 3.

* Sampling for these parameters may be within water supply zones or at supply points as specified in Table 3, subject to notes (ii) and (iii) below.

(i) Where the population is not an exact multiple of 5000, the population figure should be rounded up to the nearest multiple of 5000.

(ii) Audit monitoring in water supply zones is required only where sodium hypochlorite is added after water has left the treatment works. In other circumstances, audit monitoring is required at supply points.

(iii) To monitor for total indicative dose (for radioactivity).

(1) *Substances and parameters subject to check monitoring*	*(2)* *Estimated population of water supply zone*	*(3)* *Reduced*	*(4)* *Standard*
Gross alpha[(iii)*]			
Gross beta[(iii)*]			

Notes:

\+ See regulation 6(2) and Table 1 in Schedule 3.

***** Sampling for these parameters may be within water supply zones or at supply points as specified in Table 3, subject to notes (ii) and (iii) below.

(i) Where the population is not an exact multiple of 5000, the population figure should be rounded up to the nearest multiple of 5000.

(ii) Audit monitoring in water supply zones is required only where sodium hypochlorite is added after water has left the treatment works. In other circumstances, audit monitoring is required at supply points.

(iii) To monitor for total indicative dose (for radioactivity).

TABLE 3

ANNUAL SAMPLING FREQUENCIES: SUPPLY POINTS

(1) *Item*	*(2)* *Substances and parameters subject to check monitoring*	*(3)* *Volume of water supplied m3/d*	*(4)* *Reduced*	*(5)* *Standard*
1.	*Clostridium perfringens* (including spores)[(i)]	<20		2
		20 – 999	2	4
2.	Conductivity	1,000 – 1,999	6	12
		2,000 – 5,999	12	24
		6,000 – 9,999	18	36
		10,000 – 15,999	26	52
		16,000 – 32,999	52	104
		33,000 – 49,999	78	156
		50,000 – 67,999	104	208
		68,000 – 84,999	130	260

Notes:

(i) Check monitoring is required only in respect of surface waters (see regulation 6(2) and Table 1 in Schedule 3), otherwise audit monitoring.

(ii) Audit monitoring at supply points is permitted only where sodium hypochlorite is not added after water has left the treatment works. In other circumstances, audit monitoring is required in water supply zones.

(iii) To monitor for total indicative dose (for radioactivity).

(1) *Item*	*(2)* *Substances and parameters subject to check monitoring*	*(3)* *Volume of water supplied m3/d*	*(4)* *Reduced*	*(5)* *Standard*
		85,000 – 101,999	156	312
		102,000 – 119,999	183	365
		120,000 – 241,999	365	730
		242,000 – 484,999	730	1,460
		485,000 – 728,999	1,095	2,190
	Parameters subject to audit monitoring			
3.	Benzene			
4.	Boron			
5.	Bromate[(ii)]			
6.	Cyanide			
7.	1,2 dichloroethane			
8.	Fluoride	<20		1
9.	Mercury	20 – 999		4
10.	Pesticides	1,000 – 49,999		8
11.	Trichloroethene/ Tetrachloroethene	50,000 – 89,999		12
		90,000 – 299,999		24
12.	Tetrachloromethane	300,000 – 649,999		36
13.	Chloride	≥650,000		48
14.	Sulphate			
15.	Total organic carbon			

Notes:

(i) Check monitoring is required only in respect of surface waters (see regulation 6(2) and Table 1 in Schedule 3), otherwise audit monitoring.

(ii) Audit monitoring at supply points is permitted only where sodium hypochlorite is not added after water has left the treatment works. In other circumstances, audit monitoring is required in water supply zones.

(iii) To monitor for total indicative dose (for radioactivity).

(1) *Item*	*(2)* *Substances and parameters subject to check monitoring*	*(3)* *Volume of water supplied m3/d*	*(4)* *Reduced*	*(5)* *Standard*
16.	Tritium			
17.	Gross alpha[(iii)]			
18.	Gross beta[(iii)]			

Notes:

(i) Check monitoring is required only in respect of surface waters (see regulation 6(2) and Table 1 in Schedule 3), otherwise audit monitoring.

(ii) Audit monitoring at supply points is permitted only where sodium hypochlorite is not added after water has left the treatment works. In other circumstances, audit monitoring is required in water supply zones.

(iii) To monitor for total indicative dose (for radioactivity).

TABLE 4

ANNUAL SAMPLING FREQUENCIES: WATER TREATMENT WORKS

(1) *Item*	*(2)* *Substances and parameters subject to check monitoring*	*(3)* *Volume of water supplied m3/d*	*(4)* *Reduced*	*(5)* *Standard*
1.	*E. coli*	<20		4
2.	Coliform bacteria	20 – 1,999	12	52
3.	Colony counts	2,000 – 5,999	52	104
4.	Residual disinfectant	6,000 – 11,999	104	208
		≥12,000	104	365
5.	Nitrite[(i)]	<20		2
6.	Turbidity	20 – 999	2	4
		1,000 – 1,999	6	12
		2,000 – 5,999	12	24
		6,000 – 9,999	18	36
		10,000 – 15,999	26	52
		16,000 – 32,999	52	104
		33,000 – 49,999	78	156
		50,000 – 67,999	104	208

Notes:

(i) check monitoring at treatment works is required only when chloramination is practised. In other circumstances, audit monitoring is required.

(1) *Item*	*(2)* *Substances and parameters subject to check monitoring*	*(3)* *Volume of water supplied m3/d*	*(4)* *Reduced*	*(5)* *Standard*
		68,000 – 84,999	130	260
		85,000 – 101,999	156	312
		102,000 – 119,999	183	365
		120,000 – 241,999	365	730
		242,000 – 484,999	730	1,460
		485,000 – 728,999	1,095	2,190
	Substances and parameters subject to audit monitoring			
7.	Nitrite[(i)]	<20		1
		20 – 999		4
		1,000 – 49,999		8
		50,000 – 89,999		12
		90,000 – 299,999		24
		300,000 – 649,999		36
		>650,000		48

Notes:

(i) check monitoring at treatment works is required only when chloramination is practised. In other circumstances, audit monitoring is required.

SCHEDULE 4 Regulation 16

ANALYTICAL METHODOLOGY

TABLE A1

Parameters for which, subject to regulation 16(5), methods of analysis are prescribed

(1) *Parameter*	*(2)* *Method*
Clostridium perfringens (including spores)	Membrane filtration followed by anaerobic incubation of the membrane on m–CP agar at 44 ±1°C for 21 ± 3 hours. Count opaque yellow colonies that turn pink or red after exposure to ammonium hydroxide vapours for 20 to 30 seconds.
Coliform bacteria	ISO 9308–1
Colony count 22°C – enumeration of culturable microorganisms	prEN ISO 6222
Colony count 37°C – enumeration of culturable microorganisms	pr EN 6222
Enterocci	ISO 7899–2
Escherichia coli (E. coli)	ISO 9308–1

a The composition of m-CP agar is:

Basal medium

Tryptose	30.0g
Yeast extract	20.0g
Sucrose	5.0g
L–cysteine hydrichloride	1.0g
$MgSO_4 \cdot 7H_2O$	0.1g
Bromocresol purple	40.0mg
Agar	15.0g
Water	1,000.0ml

Dissolve the ingredients of the basal medium, adjust pH to 7.6 and autoclave at 121°C for 15 minutes. Allow the medium to cool and add:

D-cycloserine	400.0mg
Polymyxine-B sulphate	25.0mg
Indoxyl-•-D-glucoside to be dissolved in 8ml sterile water before addition	60.0mg
Filter – sterilised 0.5% phenolphthalein disphosphate solution	20.0ml
Filter – sterilised 4.5% $FeC1_3 \cdot 6H_2O$	2.0ml

TABLE A2

Parameters in relation to which methods of analysis must satisfy prescribed characteristics

(1) *Parameters*	*(2)* *Trueness % of prescribed concentration or value or specification*	*(3)* *Precision % of prescribed concentration or value or specification*	*(4)* *Limit of detection % of prescribed concentration or value or specification*
Aluminium	10	10	10
Ammonium	10	10	10
Antimony	25	25	25
Arsenic	10	10	10
Benzene	25	25	25
Benzo(a)pyrene	25	25	25
Boron	10	10	10
Bromate	25	25	25
Cadmium	10	10	10
Chloride	10	10	10
Chromium	10	10	10
Colour	10	10	10
Conductivity	10	10	10
Copper	10	10	10
Cyanide[(i)]	10	10	10
1,2-dichloroethane	25	25	10
Fluoride	10	10	10
Iron	10	10	10
Lead	10	10	10
Manganese	10	10	10
Mercury	20	10	20

Notes:

(i) The method of analysis should determine total cyanide in all forms.

(ii) The performance characteristics apply to each individual pesticide and will depend on the pesticide concerned.

(iii) The performance characteristics apply to the individual substances specified at 25% of the parametric value in Part I of Table B in Schedule 1.

(iv) The performance characteristics apply to the individual substances specified at 50% of the parametric value in Part I of Table B in Schedule 1.

(v) The performance characteristics apply to the prescribed value of 4NTU.

(vi) The performance characteristics apply to the specification of 1NTU for water leaving treatment works.

(1) *Parameters*	*(2)* *Trueness % of prescribed concentration or value or specification*	*(3)* *Precision % of prescribed concentration or value or specification*	*(4)* *Limit of detection % of prescribed concentration or value or specification*
Nickel	10	10	10
Nitrate	10	10	10
Nitrite	10	10	10
Pesticides and related products[(ii)]	25	25	25
Polycyclic aromatic hydrocarbons[(iii)]	25	25	25
Selenium	10	10	10
Sodium	10	10	10
Sulphate	10	10	10
Tetrachloroethene[(iv)]	25	25	10
Tetrachloromethane	20	20	20
Trichloroethene[(iv)]	25	25	10
Trihalomethanes: Total[(iii)]	25	25	10
Turbidity[(v)]	10	10	10
Turbidity[(vi)]	25	25	25

Notes:

(i) The method of analysis should determine total cyanide in all forms.

(ii) The performance characteristics apply to each individual pesticide and will depend on the pesticide concerned.

(iii) The performance characteristics apply to the individual substances specified at 25% of the parametric value in Part I of Table B in Schedule 1.

(iv) The performance characteristics apply to the individual substances specified at 50% of the parametric value in Part I of Table B in Schedule 1.

(v) The performance characteristics apply to the prescribed value of 4NTU.

(vi) The performance characteristics apply to the specification of 1NTU for water leaving treatment works.

SCHEDULE 5

Regulation 36

AMENDMENT OF THE WATER SUPPLY (WATER QUALITY) (SCOTLAND) REGULATIONS 1990

The amendments to the 1990 Regulations referred to in regulation 36 are–

(a) after regulation 13, the insertion of the following regulation:–

"**Frequency of sampling for particular organisms and substances**

13A.—(1) In each of the years 2001, 2002 and 2003 a water authority shall take, or cause to be taken, from its sampling points or, as the case may be, its supply points, for analysis for testing against the concentrations and values specified in column (2) of Table 4A for the organisms and substances listed in column (1) of that Table, not less than the number of samples specified in paragraphs (2) and (3).

(2) In respect of supply points and the supply of a volume of water within a range shown in column (3) of that Table, the number is that shown in column (4) of that Table as applicable to a volume within that range.

(3) In respect of sampling points and the supply of water to an estimated population within a range shown in column (5) of that Table, the number is that shown in column (6) of that Table as applicable to a population within that range.

(4) In each of those years samples shall be taken at regular intervals throughout the year.";

(b) after regulation 20, the insertion of the following regulation:

"**Additional testing for particular parameters**

20A. The samples taken in accordance with regulation 13 shall additionally be tested against the concentrations specified in column (2) of Table 4B.";

(c) in regulation 21 (collection and analysis of samples)–

(i) in paragraph (2), the insertion, after "means", of "subject to paragraph (3)"; and

(ii) the addition, at the end, of the following:–

"(3) In relation to each of the parameters and other substances listed in column (1) of Table 4B, paragraph (2)(d) shall have effect as if for sub-paragraph (iii) there were substituted the requirements of regulation 21A."

(d) after regulation 21, the insertion of the following regulation:–

"**Method of analysis of samples for particular parameters and substances**

21A.—(1) The method of analysis used for testing for a parameter or other substance listed in column (1) of Table 4B against the concentrations in column (2) of that Table must be capable, at the time of use–

(a) of measuring concentrations with the trueness and precision specified in relation to that parameter or substance in columns (3) and (4) of Table 4B; and

(b) of detecting the parameter or substance at the limit of detection specified in relation to it in column (5) of that Table.

(2) For the purposes of paragraph (1)–

"limit of detection" is to be calculated as–

(a) three times the relative within batch standard deviation of a natural sample containing a low concentration of the parameter or substance; or

(b) five times the relative within batch standard deviation of a blank sample;

"precision" (the random error); is to be calculated as twice the standard deviation (within a batch and between batches) of the spread of result about the mean; and

"trueness" (the systematic error) is to be calculated as the difference between the mean value of a large number of repeated measurements and the true value."; and

(e) the insertion, after Table 4 in Schedule 2, of the following Tables:

"**TABLE 4A**

SAMPLING FOR PARTICULAR ORGANISMS AND SUBSTANCES

(1) Organism or substance	*(2) Concentration or value and unit of measurement*	*(3) Volume of water supplied m3/d*	*(4) Annual sampling frequency*	*(5) Estimated population of water supply zone*	*(6) Annual sampling frequency*
Benzene	1.0 µg/l				
Bromate	10 µgBrO3/l				
*Clostridium perfringens** (including spores)	0 Number/100ml	• 999	2	• 4,999	2
		1,000 – 49,999	4	5,000–	4
		≥ 50,000	8	50,000	
1,2 dichloroethane	3.0 µg/l				
Enterococci	0 Number/100ml				
Nitrite	0.1 mgNO2/l				

* where the water originates from, or is influenced by, surface waters

TABLE 4B

PARAMETERS AND SUBSTANCES IN RELATION TO WHICH, SUBJECT TO REGULATION 21A, METHODS OF ANALYSIS MUST SATISFY THE PRESCRIBED CHARACTERISTICS

(1) Parameter or other substance	*(2) Concentration and unit of measurement*	*(3) Trueness % of concentration*	*(4) Precision % of concentration*	*(5) Limit of detection % of concentration*
Antimony	5.0 µgSb/l	25	25	25
Arsenic	10 µgAs/l	10	10	10
Benzene	1.0 µg/l	25	25	25
Boron	1.0 mgB/l	10	10	10
Bromate	10 µgBrO3/l	25	25	25

\+ Polycyclic Aromatic Hydrocarbons (PAH), sum of the concentrations of benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)pyrene, and indeno(1,2,3-cd)pyrene. The performance characteristics apply to the individual substances specified at 25% of the concentration in column (2).

* The performance characteristics apply to the individual substances specified at 50% of the concentration in column (2).

(1) Parameter or other substance	*(2) Concentration and unit of measurement*	*(3) Trueness % of concentration*	*(4) Precision % of concentration*	*(5) Limit of detection % of concentration*
Copper	2.0 mgCu/l	10	10	10
1, 2 dichloroethane	3.0 µg/l	25	25	10
Lead	25 µgPb/l	10	10	10
Nickel	20 µgNi/l	10	10	10
Nitrite (ex-works)	0.1 mgNO2/l	10	10	10
PAH+	0.1µg/l	25	25	25
Tetrachloroethene*	10 µg/l	25	25	10
Trichloroethene*	10 µg/l	25	25	10".

\+ Polycyclic Aromatic Hydrocarbons (PAH), sum of the concentrations of benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)pyrene, and indeno(1,2,3-cd)pyrene. The performance characteristics apply to the individual substances specified at 25% of the concentration in column (2).

* The performance characteristics apply to the individual substances specified at 50% of the concentration in column (2).

SCHEDULE 6

Regulation 37

PROGRAMMES OF WORK

A water authority's programme of work shall–

(a) identify, by reference to the parameters set out in the Table below, those parameters in respect of which the requirements of regulation 38(1)(a) and (b) are unlikely to be satisfied unless steps are taken to secure that those requirements will be met;

(b) identify whether the requirement that the formula [nitrate]/50 + [nitrite]/3 • 1, where the square brackets signify the concentrations in mg/l for nitrate (NO3) and nitrite (NO2) is unlikely to be satisfied;

(c) specify the steps that the water authority intends to take for the purpose of securing that the requirements of regulation 38(1)(a) and (b) are met;

(d) specify the date by which each of the steps specified in accordance with paragraph (c) is proposed to be completed; and

(e) specify the times at which, or the periods within which, reports will be made to the Scottish Ministers in relation to the taking and completion of the steps specified in accordance with paragraph (c).

TABLE

E. coli
Enterococci
Acrylamide

Antimony
Arsenic
Benzene
Benzo(a)pyrene
Boron
Bromate
Cadmium
Chromium
Copper
Cyanide
1,2-dichloroethane
Epichlorohydrin
Lead
Mercury
Nickel
Nitrate
Nitrite
Pesticides
Polycyclic aromatic hydrocarbons
Selenium
Tetrachloroethene/Trichloroethene (sum of concentrations)
Trihalomethanes – Total (sum of concentrations of chloroform, bromoform, dibromochloromethane and bromodichloromethane)
Vinyl chloride

EXPLANATORY NOTE

(This note is not part of the Regulations)

These Regulations supplement Part VIA of the Water (Scotland) Act 1980 ("the 1980 Act"); amend, for a limited period, the Water Supply (Water Quality) (Scotland) Regulations 1990 ("the 1990 Regulations") and, on 25th December 2003, revoke and replace those Regulations. They are primarily concerned with the quality of water supplied in Scotland for drinking, washing, cooking and food preparation, and for food production, and with arrangements for the publication of information about water quality.

The Regulations are directed at the achievement of the objective set out in Article 2 of Council Directive 98/83/EC (O.J. No. L 330, 5.12.98, p.32) ("the 1998 Directive"), namely, to protect human health from the adverse effects of any contamination of water intended for human consumption by ensuring that it is wholesome and clean. In particular, effect is given in Part III of the Regulations to Articles 4 and 5 of the 1998 Directive which relate to the quality of water intended for human consumption and, in Part V, to Article 7 (monitoring) of that Directive.

Subject to the exceptions mentioned below, the Regulations come into force on 25th December 2003. Regulations 1 and 2 (which deal with commencement, extent and interpretation), regulation 36 (which introduces the amendments to the 1990 Regulations set out in Schedule 5) and regulation 37 (a transitional provision requiring the submission of programmes of work) come into force on 21st June 2001.

Regulation 3 (which deals with water supply zones) and regulation 38 (a transitional provision enabling applications to be made for authorisations) come into force on 1st June 2003.

Regulation 4 (which prescribes new standards of wholesomeness), regulations 17 to 24 (which deal with sampling) paragraphs (4) and (5) of regulation 26 (which deals with lead pipes) and paragraph (1) of regulation 39 (which revokes provisions of the 1990 Regulations superseded by regulations 4 and 17 to 24) come into force on 25th December 2003, when the 1998 Directive takes effect.

Part I of the Regulations (regulations 1 and 2) defines terms that are used in the Regulations.

Part II (regulation 3) requires water authorities to identify annually the areas ("water supply zones") that are to be relevant for a particular year for the purposes of the application of the provisions of the Regulations. A water supply zone may not comprise an area in which the estimated population exceeds 100,000. Water authorities may not alter the boundaries of water supply zones during the year.

Part III (regulation 4) prescribes standards of wholesomeness in respect of water that is supplied by water authorities for cooking, drinking, food preparation and washing and other domestic purposes and to premises for food production purposes. These various purposes are referred to in the Regulations as "regulation 4(1) purposes". In particular, regulation 4 provides that water is to be regarded as wholesome if it contains concentrations or values in respect of various properties, elements, organisms and substances that do not contravene prescribed maximum and, in some cases, minimum concentrations or values. Some of the prescribed maximum and minimum concentrations and values are specified in regulation 4, but most are included in Tables A and B which appear in Schedule 1. They include the values specified in Part A and B of Annex 1 to the 1998 Directive. There are also specifications for indicator parameters in Schedule 2.

Part IV (regulations 5 to 10) provides for the monitoring of water supplies by reference to the analysis of samples. Regulation 5 defines two monitoring regimes; "audit" monitoring and "check" monitoring. Regulation 6 requires water authorities to take a minimum number of samples each year in respect of a variety of properties, elements, organisms and substances. It also makes special provision for monitoring supplies from tankers. Regulation 7 requires water authorities to select at random the consumers' taps from which samples are to be taken. Regulation 8 authorises the taking of samples from points other than consumers' taps ("supply points") and allows the Scottish Ministers to authorise other supply points. Regulation 9 deals with the number of samples to be taken. These are specified in Tables 1 and 2 in Schedule 3 to the Regulations, and are not less than those specified in Annex II to the 1998 Directive. Regulation 10 requires samples to be taken where water authorities have reason to believe that the quality of the water within their water supply zone has been adversely affected by the presence of certain elements, organisms or substances.

Part V (regulations 11 to 16) contains additional provisions relating to sampling. Regulations 13 and 14 require samples to be taken in respect of particular organisms and substances, at treatment works and at reservoirs which store treated water. Regulation 15 requires samples to be taken before water

is supplied from new sources and from sources which have not recently been used. Regulation 16 prescribes requirements relating to the taking, handling, storage, transport and analysis of samples.

Part VI (regulations 17 to 24) provides for the investigation of every failure to satisfy a concentration, value or state prescribed by regulation 4 and for a report to be made to the Scottish Ministers. Where a failure relates to a Table B parameter, and certain other conditions are met, the Scottish Ministers may require the water authority to apply to them for an authorisation allowing a departure from the requirements of Part III, as regards that parameter. The circumstances in which such authorisations may be issued, and the conditions to which they are subject are contained in regulations 21 and 22, respectively. (Article 9 of the 1998 Directive permits derogations from the parametric values). Provision is made in regulation 23 for publicising authorisations. Regulation 24 provides for the modification and withdrawal of authorisations.

Part VII (regulations 25 to 29) deals with the treatment of water and regulates the substances, processes and products that may be used by water authorities in connection with the supply of water. Regulation 25 imposes requirements relating to the disinfection of water and imposes additional requirements for the treatment of surface water. It prohibits the abstraction for the supply of drinking water of waters below category A3, as required by Council Directive 75/440/EEC (quality required of surface water intended for the abstraction of drinking water). Regulation 26 makes provision for securing the elimination or reduction to a minimum of the risk that water will be contaminated after supply by excessive concentrations of copper or lead. Regulation 27 specified the circumstances in which water authorities may apply or introduce substances or products into water supplied for drinking, washing or cooking. Regulation 28 enables the Scottish Ministers to require that their approval be obtained to the use of processes. Contravention of some of the requirements of regulations 27 and 28 is made a criminal offence by regulation 29, as is the making of false statements.

Part VIII deals with the provision of information by water authorities. Regulation 30 requires water authorities to prepare and maintain records containing information about the quality of water supplied in their water supply zones. Regulation 31 requires each water authority to make available for public inspection, and to supply local authorities with, information about the quality of water within its water supply zones, the extent to which Part IV of the Regulations has been complied with, details of any departures authorised under Part VI of the Regulations and of the action which has been taken to comply with default orders under section 76E of the 1980 Act. It also requires each water authority to provide local authorites, health authorities and the Water Industry Commissioner for Scotland with information relating to matters that could give rise to significant risk to public health. Regulation 32 requires water authorities to publish an annual report containing information about the quality of water in the area for which it is responsible.

Part IX (regulations 33 and 34) imposes requirements on local authorities in the performance of their duties in relation to the quality of water supplied by water authorities. Regulation 34 provides for the making of arrangements between the local authorities and the water authorities about the provision of information. It also enables local authorities to take such samples of water as they may reasonably require.

Part X (regulation 35) provides that contraventions by water authorities of duties or requirements imposed by Parts V to VIII of the Regulations are to be enforceable under section 76E of the 1980 Act by the Scottish Ministers. This provision is additional to the criminal sanctions provided by regulation 29 in relation to contraventions of regulations 27 and 28.

Part XI (regulations 36 to 39) amends the 1990 Regulations, makes transitional provision and revokes the 1990 Regulations and other related provisions subject to savings. The amendments to the 1990 Regulations, which take effect on 26th June 2001, are set out in Schedule 5 (Regulation 36). Regulation 37 requires water authorities to submit for the Scottish Ministers' approval programmes of work designed to secure that the standards of wholesomeness prescribed in Part III of the Regulations will, so far as reasonably practicable, be achieved when that Part comes into force on 25th December 2003. Programmes of work are to be prepared in accordance with Schedule 6 and are

to be submitted to the Scottish Ministers before 25th September 2001. They may be approved by the Scottish Ministers with or without modification, and may be modified where the Scottish Ministers consider it necessary to do so for the purpose of securing that wholesomeness standards will be met on and after 25th December 2003. If a water authority fails to submit a programme of works, or if the submitted programme fails to meet the requirements of Schedule 6, or is otherwise unacceptable, the Scottish Ministers may prepare one. Programmes of work will be enforceable under section 76E of the 1980 Act by the Scottish Ministers.

Regulation 38 makes further transitional provision to enable water authorities to apply in advance for the Scottish Ministers' authorisation in relation to matters which, on and after 25th December 2003, will need to be authorised under Part VI of the Regulations.

Regulation 39 revokes the 1990 Regulations, and amending regulations. Regulation 23(b) of the Private Water Supplies (Scotland) Regulations 1992 and regulation 16(1) of the Surface Waters (Abstraction for Drinking Water) (Classification) (Scotland) Regulations 1996 are also revoked to the same extent, in consequence of the revocation of the 1990 Regulations. The revocation of the 1990 Regulations does not affect the obligations of water authorities to keep records, provide information, or make reports for the year ending on 31st December 2003.

A regulatory impact assessment in respect of the Regulations may be obtained from the Scottish Executive, Rural Affairs Department, Environment Group, Water Services Unit, Victoria Quay, Edinburgh EH6 6QQ. A copy has been placed in the Scottish Parliament Information Centre.

Copies of the ISO standards referred to in Table A1 in Schedule 4 may be obtained from BSI, 389 Chiswick High Road, London W4 4AL.

SCOTTISH HOSPITALS INQUIRY

Bundle of documents for Oral hearings commencing from 19 August 2024 in relation to the Queen Elizabeth University Hospital and the Royal Hospital for Children, Glasgow

Bundle 27 – Volume 17 – Miscellaneous Documents