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

Miscellaneous Documents

Volume 16

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SCOTTISH HOSPITALS INQUIRY

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Case Note Review

Paediatric Haemato-Oncology Patients

Royal Hospital for Children

NHS Greater Glasgow and Clyde

Epidemiological Protocol

February 2020 v0.1

Introduction

Health Protection Scotland has been supporting NHS Greater Glasgow and Clyde in the investigation of a suspected increase in the number of cases of patients with Gram-negative bacteria blood cultures.

Previous epidemiological investigations have focused on microbiological laboratory data. These investigations recommended that systematic collection of clinical and epidemiological information on cases be carried out allowing the cases to be described in the context of risk including, where possible, in the context of environmental risks and incidents.

In January 2020, the Scottish Government announced that a case note review would be undertaken. An internationally validated Paediatric Trigger Tool (PTT) has been selected to undertake the review by the independent review team. In addition to the PTT review, a supplementary epidemiological review of the cases is required to collect patient, outcome and risk data systematically using agreed definitions and for the findings to support the incident investigation.

The objectives of this epidemiological investigation are to:

- Determine a timeline for each of the cases
- Characterise the cases in terms of time, place and person
    - Time: describe the episodes of BSI over time and create a timeline for outbreak, including plotting of control measures against number of cases
    - Place: describe the location of patients (hospital, ward, bed/bay) and describe their movements in the hospital
    - Person: characterise the patients with infection in terms of intrinsic and extrinsic risk factors; outcomes; antimicrobial prophylaxis and treatment; and individual infection prevention and control measures in place.
- Describe the cases in the context of environmental risks and incidents (where possible)

Methods

Study design

This protocol describes the methodology for a retrospective review of cases using patient cases notes from January 2015 to present. The epidemiological study design is descriptive rather than analytical and no control group will be selected to make comparisons between patients with and without infection to determine risk (at this time).

The study population includes all cases that meet one of the following definitions:

- A patient aged <18 years cared for in the haematology or oncology specialty in the Royal Hospital for Children, NHS GGC with:
    - at least one positive blood culture of a Gram negative organism between May 2015 and September 2019 OR
    - at least one positive blood culture of a Gram positive organism associated with the environment* between May 2015 and September 2019 OR
    - at least one positive culture of an atypical Mycobacterium between May 2015 and September 2019
    - at least one Gram negative organism isolated during post mortem

A number of datasets were available from previous epidemiological review of the microbiology data. Assessment of these datasets indicated that, whilst there were overlaps, it was necessary for optimal case ascertainment to combine the datasets and create a list of patients that appear in one or more of the datasets. These datasets were two extracts from NHS GGC's LIMS system, a central line associated bloodstream infection surveillance dataset and the Electronic Communication of Surveillance in Scotland (ECOSS). This exercised has identified 104 cases for inclusion in the case note review.

* Categories of and details of the organisms in each of the datasets are provided in Appendix 3.

Data collection methods

A team of 3-4 epidemiologists from HPS will undertake the case note review using all available patient information. It is expected that this will include:

- Current nursing notes
- Current medical notes
- Temperature charts
- Drug charts
- Surgery/operation notes
- Laboratory report e.g. microbiology results
- Other relevant charts e.g. wound charts, stool charts, care plans.

It is anticipated that several of these sources of information will be held electronically and a representative from the local GGC team will be required to assist with the extraction of these data. Development of a data capture system is being scoped e.g. paper forms, database.

Data will be collected by reviewing the case notes and extracting data relating to:

- Patient demographics and admissions
- Location data for each admission to hospital (using PAS and ICNet)
- Risk factors associated with haemato-oncology diagnosis and treatment
- Extrinsic risk factors for infection
- Infection data (defined using the ECDC case definitions for infection)
- Patient level infection prevention and control measures
- Patient outcome

Further information relating to each of these categories is provided in the table below.

Infection case definitions

The ECDC case definitions for infection are used to identify cases of infection in this patient population (Appendix 2). These definitions have previously been used in the paediatric population in European and Scottish Point Prevalence Surveys.

Infection should be determined to be hospital acquired, healthcare associated or community acquired according to the origin of infection definition included in the protocol for mandatory surveillance of bacteraemia in Scotland (Appendix 3).

Demographics and admission details	Location data (for each admission)	risk factors associated with cancer and treatment	Extrinsic risk factors	Infections	Patient level IPC measures	Outcome
Name	Date of admission	Morbidity	Indwelling device (for each insertion) - Type of devuce - Date inserted - Where inserted (theatre) - Date manipulated - Date removed	Infection type (ECDC case definitions, see appendix 2 for definitions)	Patient placement	Patient died
Date of birth	Admitted from (home, other hospital (name), other)	Diagnosis	Surgical procedure (for each procedure) - Procedure - Date of procedure - Theatre - Any other clinical information re procedure?	Date of onset	TBPs in place	Infection on death certificate
Community Health Index (CHI)	Date of discharge	Treatment phase Are there other important treatment questions?	Prophylactic antibiotics - Antibiotic name - Antibiotic start date - Antibiotic end date - Dose	Secondary source of BSI		PICU admission (dates)

			- Route of administration - Surgical/medical - Meets local policy			
Sex	Ward (all wards during admission)	Chemotherapy (+/- regime, date start and end)	Nutrition e.g. parenteral, PEG	Origin of infection e.g. healthcare associated, hospital acquired, community (see appendix 3 for definitions)		Delays to treatment
Specialty	Bed (all beds during admission)	Neutropenia (including duration of)		Device in prior to onset (24h lines, 7 days urinary catheter)		Removal of line due to infection
Reason for admission	Discharged to (home, other hospital (name), other)	Immunosuppression		Treatment of infection - Antibiotic name - Antibiotic start date - Antibiotic end date - Dose?? - Route of administration - Diagnosis Meets local policy?		Sepsis
		Palliative care				Others?

		BMT/stem cell transplant (date)				

Appendix 1: Prior microbiology groupings in the 4 datasets

Organisms included in Environmental groupings

NHSGGC CLABSI surveillance	NHSGGC ECOSS selected Gram-negative organisms (GGC Selected GNeg)	NHSGGC Microbiology LIMS Surveillance	HPS ECOSS Under18 bloods RHC HaemOnc
Gram Negative Environmental (GN ENV)	**Gram Negative Environmental (GN ENV)**	**Gram Negative Environmental (GN ENV)**	**Gram Negative Environmental (GN ENV)**
Achromobacter spp. *Acinetobacter baumannii* *Acinetobacter ursingii* *Aeromonas hydrophila* *Burkhold cepacia* *Chryseomonas indologenes* *Chryseob. spp* *Cupriavidis pauculus* *Eliz. meningoseptica* *Elizabethkingia spp.* *Delftia acidovorans* *Pseudomonas spp.* *Rhiz. radiobacter* *Roseomonas mucosa* *Sphingomonas spp.* *Steno. maltophilia*	*Acinetobacter baumannii* *Acinetobacter ursingii* *Aeromonas hydrophila* *Brevundimonas spp.* *Burkholderia cepacia* *Chryseobacterium indologenes* *Chryseobacterium spp.* *Cupriavidus pauculus* *Delftia acidovorans* *Elizabethkingia meningoseptica* *Elizabethkingia spp.* *Pseudomonas spp.* *Rhizobium radiobacter* *Sphingomonas paucimobilis* *Steno. maltophilia*	*Achromobacter sp* *Acinetobacter baumannii* *Acinetobacter ursingii* *Aeromonas spp* *Brev. spp.* *Burk. cepacia group* *Chryseob. spp* *Chryseobacterium indologenes* *Chryseomonas sp p.* *Cup. pauculus* *Del. acidovorans* *Delftia spp.* *Elizabethkingia. spp.* *Herbaspirillum sp* *Pseudomonas spp* *R. planticola* *Rh. radiobacter* *R. mucosa* *Sph. paucimobil* *Steno. maltophilia*	*Achromobacter spp.* *Acinetobacter spp.* *Aeromonas hydrophila* *Brevundimonas spp.* *Burkholderia cepacia* *Chryseobacterium indologenes* *Chryseobacterium spp.* *Cupriavidus pauculus* *Delftia acidovorans* *Elizabethkingia meningoseptica* *Elizabethkingia miricola* *Elizabethkingia spp.* *Pseudomonas spp.* *Raoultella planticola* *Rhizobium radiobacter* *Roseomonas mucosa* *Sphingomonas paucimobilis* *Steno. maltophilia*
Gram Negative Enteric /Environmental (GN ENT/ENV)	**Gram Negative Enteric /Environmental (GN ENT/ENV)**	**Gram Negative Enteric /Environmental (GN ENT/ENV)**	**Gram Negative Enteric /Environmental (GN ENT/ENV)**
Citrobacter spp. *Enterobacter cloacae* *Klebsiella spp.* *Pantoea spp.* *Serratia liquefaciens* *Serratia marcesens*	*Citrobacter spp.* *Enterobacter spp.* *Klebsiella spp.* *Pantoea spp.* *Serratia liquefaciens* *Serratia marcescens*	*Citrobacter spp.* *Enterobacter spp.* *Klebsiella spp.* *Pantoea spp.* *Ser. liquefac.* *Ser. marcescens*	*Citrobacter spp.* *Enterobacter spp.* *Klebsiella spp.* *Pantoea spp.* *Serratia liquefaciens* *Serratia marcescens*
Gram Positive Environmental (GP ENV)	**Gram Positive Environmental (GP ENV)**	**Gram Positive Environmental (GP ENV)**	**Gram Positive Environmental (GP ENV)**
Gordonia polyisoprenivorans	N/A	*Gordonia polyisoprenivorans*	*Gordonia bronchialis*
Acid Fast Environmental (AF ENV)	**Acid Fast Environmental (AF ENV)**	**Acid Fast Environmental (AF ENV)**	**Acid Fast Environmental (AF ENV)**
Mycobacterium chelonae	N/A	*Myc. chelonae group* *Myco fortuitum* *Mycobacterium chelonae*	*Mycobacterium chelonae* *Mycobacterium spp.*
Fungi Environmental (Fungi ENV)	**Fungi Environmental (Fungi ENV)**	**Fungi Environmental (Fungi ENV)**	**Fungi Environmental (Fungi ENV)**
Rhodotorula mucilaginosa	N/A	*Rhod. mucilaginosa*	*Rhodotorula mucilaginosa*

Organisms included in Non-environmental groupings

NHSGGC CLABSI surveillance	NHSGGC ECOSS selected Gram-negative organisms (GGC Selected GNeg)	NHSGGC Microbiology LIMS Surveillance	HPS ECOSS Under18 bloods RHC HaemOnc
Gram Negative Non-environmental (GN NON-ENV)	**Gram Negative Non-environmental (GN NON-ENV)**	**Gram Negative Non-environmental (GN NON-ENV)**	**Gram Negative Non-environmental (GN NON-ENV)**
Escherichia coli *Fusobacterium nucleatum* *Proteus mirabilis*	N/A	*Bact. uniformis* *Cap. sputigena* *Escherichia coli* *Fuso. nucleatum* *Haemophilus influenzae* *Mor. catarrhalis* *Moraxella nonliquefaciens* *Moraxella osloensis* *Neis. subflava* *Proteus mirabilis*	*Bacteroides uniformis* *Capnocytophaga sputigena* *Escherichia coli* *Escherichia fergusonii* *Fusobacterium nucleatum* *Haemophilus influenzae* *Moraxella spp.* *Neisseria spp.* *Ochrobactrum anthropi* *Proteus mirabilis*
Gram Positive Non-environmental (GP NON-ENV)	**Gram Positive Non-environmental (GP NON-ENV)**	**Gram Positive Non-environmental (GP NON-ENV)**	**Gram Positive Non-environmental (GP NON-ENV)**
Aerococcus viridans *Clostridium spp.* *Corynebacterium spp.* *Dermacoccus nishinomiyaens* *Diphtheroids* *Enterococcus spp.* *Gemella Sanguinis* *Gordonia polyisoprenivorans* *Gram +ve bacilli* *Gram Pos B* *Gram Pos C* *Gran Adiac* *Granulicatella Adiacens* *Kocuria rhizophilia* *Lactobacilus* *Micrococcus spp.* *Paenibacillus durus* *Propionibacterium acnes* *Rot. mucilaginosa* *Staphylococcus spp.* *STCNS* *Streptococcus spp.*	N/A	*Aerococcus viridans* *Alpha strep* *Bacillus spp.* *C. perfiringens* *Coag Neg Staph.* *Corynebacterium spp* *Derm. nishinomiyaens* *Diphtheroids* *Enterococcus spp.* *Gemella.sanguinis* *GPC-Strep* *Gram +ve bacilli* *Gram positive cocci* *Gran. adiacens* *K. rhizophila* *Lactobacillus spp* *Micrococcus spp.* *Paenibacillus spp.* *Propionibacterium acnes* *Rothia mucilaginosa* *Staphylococcus spp.* *Streptococcus spp.*	*Abiotrophia defectiva* *Aerococcus viridans* *Bacillus spp.* *Clostridium perfringens* *Clostridium septicum* *Corynebacterium spp.* *Dermacoccus spp.* *Enterococcus spp.* *Gemella sanguinis* *Granulicatella adiacens* *Kocuria spp.* *Lactobacillus spp.* *Lactococcus lactis* *Leuconostoc lactis* *Micrococcus spp.* *Paenibacillus spp.* *Propionibacterium spp.* *Rothia spp.* *Staphylococcus spp.* *Streptococcus spp.*
Acid Fast Non-environmental (AF NON-ENV)	**Acid Fast Non-environmental (AF NON-ENV)**	**Acid Fast Non-environmental (AF NON-ENV)**	**Acid Fast Non-environmental (AF NON-ENV)**
Nil	N/A	Nil	Nil
Fungi Non-environmental (Fungi NON-ENV)	**Fungi Non-environmental (Fungi NON-ENV)**	**Fungi Non-environmental (Fungi NON-ENV)**	**Fungi Non-environmental (Fungi NON-ENV)**
Candida spp. *Yeasts*	N/A	*Candida spp.*	*Candida spp.*

Appendix 2: Case definitions for infection

CRI: CATHETER-RELATED INFECTION

An aid to assist with the diagnosis of catheter-related infections is provided in Figure 4.4.2. A catheter-related infection may be related to central vascular catheters or peripheral/arterial vascular catheters.

ONSET: Catheter-related infections may develop any time after the device has been inserted.

Catheter-related infection 1 (CRI1)

CRI1-CVC: Local CVC-related infection (no positive blood culture)

- quantitative CVC culture ≥ 10^3 CFU/ml (1) or semi-quantitative CVC culture > 15 CFU(2)

And

- pus/inflammation at the insertion site or tunnel

CRI1-PVC: Local PVC-related infection (no positive blood culture)

- quantitative PVC culture ≥ 10^3 CFU/ml or semi-quantitative PVC culture > 15 CFU

And

- pus/inflammation at the insertion site or tunnel

Catheter-related infection 2 (CRI2)

CRI2-CVC: General CVC-related infection (no positive blood culture)

- quantitative CVC culture ≥ 10^3 CFU/ml or semi-quantitative CVC culture > 15 CFU

And

- clinical signs improve within 48 hours after catheter removal

CRI2-PVC: General PVC-related infection (no positive blood culture)

- quantitative PVC culture ≥ 10^3 CFU/ml or semi-quantitative PVC culture > 15 CFU

And

- clinical signs improve within 48 hours after catheter removal

Catheter-related infection 3 (CRI3)

CRI3-CVC: microbiologically confirmed CVC-related bloodstream infection

- BSI occurring 48 hours before or after catheter removal

And positive culture with the same micro-organism of either:

- quantitative CVC culture ≥ 10^3 CFU/ml or semi-quantitative CVC culture > 15 CFU
- quantitative blood culture ratio CVC blood sample/peripheral blood sample> 5 (3)
- differential delay of positivity of blood cultures (4): CVC blood sample culture positive 2 hours or more before peripheral blood culture (blood samples drawn at the same time)
- positive culture with the same micro-organism from pus from insertion site

CRI3-PVC: microbiologically confirmed PVC-related bloodstream infection

- BSI occurring 48 hours before or after catheter removal

And positive culture with the same micro-organism of either:

- quantitative PVC culture ≥ 10^3 CFU/ml or semi-quantitative PVC culture > 15 CFU
- positive culture with the same micro-organism from pus from insertion site

Note:
Central vascular catheter colonisation should not be reported

GI: GASTROENTERITIS

GI-CDI: *Clostridium difficile* infection

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 28 days prior to admission.

A *Clostridium difficile* infection (CDI) must meet at least 1 of the following criteria:

1. Diarrhoeal stools or toxic megacolon, and a positive laboratory assay for *C. difficile* toxin A and/or B in stools or a toxin-producing *C. difficile* organism detected in stool via culture or other means e.g. a positive PCR result.
2. Pseudomembranous colitis revealed by lower gastro-intestinal endoscopy
3. Colonic histopathology characteristic of *C. difficile* infection (with or without diarrhoea) on a specimen obtained during endoscopy, colectomy or autopsy

GI-GE: Gastroenteritis (excluding CDI)

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

Gastroenteritis must meet at least 1 of the following criteria:

1. Patient has an acute onset of diarrhoea (liquid stools for more than 12 hours) with or without vomiting or fever (>38°C) and no likely noninfectious cause (e.g., diagnostic tests, therapeutic regimen other than antimicrobial agents, acute exacerbation of a chronic condition, or psychological stress).

OR

2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: nausea, vomiting, abdominal pain, fever (>38°C), or headache

   And at least 1 of the following:
   - an enteric pathogen is cultured from stool or rectal swab
   - an enteric pathogen is detected by routine or electron microscopy
   - an enteric pathogen is detected by antigen or antibody assay on blood or faeces
   - evidence of an enteric pathogen is detected by cytopathic changes in tissue culture toxin assay) diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen.

BSI: BLOODSTREAM INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

Catheter-related BSI may develop any time after the device has been inserted.

BSI: Laboratory-confirmed bloodstream infection

Laboratory-confirmed bloodstream infection must meet at least 1 of the following criteria:

1. One positive blood culture for a recognised pathogen

OR

2.
   a. Patient has at least one of the following signs or symptoms: fever (>38°C.), chills, or hypotension

   AND

   b. Two positive blood cultures for a common skin contaminant (from 2 separate blood samples, usually within 48 hours).

Note:
Common skin contaminants are coagulase-negative staphylococci*, Micrococcus sp., Propionibacterium acnes, Bacillus sp., Corynebacterium sp.*

Source of bloodstream infection:

- Catheter-related: Microbiologically confirmed catheter-related BSI (the same microorganism was cultured from the catheter) should be recorded as ***CRI3-CVC*** or ***CRI3-PVC***. Non-microbiologically confirmed catheter-related BSI (symptoms improve within 48 hours of removal of the catheter) should be recorded as ***BSI*** with source ***C-CVC*** or ***C-PVC***. An aid to assist with the diagnosis of catheter-related infections is provided in Figure 4.4.2.

- Secondary to another infection: the same micro-organism was isolated from another infection site or strong clinical evidence exists that bloodstream infection was secondary to another infection site, invasive diagnostic procedure or foreign body.
  - Pulmonary (***S-PUL***)
  - Urinary tract infection (***S-UTI***)
  - Digestive tract infection (***S-DIG***)
  - SSI (***S-SSI***): surgical site infection
  - Skin and soft tissue (**S-SST**)
  - Other (***S-OTH***)
- Unknown origin (***UO***): None of the above, bloodstream infection of unknown origin (no source found)
- Unknown (***UNK***): No information available about the source of the bloodstream infection or information missing

CVC- associated BSI

A BSI is defined as CVC-associated if a CVC was present (even intermittently) in the 48 hours preceding the onset of infection. This is recorded using the “device in situ prior to onset” field.

PN: PNEUMONIA

The case definitions for pneumonia require a number of criteria to be fulfilled. These include diagnostic test results, symptoms and microbiological test. There are 5 pneumonia definitions (PN1-5) that differ depending on the microbiology results used to diagnose pneumonia.

An aid to assist with the diagnosis of pneumonia is provided in Figure 4.4.3.

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

Ventilator-associated pneumonia may develop any time after the device has been inserted.

Rx

Patients without underlying cardiac or pulmonary disease:

One definitive chest X-ray or CT-scan with a suggestive image of pneumonia

Patients with underlying cardiac or pulmonary disease:

Two or more serial chest X-rays or CT-scans with a suggestive image of pneumonia (e.g. pulmonary oedema, chronic obstructive pulmonary disease, bronchitis, right heart failure, respiratory distress syndrome, broncho-pulmonary dysplasia, pulmonary oedema).

OR

One definitive chest X-ray or CT-scan with a suggestive image of pneumonia when compared with previous chest x-rays or CT scans which have not indicated pneumonia

Symptoms

and at least one of the following:

- Fever > 38°C with no other cause
- Leukopenia (<4000 WBC[1]/mm^3) or leucocytosis (≥ 12 000 WBC/mm^3)

and at least one of the following (or at least two if clinical pneumonia only = PN 4 and PN 5):

- New onset of purulent sputum, or change in character of sputum (color, odor, quantity, consistency)
- Cough or dyspnea or tachypnea
- Suggestive auscultation (rales or bronchial breath sounds), ronchi, wheezing
- Worsening gas exchange (e.g., O_2 desaturation or increased oxygen requirements or increased ventilation demand)

[1] WBC = White Blood Cell Count

<u>And</u> according to the used diagnostic method

Microbiology

a - Bacteriologic diagnostic performed by : *Positive quantitative culture from minimally contaminated LRT[2] specimen* ***(PN 1)***

- Broncho-alveolar lavage (BAL) with a threshold of > 10^4 CFU[3]/ml or ≥5 % of BAL obtained cells contain intracellular bacteria on direct microscopic exam (classified on the diagnostic category BAL).
- Protected brush (PB Wimberley) with a threshold of >10^3 CFU/ml
- Distal protected aspirate (DPA) with a threshold of > 10^3 CFU/ml

Positive quantitative culture from possibly contaminated LRT specimen **(*PN 2*)**

- Quantitative culture of LRT specimen (e.g. endotracheal aspirate) with a threshold of 106 CFU/ml

b- Alternative microbiology methods (*PN 3*)

- Positive blood culture not related to another source of infection
- Positive growth in culture of pleural fluid
- Pleural or pulmonary abscess with positive needle aspiration
- Histologic pulmonary exam shows evidence of pneumonia
- Positive exams for pneumonia with virus or particular germs (*Legionella*, *Aspergillus*, mycobacteria, mycoplasma, *Pneumocystis carinii*)
    - Positive detection of viral antigen or antibody from respiratory secretions (e.g., EIA, FAMA, shell vial assay, PCR)
    - Positive direct exam or positive culture from bronchial secretions or tissue
    - Seroconversion (ex : influenza viruses, *Legionella, Chlamydia*)
    - Detection of antigens in urine (*Legionella)*

c - Others

- Positive **sputum** culture **or non-quantitative LRT specimen** culture ***(PN4)***
- **No positive microbiology *(PN 5)***

Note:
PN 1 and PN 2 criteria were validated without previous antimicrobial therapy

The subdivision of the pneumonia definition in 5 categories allows for the comparison of similar entities of pneumonia within and between networks. *It is essential that all networks report PN4 and PN5 (clinical pneumonia without microbiological evidence) in order to achieve overall comparability, even if a microbiological exam was performed and yielded negative results*. It is also advised, both for clinical and surveillance purposes, that networks promote as much as possible microbiological confirmation (PN1-3) as a routine practice in the ICU.

<u>Intubation-associated pneumonia (IAP)</u>
A pneumonia is defined as intubation-associated (IAP) if an invasive respiratory device was present (even intermittently) in the 48 hours preceding the onset of infection. This is recorded using the “device in situ prior to onset” field.

[2] LRT = Lower Respiratory Tract
[3] CFU= Colony Forming Unit

SST: SOFT TISSUE INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

SST-ST: Soft tissue (necrotizing fascitis, infectious gangrene, necrotizing cellulitis, infectious myositis, lymphadenitis, or lymphangitis)

Soft tissue infections must meet at least 1 of the following criteria:

1. Patient has organisms cultured from tissue or drainage from affected site.
2. Patient has purulent drainage at affected site.
3. Patient has an abscess or other evidence of infection seen during a surgical operation or histopathologic examination.
4. Patient has at least 2 of the following signs or symptoms at the affected site with no other recognized cause: localized pain or tenderness, redness, swelling, or heat

   And at least 1 of the following:
   a. organisms cultured from blood
   b. positive antigen test performed on blood or urine (e.g., *H influenzae*, *S pneumoniae*, *N meningitidis*, Group B *Streptococcus*, *Candida* spp)
   c. diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen.

Note:
Report infected decubitus ulcers as DECU-1 or DECU-2.

Report infection of deep pelvic tissues as OREP.

SST-DECU1**:** Decubitus ulcer, including both superficial and deep infections (microbiologically confirmed)

Decubitus ulcer infections must meet the following criteria:

1. Patient has at least 2 of the following signs or symptoms with no other recognized cause: redness, tenderness, or swelling of decubitus wound edges

   And at least 1 of the following:

2. organisms cultured from properly collected fluid or tissue (see Comments)
3. organisms cultured from blood.

Note:
Organisms cultured from the surface of a decubitus ulcer are not sufficient evidence that the ulcer is infected. A properly collected specimen from a decubitus ulcer involves needle aspiration of fluid or biopsy of tissue from the ulcer margin.

SST-DECU2**:** Decubitus ulcer, including both superficial and deep infections (not microbiologically confirmed)

Decubitus ulcer infections must meet the following criterion:

1. Patient has purulent drainage at affected site.

SSI: SURGICAL SITE INFECTION

ONSET: *Day of surgery onwards*

Present on admission or developing on Day 1 or 2 of admission

Superficial incisional (SSI-S)

Infection occurs within 30 days after the operation and infection involves only skin and subcutaneous tissue of the incision and at least one of the following:

1. Purulent drainage with or without laboratory confirmation, from the superficial incision
2. Organisms isolated from an aseptically obtained culture of fluid or tissue from the superficial incision.
3. At least one of the following signs or symptoms of infection: pain or tenderness, localized swelling, redness, or heat and superficial incision is deliberately opened by surgeon, unless incision is culture-negative.
4. Diagnosis of superficial incisional SSI made by a surgeon or attending physician.

Deep incisional (SSI-D)

Infection occurs within 30 days after the operation if no implant is left in place or within 90 days if implant is in place and the infection appears to be related to the operation and infection involves deep soft tissue (e.g. fascia, muscle) of the incision and at least one of the following:

1. Purulent drainage from the deep incision but not from the organ/space component of the surgical site.
2. A deep incision spontaneously dehisces or is deliberately opened by a surgeon when the patient has at least one of the following signs or symptoms: fever (>38°C), localized pain or tenderness, unless incision is culture-negative.
3. An abscess or other evidence of infection involving the deep incision is found on direct examination, during reoperation, or by histopathologic or radiologic examination.
4. Diagnosis of deep incisional SSI made by a surgeon or attending physician.

Organ/Space (SSI-O)

Infection occurs within 30 days after the operation if no implant is left in place or within 90 days if implant is in place and the infection appears to be related to the operation and infection involves any part of the anatomy (e.g. deep or organ / space SSI) other than the incision which was opened or manipulated during an operation and at least one of the following:

1. Purulent drainage from a drain that is placed through a stab wound into the organ/space.
2. Organisms isolated from an aseptically obtained culture of fluid or tissue in the organ/space.
3. An abscess or other evidence of infection involving the organ/space that is found on direct examination, during reoperation, or by histopathologic or radiologic examination.
4. Diagnosis of organ/space SSI made by a surgeon or attending physician.

UTI: URINARY TRACT INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

Catheter-associated UTI may develop any time after the device has been inserted

UTI-A: microbiologically confirmed symptomatic UTI

1.
   a. Patient has at least one of the following signs of symptoms with no other recognized cause: fever (>38°C), urgency, frequency, dysuria, or suprapubic tenderness

   And

   b. patient has a positive urine culture, that is, $\geq 10^5$ microorganisms per ml of urine with no more than two species of microorganisms.

UTI-B: not microbiologically confirmed symptomatic UTI

1. Patient has at least two of the following with no other recognized cause: fever (>38°C), urgency, frequency, dysuria, or suprapubic tenderness

And at least one of the following:

   a. Positive dipstick for leukocyte esterase and/or nitrate
   b. Pyuria urine specimen with ≥10 WBC/ml or ≥ 3 WBC/high-power field of unspun urine
   c. Organisms seen on Gram stain of unspun urine
   d. At least two urine cultures with repeated isolation of the same uropathogen (gram-negative bacteria or *S. saprophyticus*) with $\geq 10^2$ colonies/ml urine in nonvoided specimens
   e. $\leq 10^5$ colonies/ml of a single uropathogen (gram-negative bacteria or *S. saprophyticus*) in a patient being treated with effective antimicrobial agent for a urinary infection
   f. Physician diagnosis of a urinary tract infection
   g. Physician institutes appropriate therapy for a urinary infection

NOTE:
Asymptomatic bacteriuria are excluded from the survey. Bloodstream infections secondary to asymptomatic bacteriuria are reported as BSI with source (origin) S-UTI

Catheter-associated UTI

A UTI is defined as catheter-associated if a urinary catheter was present (even intermittently) in the 7 days preceding the onset of infection. This is recorded using the “device in situ prior to onset” field.

BJ: BONE AND JOINT INFECTION

ONSET: *Day 3 onwards*
Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

BJ-BONE: Osteomyelitis

Osteomyelitis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from bone.
2. Patient has evidence of osteomyelitis on direct examination of the bone during a surgical operation or histopathologic examination.
3. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), localized swelling, tenderness, heat, or drainage at suspected site of bone infection

   And at least 1 of the following:
   a. organisms cultured from blood
   b. positive blood antigen test (e.g., *H influenzae, S pneumoniae*)
   c. radiographic evidence of infection (e.g., abnormal findings on x-ray, CT scan, MRI, radiolabel scan [gallium, technetium, etc]).

Note:

- Report mediastinitis following cardiac surgery that is accompanied by osteomyelitis as surgical site infection-organ/space (SSI-O).

BJ-JNT: Joint or bursa

Joint or bursa infections must meet at least 1 of the following criteria:

1. Patient has organisms cultured from joint fluid or synovial biopsy.
2. Patient has evidence of joint or bursa infection seen during a surgical operation or histopathologic examination.
3. Patient has at least 2 of the following signs or symptoms with no other recognized cause: joint pain, swelling, tenderness, heat, evidence of effusion or limitation of motion

   And at least 1 of the following:
   a. organisms and white blood cells seen on Gram's stain of joint fluid
   b. positive antigen test on blood, urine, or joint fluid
   c. cellular profile and chemistries of joint fluid compatible with infection and not explained by an underlying rheumatologic disorder
   d. radiographic evidence of infection (e.g., abnormal findings on x-ray, CT scan, MRI, radiolabel scan [gallium, technetium, etc]).

BJ-DISC: Disc space infection

Vertebral disc space infection must meet at least 1 of the following criteria:

1. Patient has organisms cultured from vertebral disc space tissue obtained during a surgical operation or needle aspiration.
2. Patient has evidence of vertebral disc space infection seen during a surgical operation or histopathologic examination.
3.
    a. Patient has fever (>38°C) with no other recognized cause or pain at the involved vertebral disc space

    And

    b. Radiographic evidence of infection, (e.g., abnormal findings on x-ray, CT scan, MRI, radiolabel scan [gallium, technetium, etc]).
4.
    a. Patient has fever (>38°C) with no other recognized cause and pain at the involved vertebral disc space

    And

    b. Positive antigen test on blood or urine (e.g., *H influenzae, S pneumoniae, N meningitidis,* or Group B *Streptococcus*).

CNS: CENTRAL NERVOUS SYSTEM INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

CNS-IC: Intracranial infection (brain abscess, subdural or epidural infection, encephalitis)

Intracranial infection must meet at least 1 of the following criteria:

1. Patient has organisms cultured from brain tissue or dura.
2. Patient has an abscess or evidence of intracranial infection seen during a surgical operation or histopathologic examination.
3. Patient has at least 2 of the following signs or symptoms with no other recognized cause: headache, dizziness, fever (>38°C), localizing neurologic signs, changing level of consciousness, or confusion

   And at least 1 of the following:

   a. organisms seen on microscopic examination of brain or abscess tissue obtained by needle aspiration or by biopsy during a surgical operation or autopsy
   b. positive antigen test on blood or urine
   c. radiographic evidence of infection, (e.g., abnormal findings on ultrasound, CT scan, MRI, radionuclide brain scan, or arteriogram)
   d. diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen

   And

   Physician institutes appropriate antimicrobial therapy.

Note:
If meningitis and a brain abscess are present together, report the infection as ***CNS-IC***.

CNS-MEN: Meningitis or ventriculitis

Meningitis or ventriculitis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from cerebrospinal fluid (CSF).
2. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), headache, stiff neck, meningeal signs, cranial nerve signs, or irritability

   And at least 1 of the following:
   a. increased white cells, elevated protein, and/ or decreased glucose in CSF
   b. organisms seen on Gram's stain of CSF
   c. organisms cultured from blood
   d. positive antigen test of CSF, blood, or urine
   e. diagnostic single antibody titer (IgM) or 4-fold increase in paired sera (IgG) for pathogen

   And

   Physician institutes appropriate antimicrobial therapy.

Note:
Report CSF shunt infection as SSI if it occurs <=1 year of placement; if later or after manipulation/access of the shunt, report as ***CNS-MEN.***
Report meningoencephalitis as ***CNS-MEN.***
Report spinal abscess with meningitis as ***CNS-MEN.***

CNS-SA: Spinal abscess without meningitis

An abscess of the spinal epidural or subdural space, without involvement of the cerebrospinal fluid or adjacent bone structures, must meet at least 1 of the following criteria:

1. Patient has organisms cultured from abscess in the spinal epidural or subdural space.
2. Patient has an abscess in the spinal epidural or subdural space seen during a surgical operation or at autopsy or evidence of an abscess seen during a histopathologic examination.
3. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), back pain, focal tenderness, radiculitis, paraparesis, or paraplegia

   And at least 1 of the following:
   a. organisms cultured from blood
   b. radiographic evidence of a spinal abscess (e.g., abnormal findings on myelography, ultrasound, CT scan, MRI, or other scans [gallium, technetium, etc]).

   And

   Physician institutes appropriate antimicrobial therapy.

Note:
Report spinal abscess with meningitis as meningitis ***CNS-MEN***

CVS: CARDIOVASCULAR SYSTEM INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

CVS-VASC: Arterial or venous infection

Arterial or venous infection must meet at least 1 of the following criteria:

1.
   a. Patient has organisms cultured from arteries or veins removed during a surgical operation

   And

   b. blood culture not done or no organisms cultured from blood.

2. Patient has evidence of arterial or venous infection seen during a surgical operation or histopathologic examination.

3.
   a. Patient has at least 1 of the following signs or symptoms with no other recognised cause: fever (>38°C), pain, erythema, or heat at involved vascular site

   And

   b. more than 15 colonies cultured from intravascular cannula tip using semiquantitative culture method

   And

   c. blood culture not done or no organisms cultured from blood.

4.
   a. Patient has purulent drainage at involved vascular site

   And

   b. blood culture not done or no organisms cultured from blood.

Note:
Report infections of an arteriovenous graft, shunt, or fistula without organisms cultured from blood as CVS-VASC.

Report vascular catheter related infections without organisms cultured from blood as CRI1-CVC or CRI1-PVC or CRI2-CVC or CRI2-PVC.

Report vascular catheter related infections without organisms cultured from blood or the catheter tip as CVC-VASC.

An aid to assist with the diagnosis of catheter-related infections is provided in Figure 4.4.2.

CVS-ENDO: Endocarditis

Endocarditis of a natural or prosthetic heart valve must meet at least 1 of the following criteria:

1. Patient has organisms cultured from valve or vegetation.
2. Patient has 2 or more of the following signs or symptoms with no other recognized cause: fever (>38°C), new or changing murmur, embolic phenomena, skin manifestations (i.e., petechiae, splinter hemorrhages, painful subcutaneous nodules), congestive heart failure, or cardiac conduction abnormality

   And at least 1 of the following:

   a. organisms cultured from 2 or more blood cultures
   b. organisms seen on Gram's stain of valve when culture is negative or not done
   c. valvular vegetation seen during a surgical operation or autopsy
   d. positive antigen test on blood or urine (e.g., *H influenzae*, *S pneumoniae*, *N. meningitidis*, or Group B *Streptococcus*)
   e. evidence of new vegetation seen on echocardiogram

   And

   Physician institutes appropriate antimicrobial therapy.

CVS-CARD: Myocarditis or pericarditis

Myocarditis or pericarditis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from pericardial tissue or fluid obtained by needle aspiration or during a surgical operation.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), chest pain, paradoxical pulse, or increased heart size

   And at least 1 of the following:

   a. abnormal EKG consistent with myocarditis or pericarditis
   b. positive antigen test on blood (e.g., *H influenzae*, *S pneumoniae*)
   c. evidence of myocarditis or pericarditis on histologic examination of heart tissue
   d. 4-fold rise in type-specific antibody with or without isolation of virus from pharynx or faeces
   e. pericardial effusion identified by echocardiogram, CT scan, MRI, or angiography.

Note:
Most cases of postcardiac surgery or postmyocardial infarction pericarditis are not infectious.

CVS-MED: Mediastinitis

Mediastinitis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from mediastinal tissue or fluid obtained during a surgical operation or needle aspiration.
2. Patient has evidence of mediastinitis seen during a surgical operation or histopathologic examination.
3. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), chest pain, or sternal instability

   And at least 1 of the following:

   a. purulent discharge from mediastinal area
   b. organisms cultured from blood or discharge from mediastinal area
   c. mediastinal widening on x-ray.

Note:
Report mediastinitis following cardiac surgery that is accompanied by osteomyelitis as SSI-O

EENT: EYE, EAR, NOSE, THROAT, OR MOUTH INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

EENT-CONJ: Conjunctivitis

Conjunctivitis must meet at least 1 of the following criteria:

1. Patient has pathogens cultured from purulent exudate obtained from the conjunctiva or contiguous tissues, such as eyelid, cornea, meibomian glands, or lacrimal glands.
2. Patient has pain or redness of conjunctiva or around eye

   And at least 1 of the following:

   a. WBCs and organisms seen on Gram stain of exudate
   b. purulent exudate
   c. positive antigen test (e.g., ELISA or IF for *Chlamydia trachomatis*, herpes simplex virus, adenovirus) on exudate or conjunctival scraping
   d. multinucleated giant cells seen on microscopic examination of conjunctival exudate or scrapings
   e. positive viral culture
   f. diagnostic single antibody titer (IgM) or 4-fold increase in paired sera (IgG) for pathogen.

Note:
Report other infections of the eye as EYE.
Do not report chemical conjunctivitis caused by silver nitrate ($AgNO_3$) as a health care–associated infection.
Do not report conjunctivitis that occurs as a part of a more widely disseminated viral illness (such as measles, chickenpox, or a URI).

EENT-EYE: Eye, other than conjunctivitis

An infection of the eye, other than conjunctivitis, must meet at least 1 of the following criteria:
1. Patient has organisms cultured from anterior or posterior chamber or vitreous fluid.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: eye pain, visual disturbance, or hypopyon

   And at least 1 of the following:

   a. physician diagnosis of an eye infection
   b. positive antigen test on blood (e.g., *H influenzae, S pneumoniae*)
   c. organisms cultured from blood.

EENT-EAR: Ear mastoid

Ear and mastoid infections must meet at least 1 of the following criteria:

Otitis externa must meet at least 1 of the following criteria:

1. Patient has pathogens cultured from purulent drainage from ear canal.
2.
    a. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), pain, redness, or drainage from ear canal

    And

    b. Organisms seen on Gram's stain of purulent drainage.

Otitis media must meet at least 1 of the following criteria:

1. Patient has organisms cultured from fluid from middle ear obtained by tympanocentesis or at surgical operation.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), pain in the eardrum, inflammation, retraction or decreased mobility of eardrum, or fluid behind eardrum.

Otitis interna must meet at least 1 of the following criteria:

1. Patient has organisms cultured from fluid from inner ear obtained at surgical operation.
2. Patient has a physician diagnosis of inner ear infection.

Mastoiditis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from purulent drainage from mastoid.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), pain, tenderness, erythema, headache, or facial paralysis

    And at least 1 of the following:
    a. organisms seen on Gram's stain of purulent material from mastoid
    b. positive antigen test on blood.

EENT-ORAL: Oral cavity (mouth, tongue, or gums)
Oral cavity infections must meet at least 1 of the following criteria:

1. Patient has organisms cultured from purulent material from tissues of oral cavity.
2. Patient has an abscess or other evidence of oral cavity infection seen on direct examination, during a surgical operation, or during a histopathologic examination.
3. Patient has at least 1 of the following signs or symptoms with no other recognized cause: abscess, ulceration, or raised white patches on inflamed mucosa, or plaques on oral mucosa

   And at least 1 of the following:
   a. organisms seen on Gram stain
   b. positive KOH (potassium hydroxide) stain
   c. multinucleated giant cells seen on microscopic examination of mucosal scrapings
   d. positive antigen test on oral secretions
   e. diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen
   f. physician diagnosis of infection and treatment with topical or oral antifungal therapy.

Note:
Report health care–associated primary herpes simplex infections of the oral cavity as ***EENTORAL***; recurrent herpes infections are not healthcare–associated.

EENT-SINU**:** Sinusitis
Sinusitis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from purulent material obtained from sinus cavity.
2. Patient has at least 1 of the following signs or symptoms with no other recognized cause: fever (>38°C), pain or tenderness over the involved sinus, headache, purulent exudate, or nasal obstruction

   And at least 1 of the following:
   a. positive transillumination
   b. positive radiographic examination (including CT scan).

EENT-UR: Upper respiratory tract, pharyngitis, laryngitis, epiglottitis
Upper respiratory tract infections must meet at least 1 of the following criteria:

1. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), erythema of pharynx, sore throat, cough, hoarseness, or purulent exudate in throat

   And at least 1 of the following:
   a. organisms cultured from the specific site
   b. organisms cultured from blood
   c. positive antigen test on blood or respiratory secretions

d. diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen
e. physician diagnosis of an upper respiratory infection.

2. Patient has an abscess seen on direct examination, during a surgical operation, or during a histopathologic examination.

LRI: LOWER RESPIRATORY TRACT INFECTION, OTHER THAN PNEUMONIA

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

LRI-BRON: Bronchitis, tracheobronchitis, bronchiolitis, tracheitis, without evidence of pneumonia

Tracheobronchial infections must meet at least 1 of the following criteria:

1.
   a. Patient has no clinical or radiographic evidence of pneumonia

   And

   b. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), cough, new or increased sputum production, rhonchi, wheezing

And at least 1 of the following:
   a. positive culture obtained by deep tracheal aspirate or bronchoscopy
   b. positive antigen test on respiratory secretions.

Note:
Do not report chronic bronchitis in a patient with chronic lung disease as an infection unless there is evidence of an acute secondary infection, manifested by change in organism.

LRI-LUNG: Other infections of the lower respiratory tract

Other infections of the lower respiratory tract must meet at least 1 of the following criteria:

1. Patient has organisms seen on smear or cultured from lung tissue or fluid, including pleural fluid.
2. Patient has a lung abscess or empyema seen during a surgical operation or histopathologic examination.
3. Patient has an abscess cavity seen on radiographic examination of lung.

Note:
Report lung abscess or empyema without pneumonia as ***LRI- LUNG.***

GI: GASTROINTESTINAL TRACT INFECTION. OTHER THAN GASTROENTERITIS

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

GI-GIT: Gastrointestinal tract (oesophagus, stomach, small and large bowel, and rectum) excluding gastroenteritis and appendicitis

Gastrointestinal tract infections, excluding gastroenteritis and appendicitis, must meet at least 1 of the following criteria:

1. Patient has an abscess or other evidence of infection seen during a surgical operation or histopathologic examination.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause and compatible with infection of the organ or tissue involved: fever (>38°C), nausea, vomiting, abdominal pain, or tenderness

   And at least 1 of the following:

   a. organisms cultured from drainage or tissue obtained during a surgical operation or endoscopy or from a surgically placed drain
   b. organisms seen on Gram's or KOH stain or multinucleated giant cells seen on microscopic examination of drainage or tissue obtained during a surgical operation or endoscopy or from a surgically placed drain
   c. organisms cultured from blood
   d. evidence of pathologic findings on radiographic examination
   e. evidence of pathologic findings on endoscopic examination (e.g., Candida esophagitis or proctitis).

GI-HEP: Hepatitis

Hepatitis must meet the following critera:

1. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), anorexia, nausea, vomiting, abdominal pain, jaundice, or history of transfusion within the previous 3 months

   And at least 1 of the following:
   a. positive antigen or antibody test for hepatitis A, hepatitis B, hepatitis C, or delta
   b. hepatitis
   c. abnormal liver function tests (e.g., elevated ALT/ AST, bilirubin)
   d. cytomegalovirus (CMV) detected in urine or oropharyngeal secretions.

Note:
Do not report hepatitis or jaundice of non-infectious origin (alpha-1 antitrypsin deficiency, etc).

Do not report hepatitis or jaundice that results from exposure to hepatotoxins (alcoholic or acetaminophen-induced hepatitis, etc).

Do not report hepatitis or jaundice that results from biliary obstruction (cholecystitis).

GI-IAB: Intraabdominal, not specified elsewhere including gallbladder, bile ducts, liver (excluding viral hepatitis), spleen, pancreas, peritoneum, subphrenic or subdiaphragmatic space, or other intraabdominal tissue or area not specified elsewhere

Intraabdominal infections must meet at least 1 of the following criteria:

1. Patient has organisms cultured from purulent material from intraabdominal space obtained during a surgical operation or needle aspiration.
2. Patient has abscess or other evidence of intraabdominal infection seen during a surgical operation or histopathologic examination.
3. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), nausea, vomiting, abdominal pain, or jaundice

And at least 1 of the following:

   a. organisms cultured from drainage from surgically placed drain (e.g., closed suction drainage system, open drain, T-tube drain)
   b. organisms seen on Gram stain of drainage or tissue obtained during surgical operation or needle aspiration
   c. organisms cultured from blood and radiographic evidence of infection (e.g., abnormal findings on ultrasound, CT scan, MRI, or radiolabel scans [gallium, technetium, etc] or on abdominal x-ray).

Note:
Do not report pancreatitis (an inflammatory syndrome characterized by abdominal pain, nausea, and vomiting associated with high serum levels of pancreatic enzymes) unless it is determined to be infectious in origin.

REPR: REPRODUCTIVE TRACT INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

REPR-EMET: Endometritis

Endometritis must meet at least 1 of the following criteria:

1. Patient has organisms cultured from fluid or tissue from endometrium obtained during surgical operation, by needle aspiration, or by brush biopsy.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), abdominal pain, uterine tenderness, or purulent drainage from uterus.

Note:
Report postpartum endometritis as a health care–associated infection unless the amniotic fluid is infected at the time of admission or the patient was admitted 48 hours after rupture of the membrane.

REPR-EPIS: Episiotomy

Episiotomy infections must meet at least 1 of the following criteria:

1. Postvaginal delivery patient has purulent drainage from the episiotomy.
2. Postvaginal delivery patient has an episiotomy abscess.

REPR-VCUF: Vaginal cuff

Vaginal cuff infections must meet at least 1 of the following criteria:

1. Posthysterectomy patient has purulent drainage from the vaginal cuff.
2. Posthysterectomy patient has an abscess at the vaginal cuff.
3. Posthysterectomy patient has pathogens cultured from fluid or tissue obtained from the vaginal cuff.

Note:
Report vaginal cuff infections as ***SSI-O*** if occurring within 30 days of surgery.

REPR-OREP: Other infections of the male or female reproductive tract (epididymis, testes, prostate, vagina, ovaries, uterus, or other deep pelvic tissues, excluding endometritis or vaginal cuff infections)

Other infections of the male or female reproductive tract must meet at least 1 of the following criteria:

1. Patient has organisms cultured from tissue or fluid from affected site.
2. Patient has an abscess or other evidence of infection of affected site seen during a surgical operation or histopathologic examination.
3. Patient has 2 of the following signs or symptoms with no other recognized cause: fever (>38°C), nausea, vomiting, pain, tenderness, or dysuria

   And at least 1 of the following:
   a. organisms cultured from blood
   b. physician diagnosis.

Note:
Report endometritis as ***REPR-EMET***.

Report vaginal cuff infections as ***REPR-VCUF.***

SST: OTHER SKIN AND SOFT TISSUE INFECTIONS, OTHER THAN SOFT TISSUE AND DECUBITUS ULCER

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

SST-SKIN: Skin infection

Skin infections must meet at least 1 of the following criteria:

1. Patient has purulent drainage, pustules, vesicles, or boils.
2. Patient has at least 2 of the following signs or symptoms with no other recognized cause: pain or tenderness, localized swelling, redness, or heat

And at least 1 of the following:

   a. organisms cultured from aspirate or drainage from affected site; if organisms are normal skin flora (ie, diphtheroids [*Corynebacterium* spp], *Bacillus* [not *B anthracis*] spp, P*ropionibacterium* spp, coagulase-negative staphylococci [including *S epidermidis*], viridans group streptococci, *Aerococcus* spp, *Micrococcus* spp), they must be a pure culture
   b. organisms cultured from blood
   c. positive antigen test performed on infected tissue or blood (e.g., herpes simplex, varicella zoster, *H influenzae*, *N meningitidis*)
   d. multinucleated giant cells seen on microscopic examination of affected tissue
   e. diagnostic single antibody titer (IgM) or 4fold increase in paired sera (IgG) for pathogen.

Note:
Report infected decubitus ulcers as ***DECU-1*** or ***DECU-2***.

Report infected burns as ***BURN***.

Report breast abscesses or mastitis as ***BRST***.

SST-BURN: Burn

Burn infections must meet at least 1 of the following criteria:

1.
   a. Patient has a change in burn wound appearance or character, such as rapid eschar separation, or dark brown, black, or violaceous discoloration of the eschar, or oedema at wound margin

   And

   b. histologic examination of burn biopsy shows invasion of organisms into adjacent viable tissue.

2. Patient has a change in burn wound appearance or character, such as rapid eschar separation, or dark brown, black, or violaceous discoloration of the eschar, or edema at wound margin

   And at least 1 of the following:

   a. organisms cultured from blood in the absence of other identifiable infection
   b. isolation of herpes simplex virus, histologic identification of inclusions by light or electron microscopy, or visualization of viral particles by electron microscopy in biopsies or lesion scrapings.

3. Patient with a burn has at least 2 of the following signs or symptoms with no other recognized cause: fever (>38°C) or hypothermia (< 36°C), hypotension, oliguria (< 20 cc/hr), hyperglycemia at previously tolerated level of dietary carbohydrate, or mental confusion

   And at least 1 of the following:

   a. histologic examination of burn biopsy shows invasion of organisms into adjacent viable tissue
   b. organisms cultured from blood
   c. isolation of herpes simplex virus, histologic identification of inclusions by light or electron microscopy, or visualization of viral particles by electron microscopy in biopsies or lesion scrapings.

Notes:
Purulence alone at the burn wound site is not adequate for the diagnosis of burn infection; such purulence may reflect incomplete wound care.

Fever alone in a burn patient is not adequate for the diagnosis of a burn infection because fever may be the result of tissue trauma or the patient may have an infection at another site.

Surgeons in Regional Burn Centres who take care of burn patients exclusively may require Criterion 1 for diagnosis of burn infection.

Hospitals with Regional Burn Centres may further divide burn infections into the following: burn wound site, burn graft site, burn donor site, burn donor site-cadaver; NHSN, however, will code all of these as BURN.

***SST-BRST*:** Breast abscess or mastitis

A breast abscess or mastitis must meet at least 1 of the following criteria:

1. Patient has a positive culture of affected breast tissue or fluid obtained by incision and drainage or needle aspiration.
2. Patient has a breast abscess or other evidence of infection seen during a surgical operation or histopathologic examination.
3.
    a. Patient has fever (>38°C) and local inflammation of the breast

    And

    b. physician diagnosis of breast abscess.

Note:
Breast abscesses occur most frequently after childbirth. Those that occur within 7 days after childbirth should be considered healthcare associated.

SYS: SYSTEMIC INFECTION

ONSET: *Day 3 onwards*

Present on admission or developing on Day 1 or 2 of admission in patients that have been discharged from hospital, acute or non-acute, in the 48 hours prior to admission.

SYS-DI: Disseminated infection

Disseminated infection is infection involving multiple organs or systems, without an apparent single site of infection, usually of viral origin, and with signs or symptoms with no other recognized cause and compatible with infectious involvement of multiple organs or systems.

Note:
Use this code for viral infections involving multiple organ systems (e.g., measles, mumps, rubella, varicella, erythema infectiosum). These infections often can be identified by clinical criteria alone.
Do not use this code for healthcare–associated infections with multiple metastatic sites, such as with bacterial endocarditis; only the primary site of these infections should be reported.

Do not report fever of unknown origin (FUO) as DI.

Report viral exanthems or rash illness as DI.

SYS-CSEP: Treated unidentified severe infection

1. Patient has at least one of the following clinical signs or symptoms with no other recognised cause
    a. fever (38°C)
    b. hypotension (systolic pressure <90 mm),
    c. or oliguria (20 cm^3(ml)/hr)

    And

2. Blood culture not done or no organisms or antigen detected in blood

    And

3. With no apparent infection at another site

    And

4. Physician institutes treatment for sepsis

Note:

- **Do not use this code unless absolutely needed (last resort definition)**
- For ***SYS-CSEP*** in neonates, use ***NEO-CSEP*** case definition (see below)

Appendix 3. Origin of Infection (mandatory enhanced surveillance protocol)

Hospital acquired infection (HAI): Positive blood culture obtained from a patient who has been hospitalised for ≥48 hours. If the patient was transferred from another hospital, the duration of in-patient stay is calculated from the date of the first hospital admission. If the patient was a neonate/baby who has never left hospital since being born.

OR

The patient was discharged from hospital in the 48hr prior to the positive blood culture being taken.

OR

A patient who receives regular haemodialysis as an out-patient.

OR

Contaminant if the blood aspirated in hospital.

OR

If infection source/entry point is surgical site infection (SSI).

Healthcare associated infection (HCAI): Positive blood culture obtained from a patient within 48 hours of admission to hospital and fulfils one or more of the following criteria:

Was hospitalised overnight in the 30 days prior to the positive blood culture being taken.

OR

Resides in a nursing, long term care facility or residential home.

OR

IV, or intra-articular medication in the 30 days prior to the positive blood culture being taken, but **excluding IV illicit drug use**.

OR

Had the use of a registered medical device in the 30 days prior to the positive blood culture being taken e.g. intermittent self-catheterisation or percutaneous endoscopic gastrostomy (PEG) tube with or without the direct involvement of a healthcare worker (excludes haemodialysis lines see HAI).

OR

Underwent any medical procedure which broke mucous or skin barrier i.e. biopsies or dental extraction in the 30 days prior to the positive blood culture being taken.

OR

Underwent care for a medical condition by a healthcare worker in the community which involved contact with non-intact skin, mucous membranes or the use of an invasive device in the 30 days prior to the positive blood culture being taken e.g. podiatry or dressing of chronic ulcers, catheter change or insertion.

Community infection: Positive blood culture obtained from a patient within 48 hours of admission to hospital who does not fulfil any of the criteria for healthcare associated bloodstream infection.

Not known: Only to be used if the bacteraemia is not an HAI, and unable to determine if Community or HCAI

From: Bowman D (David)
Sent: 14 March 2019 15:24:53
To: Public Engagement Unit
Cc: Cabinet Secretary for Health and Sport
Subject: FW: [redacted]

AO ? Charlotte Jack

PEU

Please could you scan this on to MACCs as an OR.

Thanks

David Bowman
Deputy Private Secretary
Ministerial Private Office (Health)
St Andrew?s House
Edinburgh

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From: Beth Armstrong
<beth.armstrong100[redacted]
Sent: 14 March 2019 14:51
To: Cabinet Secretary for Health and Sport
[redacted]
Subject: Fw: [redacted]

Dear Jeanne Freeman OBE,

I am forwarding an email to you that was sent by me today on behalf of my family regarding the death of my [redacted], at the QEU Hospital on 7th January 2019.

[redacted] cause of death was recorded as Lymphoma which was diagnosed just over 2 years ago. [redacted]r physical deterioration was rapid following the unfortunate diagnosis of Cryptococcus in December, and understandibly the family has many questions regarding the impact of this on [redacted] length of life, her treatment and her death.

We have been frustrated by the lack of communication from the hospital, and the innacuracies contained within the press releases causing further distress to us during this difficult time. The details of this are contained in my email below.

I understand that there are currently 3 inquiries being undertaken by the hospital, the Procurator Fiscal and the Scottish Parliament. We have received little communication

from the hospital during it's inquiry - the family were not interviewed and as you will see from my email to Teresa Inkster, we were not even informed of it's publication despite being reassured that we would be. The PF, in contrast, has conducted lengthy interviews with key family members and have been keeping in touch with us by phone to inform us of any developments with their inquiry.

I am writing to you to inform you of our disappointment with the way that the QEU management has handled this very distressing situation, and also to request that we are kept informed of the parliamentary inquiry, including being available to give statements or interviews regarding our experiences at the QEU following ████ death. As I have stated below, we have no complaints regarding the excellent care my mother received for her lymphoma. We are huge supporters of the NHS and will do anything that we can to assist your enquiry to ensure that no other families have to go through this again.

Yours Sincerely,
Beth Armstrong

----- Forwarded Message -----
From: Beth Armstrong
<beth.armstrong100████
To: Teresa Inkster
<teresa.inkster████
Cc: Alistair Hart
████; Ian
Macdonald
<ian.macdonald████
Sent: Wednesday, March 13, 2019, 5:35:11 PM GMT
Subject: Re: ████

Dear Dr Inskter,

I?m getting back in touch following a family meeting to consider all the issues raised in the wake of ████ death to focus on the principal issues.

Firstly, the family feels it would be helpful if only one person corresponded with various agencies who have contacted the family. Accordingly, I have agreed to act as the principal point of contact. I will speak regularly with all family members and copy them into emails and other discussions.

Secondly, the family feels it would be of benefit for a Family Liaison Officer (FLO) to be appointed. I understand the hospital is keen to facilitate that process. Can I leave it to you to forward a copy of this email to any FLO appointed and ask that they contact me direct?

Thirdly, the family would like to record its appreciation of the hard work and dedication by the medical and nursing staff at the hospital. There can be no criticism of the care and treatment that ████ received for her lymphoma. It would have been better that ████ final months were not complicated by infection arising from Cryptococcus and the family still has a number of unanswered questions regarding the circumstances in which Cryptococcus spores managed to infiltrate a sterile area and the impact this had on ████ treatment, her length of life and ultimately her death.

Clearly, there were issues regarding the presence of pigeons on the roof overlooked by the 4th floor and also a hole in the 12th floor roof. These potential breaches of the sterile integrity of the hospital were presumably risks addressed by the people responsible for building maintenance. Is the hospital?s risk assessment policy

document clear in identifying such risks and the steps to be taken to address those risks?

In terms of the press releases and the hospital?s failure to communicate effectively with the family, a couple of concerns arose at the meeting. As we have previously discussed, the press releases were inaccurate and distressing in terms of wrongly detailing [redacted] discharge history and also the uncomplimentary reference to elderliness. Further, the press releases were issued without reference to any family members and in the absence of consultation and had a consequence in terms of stress to the grieving. When we last spoke I requested that we should be kept informed on any developments with the inquiry, and also any further press releases in order to minimise the distress caused.

We were therefore very disappointed to read an article in the Independent ?i? newspaper on Saturday 9th March ?Superhospital Criticised For Failings On Cleanliness? that the HIS report was released on Friday 8th March. Is this the hospital inquiry that we have previously discussed? My recollection of our last conversation was that we would be informed before the report came out and the families involved would be sent it before it was publicly released. The article also raises significant questions regarding hospital management not reacting ?to staff concerns about the patients? environment?. Were such concerns raised about [redacted] environment and not reacted to? I would be very grateful if you could forward a copy of the report to me along with any further press releases.

I would appreciate a response either directly from yourself, or via a FLO on the points raised above and hope that the hospital will work to improve its communication with us on such matters moving forward.

Kind regards

Beth Armstrong

CC Dr Alistair Hart

Dr Iain MacDonald

Jeane Freeman MSP

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From: Goodfellow M (Melanie) on behalf of AMR/HCAI
To: beth.armstrong[redacted]
Subject: MACCS reply
Attachments: MACCS - 20190008417 - reply.pdf

Dear Ms Armstrong

Please find our reply to your recent correspondence attached above.

Kind regards

Melanie Goodfellow | Policy Manager: Healthcare Associated Infections/Antimicrobial Resistance and Excellence in Care | Chief Nursing Officer's Directorate | The Scottish Government | 2ER | St Andrew's House | Regent Road | Edinburgh | EH1 3DG

Chief Nursing Officer Directorate
Diane Murray, Associate Chief Nursing Officer

T: [redacted]
E: diane.murray[redacted]

Scottish Government
Riaghaltas na h-Alba
gov.scot

Beth Armstrong
beth.armstrong[redacted]

Our ref: 2019/0008417
11 April 2019

Dear Ms Armstrong,

I was very sorry to read your email of 14 March 2019 addressed to Jeane Freeman MSP, the Cabinet Secretary for Health and Sport, regarding the death of your [redacted] while she was being cared for at the Queen Elizabeth University Hospital (QEUH), NHS Greater Glasgow and Clyde (NHSGGC). As Associate Chief Nursing Officer for Scotland, I wanted to respond to you personally.

I would like to start by offering my sincere condolences to you and your family for the loss of your [redacted]. I fully understand that this will be a very distressing time for you all and I would like to reassure you that both Ms Freeman and I take this matter very seriously.

I am sorry that NHSGGC has not communicated with you and your family in the way you had expected. To ensure effective future communication, I have contacted NHSGGC and received assurance from the Board that a dedicated point of contact is being arranged and I expect the Board to provide you with regular updates and information relating to the internal investigations. Indeed I would expect the Board to follow guidance on reviewing adverse events and that you would be involved in the investigation. Ms Freeman and I also continue to receive updates from the Board to ensure patients, families and visitors at the hospital are safe.

Separately to the reports published by Health Protection Scotland (HPS) and the Healthcare Environment Inspectorate (HEI), you are aware that Ms Freeman asked for an independent review to consider the design, commissioning, construction, handover and maintenance of the QEUH estate.

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Earlier this month, Ms Freeman announced that Dr Andrew Fraser and Dr Brian Montgomery would co-chair this independent expert review. The co-chairs are currently establishing their team and identifying how they will seek the information needed to ensure a robust, evidence-based assessment. It will be very important that families such as yours have the opportunity to have their views heard, and the co-chairs will establish and publicise, in due course, how this information will be collected.

Finally, I noticed, and was grateful for your praise of the clinical teams who cared for your [redacted] and I appreciate that in this difficult period you were able to recognise the skill and dedication of her doctors and nurses.

I hope I have reassured you about the independent review process and that every effort is being made to ensure the QEUH provides safe, effective and person-centred care as well as learning lessons for the future.

I appreciate you have questions to be answered and I expect that the team at NHSGGC will keep you involved and informed of their investigation so that you have all the information you need. If you have any further questions, please don’t hesitate to contact me.

Yours sincerely

[redacted]

DIANE MURRAY

Associate Chief Nursing Officer

St Andrew’s House, Regent Road, Edinburgh EH1 3DG
www.gov.scot

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POSITIVE ABOUT DISABLED PEOPLE

From: Goodfellow M (Melanie) on behalf of AMR/HCAI
To: Beth Armstrong
Subject: RE: MACCS reply
Attachments: image003.png
HEI inspection report - QEUH - 2019.pdf
HPS water contamination report - QEUH - 2019.pdf

SENT ON BEHALF OF DIANE MURRAY, ASSOCIATE CHIEF NURSING OFFICER

Dear Ms Armstrong,

Thank you for your response to my letter of 11 April 2019. I am pleased to hear that the information I provided has been useful and that you now have a dedicated point of contact within the health board. However, I am sorry to hear that some of your concerns have not been addressed and following a discussion with the Executive Nurse Director for NHS Greater Glasgow and Clyde I have been assured that she will investigate these for you and contact you directly to address your concerns.

I can confirm that the Health Protection Scotland and the Healthcare Environment Inspectorate reports have now been published. On 22 February 2019, the Scottish Government published Health Protection Scotland's report into the water contamination issue at the Royal Hospital for Children, located on the Queen Elizabeth University Hospital site. Following this, on 8 March 2019, the Healthcare Environment Inspectorate published their safety and cleanliness inspection report of the Queen Elizabeth University Hospital site. I have attached a copy of both of these reports above for your information.

Both of these reports are separate to the external independent review that was announced by the Cabinet Secretary in January 2019. I would like to reiterate again how important it is that you and your family have an opportunity to contribute to this.

The reviews and reports that I have mentioned above are also separate to the board's internal review that is being undertaken by the board and I have asked that you are kept fully informed on the progress of this.

I hope that this information has helped to answer your questions and has provided clarity on the published reports. However, if you or your family do have any further enquiries please don't hesitate to contact me.

Yours sincerely

DIANE MURRAY

From: Beth Armstrong [redacted]
Sent: 14 April 2019 12:36
To: AMR/HCAI <HAI_Policy_Unit@[redacted]
Subject: Re: MACCS reply

Dear Ms Murray,
Thank you very much for your letter of 11th April ref: 2019/0008417 which I have forwarded to other family

members to keep them informed. It's very useful to have a little more information regarding the process of the various enquiries, and reassuring to know that at some point our family will have an opportunity to relate our experiences at the QEUH, as well as my mother's experience following her diagnosis of Cryptochoccus.

The QEUH did finally appoint us a point of contact for family liaison, Jennifer Haynes, jennifer.haynes[redacted] but despite her willingness to help us it appears that she doesn't have any authority to answer any of our questions, and so thus far it has made no difference to the situation for us.

Are you able to clarify for us if both of the HPS and HEI reports are now complete? We were informed at the time of my mother's death that we would be sent a copy of the hospital's internal inquiry ahead of it's publication (as well as any press releases), but neither have been forthcoming. Despite asking repeatedly I still haven't been able to establish if either of these inquiries are, in fact, the internal inquiry that we were told about. Are you able to clarify this for me? Also, are you able to send me the reports?

Thank you for your kind condolences, and we look forward to being kept informed of the progress of the Scottish Government independent inquiry and having the opportunity to be part of it. If there are any additional questions arising from your letter once my family has had a chance to read it I will get back to you.

Kind Regards,
Beth Armstrong

On Thursday, April 11, 2019, 8:37:06 AM GMT+1, HAI Policy Unit[redacted] wrote:

Dear Ms Armstrong

Please find our reply to your recent correspondence attached above.

Kind regards

Melanie Goodfellow | Policy Manager: Healthcare Associated Infections/Antimicrobial Resistance and Excellence in Care | Chief Nursing Officer's Directorate | The Scottish Government | 2ER | St Andrew's House | Regent Road | Edinburgh | EH1 3DG

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Dh'fhaodadh gum bi teachdaireachd sam bith bho Riaghaltas na h-Alba air a chlàradh neo air a sgrùdadh airson dearbhadh gu bheil an siostam ag obair gu h-èifeachdach neo airson adhbhar laghail eile. Dh'fhaodadh nach eil beachdan anns a' phost-d seo co-ionann ri beachdan Riaghaltas na h-Alba.
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Healthcare Improvement Scotland | Inspections and reviews
To drive improvement

Unannounced Inspection Report

Acute Hospital Safe Delivery of Care Inspection

The Queen Elizabeth University Hospital campus
NHS Greater Glasgow and Clyde

22 – 24 March 2022

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About our inspection

1. In December 2021, the Scottish Government commissioned Healthcare Improvement Scotland (HIS) to provide wider independent assurance of infection prevention and control measures at the Queen Elizabeth University Hospital campus, NHS Greater Glasgow and Clyde. This wider independent assurance will focus on systems and processes in place for infection prevention and control, including their implementation and to assess and determine if there are any current broader concerns requiring action.
2. We attempted to undertake an independent and unannounced inspection of infection prevention and control measures at the Queen Elizabeth University Hospital campus on Tuesday 22 March 2022. However, due to the unprecedented pressures being experienced throughout the hospital campus at the time, we made a decision to postpone the more detailed inspection and revert to our safe delivery of care inspection methodology. The safe delivery of care inspection methodology is designed to take account of changing risk considerations and sustained service pressures associated with the COVID-19 pandemic. We will return at a later date to undertake a full and wider independent assurance of infection prevention and control measures at the Queen Elizabeth University Hospital campus.
3. Further information about the methodology for safe delivery of care inspections can be found on our website.

Background

4. All of Healthcare Improvement Scotland's inspection programmes have been adapted during the COVID-19 pandemic. Since the beginning of 2021, we have been carrying out COVID-19 focused inspections of acute hospitals, using methodology adapted from our previous 'safe and clean' inspections.
5. Taking account of the changing risk considerations and sustained service pressures, in November 2021, the Cabinet Secretary for Health and Social Care approved further adaptations to our inspections of acute hospitals across NHS Scotland to focus on the safe delivery of care. To minimise the impact of our inspections on staff delivering care to patients, our inspection teams are carrying out as much of their inspection activities as possible through observation of care and virtual discussion sessions with senior managers. We will keep discussions with clinical staff to a minimum and reduce the time spent looking at care records.

Our focus

6. Our inspections consider the factors that contribute to the safe delivery of care. In order to achieve this, we:

- observe the delivery of care within the clinical areas in line with current standards and best practice
- attend hospital safety huddles
- engage with staff where possible, being mindful not to impact on the delivery of care
- engage with management to understand current pressures and assess the compliance with the NHS board policies and procedures, best practice statements or national standards, and
- report on the standards achieved on the day of our inspection and ensure the NHS board produces an action plan to address the areas for improvement identified.

About the hospital we inspected

7. The Queen Elizabeth University Hospital, Glasgow, opened in April 2015. The campus has 1,860 beds with a full range of healthcare specialities, including a major emergency department. In addition to the 14-floor hospital building, the hospital campus retains a number of other services in adjacent facilities. This includes maternity services, the Royal Hospital for Children, the Institute of Neurological Sciences, and the Langlands Building for medicine of the elderly and rehabilitation.

About this inspection

8. We carried out an unannounced inspection of the Queen Elizabeth University Hospital campus on Tuesday 22 – Thursday 24 March 2022.

9. In the **Queen Elizabeth University Hospital,** we inspected the following areas:

   - acute receiving medicine for the elderly (ARU 4)
   - emergency department
   - immediate assessment unit
   - high dependency unit (critical care unit 1)
   - specialist assessment and treatment area (SATA)
   - ward 5A, 5B, 5C, 5D, and
   - ward 6C, 8A, 8D, 9A, 9D, 10B.

10. In the **Institute of Neurological Sciences**, we inspected the following areas:

    - ward 66 and 67.

11. In the **Langlands building**, we inspected the following areas:

    - ward 57.

12. In the **maternity unit**, we inspected the following areas:

- ward 49.

13. In the **Royal Hospital for Children**, we inspected the following areas:

    - emergency department, and
    - ward 3C.

14. During our inspection, we:

    - inspected the ward and hospital environment
    - observed staff practice and interactions with patients, such as during patient mealtimes
    - spoke with ward staff (where appropriate), and
    - accessed patients' health records, monitoring reports, policies and procedures.

15. As part of our inspection, we also asked NHS Greater Glasgow and Clyde to provide evidence of its policies and procedures relevant to this inspection. The purpose of this is to limit the time the inspection team is onsite, reduce the burden on staff and inform the virtual discussion session.

16. On Monday 28 March 2022, we held a virtual discussion session with key members of NHS Greater Glasgow and Clyde staff to discuss the evidence provided and the findings of the inspection.

17. On Wednesday 13 April 2022, we carried out a follow up visit. This included a visit to the special assessment and treatment area (SATA) to ensure concerns raised during the inspection had been addressed.

18. The findings detailed within this report relate to our observations of the areas we inspected at the time of this inspection.

19. We would like to thank NHS Greater Glasgow and Clyde and, in particular, all staff at the Queen Elizabeth University Hospital campus for their assistance during our inspection.

A summary of our findings

20. Our summary findings from the inspection, areas of good practice and any requirements identified are highlighted as follows. Detailed findings from the inspection are included in the section 'What we found during this inspection'.

21. At the time of inspection, the Queen Elizabeth University Hospital campus, like much of NHS Scotland, was experiencing a significant range of pressures associated with COVID-19, including increased hospital admissions, increased waiting times in emergency departments and reduced staff availability.

22. All hospital sites in NHS Scotland are required to submit a daily situation report to the Scottish Government by 11.00am each day. On the first day of inspection, the hospital's daily site situation report detailed the extent of the pressures across the hospital campus. This included:

    - high staff absence due to sickness and requirements to self-isolate
    - long patient waiting times in the emergency department, and
    - a high hospital occupancy rate with just over 5% available 'empty beds'.

23. There were 33 wards across the hospital campus, scoring a nurse staffing risk rating of red at the start of the day. This means that nursing staff numbers or skill mix may be creating a risk to patient safety, or there are issues affecting patient safety that requires immediate attention. Further detail on how this assessment is made is provided within the report.

24. The evening before our inspection, the emergency department had put 'a divert' in place for 1 hour. Patients were redirected to another nearby emergency department to allow the Queen Elizabeth University Hospital to reduce the pressure in their emergency department.

25. During our inspection, we observed lead nurses, site managers and chief nurses working together, communicating and problem solving to try to reduce the identified risks and improve safety. However, the volume of staff absence meant that even with attempts to mitigate the risks, many clinical areas continued to score a red risk rating.

26. We found that some areas within the hospital campus were working with a less than optimum level of staffing due to staff absences and lack of available supplementary staff.

27. We observed multidisciplinary staff in clinical areas working very hard to ensure the patients were well cared for and their care needs were met. There was good leadership directing and supporting the staff teams in many areas. The

majority of patients we spoke with described the care they received as very good, with high admiration for the staff delivering the care.

28. Our inspection findings highlighted requirements and areas for improvement. However, we recognise the unprecedented pressures on NHS Scotland. We observed the effects of these pressures at the Queen Elizabeth University Hospital campus during our inspection as the multidisciplinary team worked collaboratively to provide care.

What action we expect the NHS board to take after our inspection

29. This inspection resulted in six areas of good practice and five requirements.

30. We expect NHS Greater Glasgow and Clyde to address the requirements. The NHS board must prioritise the requirements to meet national standards. An improvement action plan has been developed by the NHS board and is available on the Healthcare Improvement Scotland website: www.healthcareimprovementscotland.org

Areas of good practice

Domain 1	
1	There was a dedicated respiratory pathway entry point to separate the point of admission to the hospital for patients who have respiratory infection symptoms (see page 14).

Domain 2	
2	We observed positive and caring interactions between staff and patients (see page 16).

Domain 5	
3	Good leadership and teamwork across the clinical areas between all staff groups to support the delivery of care (see page 20).

Domain 7	
4	We observed an open and transparent approach from both the hospital senior management team and ward staff throughout the inspection, including at safety huddles (see page 23).
5	There was documented evidence of keeping staff updated as well as a focus on their wellbeing (see page 23).

Domain 9	
6	We observed responsive leadership demonstrated by senior managers and clinical staff to address real time pressures with good communication and supportive working (see page 24).

Requirements

Domain 1	
1	NHS Greater Glasgow and Clyde must ensure the environment in SATA continues to be suitable for the provision of care in a respiratory pathway, ensuring the improvements made during the inspection are maintained, including: • sufficient hand hygiene facilities • appropriate storage and access to personal protective equipment, and • adequate placement of patients (see page 15). This is to comply with the National Infection Prevention and Control Manual (2022).

Domain 5	
2	NHS Greater Glasgow and Clyde must ensure the good practice observed in some areas utilising a mealtime coordinator is practised consistently and patients receive adequate support at mealtimes (see page 20). This is to comply with the Food, Fluid and Nutritional Care Standards (2014): Standard 4, provision of food and fluid to patients in hospital.
3	NHS Greater Glasgow and Clyde must ensure that patient equipment is cleaned effectively (see page 20). This is to comply with the National Infection Prevention and Control Manual (2022).
4	NHS Greater Glasgow and Clyde must ensure that all staff carry out hand hygiene at appropriate moments and the correct use of personal protective equipment in line with current guidance (see page 20). This is to comply with the National Infection Prevention and Control Manual (2022)
5	NHS Greater Glasgow and Clyde must ensure wash hand basins are dedicated and used only for hand hygiene (see page 20).

This is to comply with the National Infection Prevention and Control Manual (2022).

What we found during this inspection

Domain 1 – Key organisational outcomes

Quality indicator 1.2 - Fulfilment of statutory duties and adherence to national guidelines

We observed the hospital team working together to try and achieve good care outcomes for patients receiving care. However, reducing the risks associated with staff shortages, increased demand for beds, and increased demand for assessment and treatment through accident and emergency and other admission routes created a significant challenge.

31. NHS Scotland was experiencing a wide range of considerable pressures associated with COVID-19 cases, staff absences, an increased demand for beds and high attendance rates at the emergency departments during our inspection.

32. In the hospital's daily situation report, we saw that the emergency department met the 4-hour waiting time target in 48.5% of cases. It was noted that the longest wait time for a patient was approximately 17 hours. We saw that the NHS board had taken steps to try and alleviate the pressure and demand on the emergency department with a series of statements released through social media, local radio and news stations. This included asking the public only to attend the emergency department if their condition was urgent or a life-threatening situation. The hospital also diverted patients to another hospital's accident and emergency department within NHS Greater Glasgow and Clyde for a period of time in the evening prior to the inspection.

33. Each ward and clinical area carried out a risk assessment each day to understand any staffing problems or patient safety risks. The risk assessment is completed by the nurse in charge of the area. Senior managers explained to us that this risk assessment is made on the professional judgement of the nurse in charge, which results in a green, amber or red score. A green score reflects business as usual, amber highlights where some actions may be required to reduce risks and a red score evidences the highest level of risk. The red score may result from concerns about staffing levels or other patient safety considerations. Once the level of risk has been decided, the lead nurse will work with the nurse in charge and other colleagues across the hospital to mitigate these risks, for example, the possible provision of additional staff where this is available.

34. Other methods for measuring acuity and risk are available, such as the nationally available real-time staffing tools. Senior managers explained to us that these tools were currently only used in their critical care areas. They are waiting for these tools to be digitalised before they are put into use in other areas across the hospital campus. During the inspection, we observed senior managers and lead nurses working together at regular site safety huddles to consider possible actions to mitigate risks where a red score was reported. These options included redeploying staff from areas, such as theatres, where scheduled procedures had been cancelled. In addition to this, efforts were made to support discharging patients who would be able to go home. However, we observed that there were still not enough staff available to reduce the risk in all of the red areas and a number of clinical areas continued to be a red risk score.

35. NHS Greater Glasgow and Clyde demonstrated that it has systems and processes in place to implement and follow the national guidelines. Respiratory pathways were in place in line with the Winter (21/22), Respiratory Infections in Health and Care Settings Infection Prevention and Control (IPC) Addendum.

36. We observed staff members and volunteers at the entrances and public areas in the hospital who were directing people to the correct place. Staff were also able to direct anyone with respiratory symptoms to the correct admission route. Posters and information were displayed, encouraging physical distancing and hand hygiene. In addition, there was good access to face masks and disposal bins.

37. The point of admission for patients with respiratory symptoms who have been referred to the hospital, for example, by their GP, is through the area known as SATA. This is in line with the national guidance and separates the point of admission to the hospital for patients who have symptoms of respiratory infection. During the pandemic, this area was used to reduce the possibility of people with COVID-19 entering the main admission units within the hospital. We observed and recognised the efforts to maintain two separate pathways for those with respiratory symptoms and those without symptoms. However, we observed a number of issues within this area that were raised to the senior site managers for their immediate attention. These issues included:

- the placement and availability of hand hygiene basins
- the storage and availability of personal protective equipment (PPE) for staff to use
- the lack of a call bell system for patients being cared for in both cubicles and side rooms, and
- patients' cared for in non-standard care areas. We saw a patient being cared for in a room previously used as a store room that was being utilised as a patient side room at the time of our inspection. As this room

was not designed as a patient side room, the appropriate facilities were not in place. This included the ability to turn off the lights, and staff could not see the patients cared for in this area as there was no viewing pane.

38. We escalated these concerns to senior management, and we were quickly provided with a detailed risk assessment with planned actions. We observed:

- that temporary hand hygiene basins were installed with a stated time for the installations of permanent basins.
- personal protective equipment dispensers were installed
- call bells were installed in both the care areas and toilet for patient use, and
- the room previously used as a store room was no longer used for patient care.

39. We were told about plans to consider relocating the SATA to a more suitable environment within the building. However, due to the current pressures, this plan could not be put in place at this time.

40. The layout of the SATA meant that although some side rooms were available, most patients were placed in a seating area that was designed and previously used as a discharge lounge. This meant that patients with different respiratory infections might be seated here before being admitted to the ward areas.

41. The national guidance recognises that it may be necessary to care for patients with different respiratory infections in one multi-bed care area before being admitted to a single room. However, it states this does carry the risk of different infections being spread, should be avoided wherever possible and only used as a last resort during times of extreme bed pressures. Our observations during this inspection were that this hospital was experiencing extreme pressures.

42. We observed many patients who were waiting for admission being cared for on chairs in a lounge area. This was due to the demand for inpatient beds exceeding the number of available beds. Patients were waiting for long periods of time before being transferred to the appropriate care area or wards. When a patient has remained in the department for longer than normal, this was reported by staff and evidenced by the incident reports provided by senior managers. Clinical staff shared concerns with our inspection team on the level of care that could be provided in these areas, as they were not designed to accommodate patients for long periods of time. We observed senior clinical staff and senior managers discussing and escalating these issues through the hospital campus safety huddles. The lengths of stay in the areas were discussed, seeking ways to move patients to wards as quickly as possible.

43. Also in SATA, senior staff raised concerns that occasions may occur where clinically vulnerable patients with respiratory symptoms, including those who

would have previously been shielding, would be cared for in the same lounge area as those patients with unknown or confirmed respiratory infections.

44. During our follow up visit to SATA, we wanted to understand the pathway for patients who are clinically vulnerable to respiratory infections. We were shown a procedure for the management of these patients who are required to be admitted through SATA. This described patients being admitted straight to one of the available side rooms. If these were not available, the next step is to ensure the patient is transferred to an appropriate care area within the hospital. This process is in line with national guidance, and the staff we spoke with understood the process that should be followed. We also observed that the improvements made during the original inspection remained in place.

45. The national guidance for ventilation recommends six air changes every hour that can be achieved by mechanical ventilation or by opening windows. The Queen Elizabeth University Hospital is entirely mechanically ventilated. Clean air is brought in from outside through their system, which means that windows cannot be opened. The ventilation system throughout the hospital has three air changes per hour.

46. For areas used as planned respiratory pathways, such as SATA, the guidance states that if six air changes per hour cannot be achieved:

- it must comply with 2 meters spacing between patients' beds, and
- patients are provided with facemasks and adequate hand hygiene facilities.

47. We observed that chair spacing was in place, and patients wore face masks when tolerated. However, as described above, hand hygiene facilities were inadequate, although this was addressed immediately during our inspection. Senior management highlighted that they were seeking other possible solutions to increase the ventilation in SATA.

Area of good practice

Domain 1	
1	There was a dedicated respiratory pathway entry point to separate the point of admission to the hospital for patients who have respiratory infection symptoms.

Requirement

Domain 1
1 NHS Greater Glasgow and Clyde must ensure the environment in SATA continues to be suitable for the provision of care in a respiratory pathway, ensuring the improvements made during the inspection are maintained, including: • sufficient hand hygiene facilities • appropriate storage and access to personal protective equipment, and • suitable placement of patients. This is to comply with This is to comply with the National Infection Prevention and Control Manual (2022).

Domain 2 – Impact on people experiencing care, carers and families

Quality indicator 2.1 - People's experience of care and the involvement of carers and families

We observed positive and respectful interactions between patients and staff. Patients described a positive experience of care and that their needs were being met.

48. During our inspection, the majority of patients experiencing care appeared to be treated with kindness and compassion in how they were supported and cared for. We observed positive interactions between patients and staff. Patients experiencing care told us that their needs were met. Although patients described good care, they told us staff were busy and, on occasions, this reduced their ability to respond to call bells in a timely manner. SATA did not have any call bells in place. However, senior management immediately rectified this after the inspection team raised this as a concern.

49. In the admission areas, such as SATA and the immediate assessment unit (IAU), staff expressed concerns about the care that could be provided and the lack of dignity for patients required to be cared for in these areas for longer than intended. For example, patients would be sitting in a chair rather than a bed space. We saw that this had been raised with senior clinical colleagues and senior managers through incident reporting systems and emails between clinical staff and senior managers. During our discussion session, senior

managers acknowledged the issues raised. However, we were told the current pressures limited available solutions, and this remains an ongoing challenge.

50. During our follow up visit to SATA, we observed it to be quieter and only a small number of patients waiting in the lounge area. We will follow this up at future inspections.

Area of good practice

Domain 2	
2	We observed positive and caring interactions between staff and patients.

Domain 5 – Delivery of safe, effective, compassionate and person-centred care

Quality indicator 5.1 - Safe delivery of care

We observed good leadership and team work across the clinical areas to provide patient care. We saw teams communicating well with a good understanding of their patient's health needs. However, we noted some missed opportunities for hand hygiene, inappropriate use of PPE and ineffective cleaning of patient equipment.

51. All wards and departments were very busy. The majority of areas had good leadership and appeared to be well organised. Staff from all disciplines were seen to be working together to support the safe delivery of care. We observed staffing shortages across all of the areas we inspected. Staff and senior managers expressed concerns about the impact of increased pressures across the hospital campus. At busier times, we saw that it was more challenging for staff to meet patient needs in a timely manner.

52. In the newer parts of the hospital campus, the majority of patient rooms are single rooms. In the areas with multi-bedded bay areas, we observed that bed and chair spacing was kept in line with the current guidance.

53. We observed staff working hard to deliver routine care, although we saw that this was affected by the number of staff available. We saw some good examples of mealtimes being well managed, particularly when the ward had a meal coordinator to help manage this effectively. On some wards, we saw that staff checked that each patient had a meal and that an alternative choice was available. Staff were seen to proactively organise appropriate meals for the patient's dietary needs, for example, a textured modified diet. In one area, staff

were helping patients with their meals, and a patient described the staff as 'fantastic'.

54. However, in some wards, due to a shortage of staff, we observed that patients had to wait for support with meals. For example, if a patient required help to sit up and eat, their meals may become cold before they get to eat them. This is not effective mealtime practice. Patients in the admission areas, such as the emergency department, had a limited choice of food at mealtimes, such as soup and sandwiches.

55. In order to minimise the risk of cross-infection, standard infection control precautions should be used by all staff at all times.

56. One of the key precautions is practising good hand hygiene. This will help reduce the risk of the spread of infection. We observed mixed practice with hand hygiene. In some areas, we observed staff carried it out well. However, we saw:

- staff not performing hand hygiene before putting on gloves, and
- some staff wearing gloves, when not required, preventing hand hygiene from being performed at the correct times.

57. We also observed staff wearing nail varnish and jewellery that can prevent hand hygiene from being carried out effectively.

58. We saw that alcohol-based hand rub was available in corridors and patient rooms. As previously reported, due to the lack of hand hygiene facilities in SATA, additional alcohol-based hand rub dispensers were added to the area during our inspection.

59. In some areas, we observed clinical hand wash basins being used for other things, such as disposing of other liquids and teeth brushing. Clinical hand wash basins should only be used for hand hygiene to reduce the risk of contamination to the clinical hand wash basin.

60. We observed some staff were using PPE, such as aprons and gloves, appropriately. However, in SATA, there were no PPE storage units, which meant that PPE was not stored correctly to prevent contamination. We also observed staff moving between different care areas without changing their PPE. During our follow up visit to SATA, we observed PPE was stored appropriately, and staff were changing it at appropriate times. We also observed new posters had been placed beside the PPE dispensers instructing staff of the correct use of PPE, and to carry our hand hygiene at the correct times.

61. In some areas, we observed the reuse of single-use visors. These are used to protect the staff member from the risk of blood or body fluid splashes. Although staff had attempted to decontaminate the visors, they are not

designed to be cleaned and reused. Senior management confirmed that this was not an approved process.

62. In discussion with senior management, we were told that within 2 weeks of the inspection, every area in the hospital was going to have an infection control visit. This will include education on hand hygiene, the use of visors, and the correct use of hand wash basins. We anticipate an update on this within the improvement action plan that the NHS board will supply to address the requirements made.

63. Throughout the hospital campus, we observed some issues with the storage of waste. For example, there was not a suitable storage area in SATA. This was addressed during the inspection by turning a store cupboard into a waste hold.

64. We saw other waste holds were unlocked, and this is not in line with waste storage guidance. This guidance stated that waste should be stored in a dedicated lockable area. We raised this with senior management. The NHS board explained that this was an ongoing issue across the hospital site and the original waste hold doors were not fit for purpose. We were provided with evidence this had already been recognised, and action was being taken to address this problem. A new lock system had been trialled and there was a three-year improvement programme in place to improve all waste hold doors within the Queen Elizabeth University Hospital and Royal Hospital for Children by March 2024.

65. We observed that transmission-based precautions were in place for the majority of patients with a suspected or confirmed infection. These are the additional infection control precautions that should be used by staff when caring for a patient with a known or suspected infection. The exception to this was in SATA, as we have previously described.

66. At the time of our inspection, physical distance guidance was in place. We observed that areas had been set up to allow physical distancing. Rooms had maximum numbers of people allowed signs on the door. We observed the majority of staff complying with physical distancing where it was possible to do so. We saw evidence of spot checks to assure management that guidance was displayed, controls were in place, and that staff complied with and understood the guidance.

67. In public areas such as entrances, shops, dining areas and waiting areas, we observed signs and information to help people remain physically distanced.

68. We observed that the cleaning products being used were in line with local policy and national guidance.

69. We observed that staff were cleaning patient care equipment between patient use. However, we observed that the areas of the equipment that would take

longer to clean were not effectively cleaned. This included the lower wheels, the underside and footplates of some equipment, such as trollies and walking frames. Care equipment is easily contaminated. Therefore, it can be a source of transferring infectious agents from shared care equipment if it has not been effectively cleaned.

70. The cleanliness of the environment within the hospital campus inspected was mostly good.

71. The majority of wards, patient rooms, corridors and storage rooms in the newer buildings were well organised and uncluttered, allowing for effective cleaning. However, in SATA, and the wards within some of the older parts of the hospital campus, areas were cluttered due to a lack of storage space. This can make it difficult to allow for efficient and effective cleaning. On our follow up visit to SATA, we observed the area was clutter free, and the environment and the cleanliness of the area was good.

72. At the time of our inspection, we observed some issues that were quickly rectified. For example, there was a water leak in a ward day room resulting in a loose section of the ceiling. When we raised this with the facilities team, they were already aware of this and informed us the ceiling had been repaired that day. Both the facilities team and the infection prevention and control team described a close working relationship, which has helped them develop procedures for quickly rectifying issues, such as the damaged ceiling.

73. We observed issues with faulty fitted bathroom equipment, such as drop-down rails and hand rails in several wards within the newer buildings. We raised this with senior management, who confirmed this was an issue they were aware of. We saw the NHS board had circulated a safety action notice to all affected areas, and the completion date for the work is due within the coming weeks. We will follow this up at future inspections.

74. To ensure effective cleaning, the environment must be well maintained and in a good state of repair. In some of the older areas of the hospital campus, including the Institute of Neurosciences, we saw that the environment was damaged, and surfaces were worn. This has been raised at previous inspections. For example, we saw black marking on the shower seals, and we raised this with the nurse in charge. They confirmed it had been appropriately cleaned; however, cleaning does not remove the staining, and this will be replaced during a full renovation of the ward. We were told that the refurbishment of the ward was due to commence in the summer of 2022.

75. We discussed the refurbishment plans for the Institute with senior managers. We were told that two general wards had already been refurbished, and one ward was about to be completed. The plan is for all the general wards within the Institute to be fully refurbished by mid-2024. Following the refurbishment

of the general wards, the plan is to start the refurbishment of critical care areas. We will follow the condition of the environment and the programme of refurbishment at future inspections.

Area of good practice

Domain 5	
3	Good leadership and team work across the clinical areas between all staff groups to support the delivery of care.

Requirements

Domain 5	
2	NHS Greater Glasgow and Clyde must ensure the good practice observed in some areas utilising a mealtime coordinator is practised consistently and patients receive adequate support at mealtimes. This is to comply with Food, Fluid and Nutritional Care Standards (2014): Standard 4, provision of food and fluid to patients in hospital.
3	NHS Greater Glasgow and Clyde must ensure that patient equipment is cleaned effectively. This is to comply with the National Infection Prevention and Control Manual (2022).
4	NHS Greater Glasgow and Clyde must ensure that all staff carry out hand hygiene at appropriate moments and the correct use of personal protective equipment in line with current guidance. This is to comply with This is to comply with the National Infection Prevention and Control Manual (2022).
5	NHS Greater Glasgow and Clyde must ensure wash hand basins are dedicated and used only for hand hygiene. This is to comply with the National Infection Prevention and Control Manual (2022).

Domain 7 – Workforce management and support

- Quality indicator 7.2 - Workforce planning, monitoring and deployment
- Quality indicator 7.3 - Communication and team working

NHS Scotland is experiencing significant pressures associated with staffing vacancies, as well as continued challenges relating to staff absence. We observed real-time staffing decisions, escalation and mitigation of risk, and evidence of allocation of staffing to the areas with highest risk when there were staffing shortfalls. We witnessed an open, honest and transparent approach, with visible and supportive leadership at a senior level and within the wards across the hospital campus.

76. At the time of our inspection, staffing pressures associated with COVID-19 were being experienced across NHS Scotland. We found that some areas within the hospital campus were working with a less than optimum level of staffing due to staff absences and lack of available supplementary staff. It was indicated at the morning safety huddle that a large number of wards were given a risk rating of red.

77. There was a 10.1% vacancy rate within both the registered nursing and allied health professional staff in the acute adult hospital setting. There were 1.6% vacancies within the healthcare support worker workforce for the last reported monthly data. The budgeted registered nurse and healthcare support worker workforce has increased by 3.5%. The highest number of vacancies in adult services were within the registered nursing and allied health professional teams; both have a 10.1% vacancy rate. It is noted that the allied health professional workforce in this area has increased by 65%.

78. The overall absence level for nursing staff groups was 9.3%. This sickness absence level was 6.6%, with COVID-19 special leave accounting for an additional absence of 2.7%.

79. Supplementary staff are additional staff who cover absences and/or provide additional support due to increased service demands to support the delivery of safe and effective care. This includes staff working additional hours, overtime, and bank and agency workers. Supplementary registered nurses accounted for 7.5% of the staff and 16.7% of healthcare support worker staff. Adult services had the highest use of supplementary staff.

80. The overall absence level was 5.6% within the allied healthcare workforce. The sickness level was 3.9%, with COVID-19 special leave accounting for an additional absence of 1.7%.

81. There was a 1.4% vacancy rate for medical staff. The medical workforce has increased by 4.2%. Sickness and COVID-19 related absences are 2.2%

82. Domestic Services had vacancies at the time of inspection, however additional funding was made available to increase fixed-term staffing levels in order to support the enhanced cleaning schedules as required by the addendum. Evidence supported over-recruitment; however, sickness absence of rates of 11.9% and COVID-19 related absences at 1.9% was affecting the availability of staff.

83. Although there were significant staff shortages throughout the hospital campus, we observed effective leadership, clear communication and effective levels of care.

84. The NHS board provided evidence that demonstrated good practice in mitigating and prioritising services to support the delivery of care and highlighting the structure of strategic, tactical and operational groups that have been and continue to be responsive to emerging staffing and safety risks.

85. At the safety huddles, workforce staffing availability for all staff groups was discussed. Availability and levels of nursing staff were discussed in more detail with a focus on staffing numbers. This assessment was carried out using the nurse in charge's professional judgement, such as falls risk and enhanced observation requirements. The critical care areas are currently using the Scottish Government's real-time staffing tool. The utilisation of the Scottish Government acute in-patient real time staffing resource would further enhance staffing decisions. The hospital lead nurse of the day was able to make an informed decision and assign available resources to each department. The lead nurses in the department would aim to assign the staff resource to the areas of highest risk need and priority. However, we observed during our inspection that despite efforts to reduce the number of areas with the highest risk rating, significant staff shortages meant that this was not possible in the majority of identified high-risk areas.

86. Despite these significant staff shortages, it was observed that wards were well managed, communication was effective, and staff were focused on the provision of safe and compassionate care for the patients. The lead nurses and senior charge nurses were visibly supporting staff in the ward areas.

87. Staffing risks are reported using the NHS electronic incident reporting system. NHS Greater Glasgow and Clyde has received a high level of reports on identified staffing risks. Staff are encouraged to report their concerns through this system. There is also a process in place to identify reporting themes.

    These reports are sent weekly to the chief nurses and monthly to the charge nurses/midwives for local dissemination and discussion through staff governance forums.

88. One initiative described in the staff newsletter was the plan to develop an improved incident reporting system to better support staff to report concerns and receive feedback. This will continue to ensure effective communication.

89. We observed a number of initiatives to promote an open and transparent culture within the hospital campus. For example:

- encouraging staff to report concerns on the NHS electronic incident reporting system
- staff newsletters
- honesty around the assessment of risk each day, even when they are not able to reduce this, and
- partnership working with the staff and inclusion of partnership representatives.

90. We also saw there are both internal and external resources available for staff to access around staff wellbeing.

Areas of good practice

Domain 7	
4	We observed an open and transparent approach from both the hospital senior management team and ward staff throughout the inspection, including at safety huddles.
5	There was documented evidence of updating staff, as well as a focus on their wellbeing.

Domain 9: Quality improvement-focused leadership

Quality indicator 9.2 - Motivating and inspiring leadership

Safety briefings and huddles were attended by representatives of a wide range of wards and departments. We observed senior managers and clinical staff from across the hospital campus working together to try to reduce risks associated with the unprecedented increased pressures.

91. At the Queen Elizabeth University Hospital campus, several patient safety huddles are carried out throughout the day for the various sites/directorates. The purpose of the huddles is to review staffing, safety risks and patient flow. The hospital campus was under exceptional pressures at the time of our inspection, and we observed how this affected the ability to reduce all of the identified risks. We saw evidence where the risks could not be reduced safely,

and this was escalated through the NHS board with senior clinical staff raising to the NHS board's executive team. We also saw this being reported in the hospital's daily situation report to the Scottish Government.

92. The NHS board was trying to relieve some of the campus's pressures. Measures included non-essential procedures being cancelled to free up staff who were then redeployed to other areas to reduce risks. Media press releases were issued using social media, local radio, and news stations to remind people to only attend the emergency department if it was very urgent or life-threatening. Staff from the hospital were also going to another nearby minor injuries unit to support that unit to stay open and reduce the flow of patients attending the emergency department at the Queen Elizabeth University Hospital.

93. To support staff working in such pressure, we saw a letter dated December 2021 to all nursing, midwifery and allied health professional staff from the nurse director of NHS Greater Glasgow and Clyde. The letter detailed the guiding principles for staff to follow when they are working in situations unlike anything they have experienced before. The principles were developed to support decision-making and help with difficult situations health professional staff may face, and reassure them that, on occasions, the provision of care is the best possible care that can be delivered under the circumstances that they are working within. The purpose of the letter was also to reassure staff that they have they have the support and understanding of their professional regulator and NHS Greater Glasgow and Clyde as an organisation. We saw this as an important message to staff who may feel they are unable to deliver care to the standard they would normally deliver due to the unprecedented pressures and demands on staff.

Area of good practice

Domain 9	
6	We observed responsive leadership demonstrated by senior managers and clinical staff to address real time pressures with good communication and supportive working.

Appendix 1 – List of national guidance

The following national standards, guidance and best practice were current at the time of this inspection. This list is not exhaustive.

- Winter (21/22), Respiratory Infections in Health and Care Settings Infection Prevention and Control (IPC) Addendum (NHS National Services Scotland, April 2022)
- National Infection Prevention and Control Manual (NHS National Services Scotland, March 2022)
- COVID-19: Guidance for maintaining services within health and care settings Infection prevention and control recommendations (Public Health England, December 2021)
- COVID-19: Endorsed Guidance For NHS Scotland Staff and Managers on Coronavirus (NHS Scotland, November 2021)
- Health and Social Care Standards (Scottish Government, June 2017)
- Healthcare Associated Infection (HAI) standards (Healthcare Improvement Scotland, February 2015)
- The Code: Professional Standards of Practice and Behaviour for Nurses and Midwives (Nursing and Midwifery Council, October 2018)
- Generic Medical Record Keeping Standards (Royal College of Physicians, November 2009)
- Allied Health Professions (AHP) Standards (Health and Care Professionals Council Standards of Conduct, Performance and Ethics, January 2016)
- Food Fluid and Nutritional Care Standards (Healthcare Improvement Scotland, November 2014)
- Prevention and Management of Pressure Ulcers - Standards (Healthcare Improvement Scotland, October 2020)
- Health and Care (Staffing) (Scotland) Act (Acts of the Scottish Parliament, 2019)
- Care of Older People in Hospital Standards (Healthcare Improvement Scotland, June 2015)
- Quality of Care Approach – The Quality Framework First Edition: September 2018 (Healthcare Improvement Scotland, September 2018)

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B.cult-Hickman line

Performed	15-May-2021 04:57	Received	15-May-2021 12:05
Reported	21-May-2021 10:42	Order Number	[redacted]
Status	Final	Source System	Telepath

Microbiology Final

Report issued by NHS GG&C Microbiology South Sector
Enquiries 0141 354 9132

** FINAL REPORT **

INVESTIGATION: Blood Culture
SPECIMEN TYPE: B.cult-Hickman line
Red lumen

CONS/GP: Dr Andrew D Clark Order No [redacted]
LOCATION: Bone Marr Transplant QEUH

Aerobic Bottle: POSITIVE
Anaerobic Bottle: POSITIVE

CULTURE RESULTS:	FROM BOTTLE:
a) Staphylococcus epidermidis	Both
b) Stenotrophomonas maltophilia	Aerobic
c)	
d)	
e)	
f)	

ANTIBIOTIC	a)	b)	c)	d)	e)	f)
Co-trimoxazole		S				
Levofloxacin		S				
Ceftazidime		S				
Vancomycin	S					
Teicoplanin	S					

Clinical microbiology advice can be obtained by calling
0141 354 (8)9132 or the on-call Microbiologist

Senders ref. no.

Authorised by: Dr Nitish Khanna
Date/Time authorised: 21.05.2021 10:39
** END OF REPORT **

Procedures to Investigate Waterborne Illness

Introduction

Humanity could not survive without a reliably clean, safe, and steady flow of drinking water. Since the early 1900s when typhoid fever and cholera were frequently causes of waterborne illness in developed countries, drinking water supplies have been protected and treated to ensure water safety, quality, and quantity. Having access to safe drinking water has always been one of the cornerstones of good public health. Safe water is not limited to drinking water, since recreational water and aerosolized water can also be sources for waterborne illness, from treated waters such as in swimming pools, whirlpools, or splash pads and from non-treated surface waters such as lakes, rivers, streams and ponds. Recreational waters may cause illness not only from ingestion of pathogens, but also when in contact with eyes, ears, or skin. Some pathogens in water can be acquired by inhalation of aerosols from water that is agitated or sprayed such as in humidifiers, fountains, or misting of produce. This poses a potential risk to those exposed, particularly if they are immunocompromised.

Often when an outbreak is first suspected, the source is not clear, i.e., food, water, animal contact. Investigation is usually needed to find the common source. In some outbreaks the food may first be identified as the source, such as with produce, but the ultimate source could be contaminated irrigation water. Investigators have to keep an open mind until laboratory and/or epidemiologic evidence links cases to the primary source.

Although we frequently think of waterborne illness originating from a microbiological agent, we should be aware that water may also be contaminated by pesticides, fertilizers, and other chemicals which may enter through industrial discharge, agriculture runoff, or deliberate contamination.

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Waterborne illness acquired from microorganisms may be classified as:

- Toxin-mediated infections caused by bacteria that produce enterotoxins or emetic toxins that affect water, glucose, and electrolyte transfer during their colonization and growth in the intestinal tract;
- Infections caused when microorganisms invade and multiply in the intestinal mucosa, eyes, ears, or respiratory tract, or contact the skin;
- Intoxications caused by ingestion of water containing poisonous chemicals or toxins produced by other microorganisms

Manifestations range from slight discomfort to acute illness to severe reactions that may terminate in death or chronic sequelae, depending on the nature of the causative agent, number of pathogenic microorganism or concentration of poisonous substances ingested, and host susceptibility and reaction.

The public relies on public health regulators to investigate and mitigate waterborne illness. Mitigation depends upon rapid detection of outbreaks and a thorough knowledge of the agents and factors responsible for waterborne illness. Public health and law enforcement agency officials should always be alert to the rare possibility of an intentional contamination of water supplies by disgruntled employees or terrorists.

The purposes of a waterborne illness investigation are to stop the outbreak or prevent further exposure by:

- Identifying illness associated with an exposure and verifying that the causative agent is waterborne
- Detecting all cases, the causative agent, and the place of exposure
- Determining the water source, mode of contamination, processes, or practices by which proliferation and/or survival of the etiological agent occurred
- Implementing emergency measures to control the spread of the outbreak
- Gathering information on the epidemiology of waterborne diseases and the etiology of the causative agents that can be used for education, training, and program planning, thereby impacting on the prevention of waterborne illness
- Determining if the outbreak under investigation is part of a larger outbreak by immediately reporting to state/provincial/national epidemiologists

In the instance of a bottled water outbreak, halting of distribution and sale of product and recall of product, some of which may already be in consumers' homes, are necessary to prevent further illness.

As epidemiologic data accumulate, information will indicate the source of the problem, whether a municipal water treatment plant, bottled water manufacturing plant, or recreational water exposure, and suggest methods for controlling and preventing waterborne illness. This information will guide administrators in making informed decisions to provide the highest degree of waterborne safety.

A flowchart, *Sequence of events in investigating a typical outbreak of waterborne illness* (Fig. 1) shows the sequential steps, as presented in this manual, in investigating a typical outbreak of waterborne illness and illustrates their relationships. A description of each step is presented in this manual.

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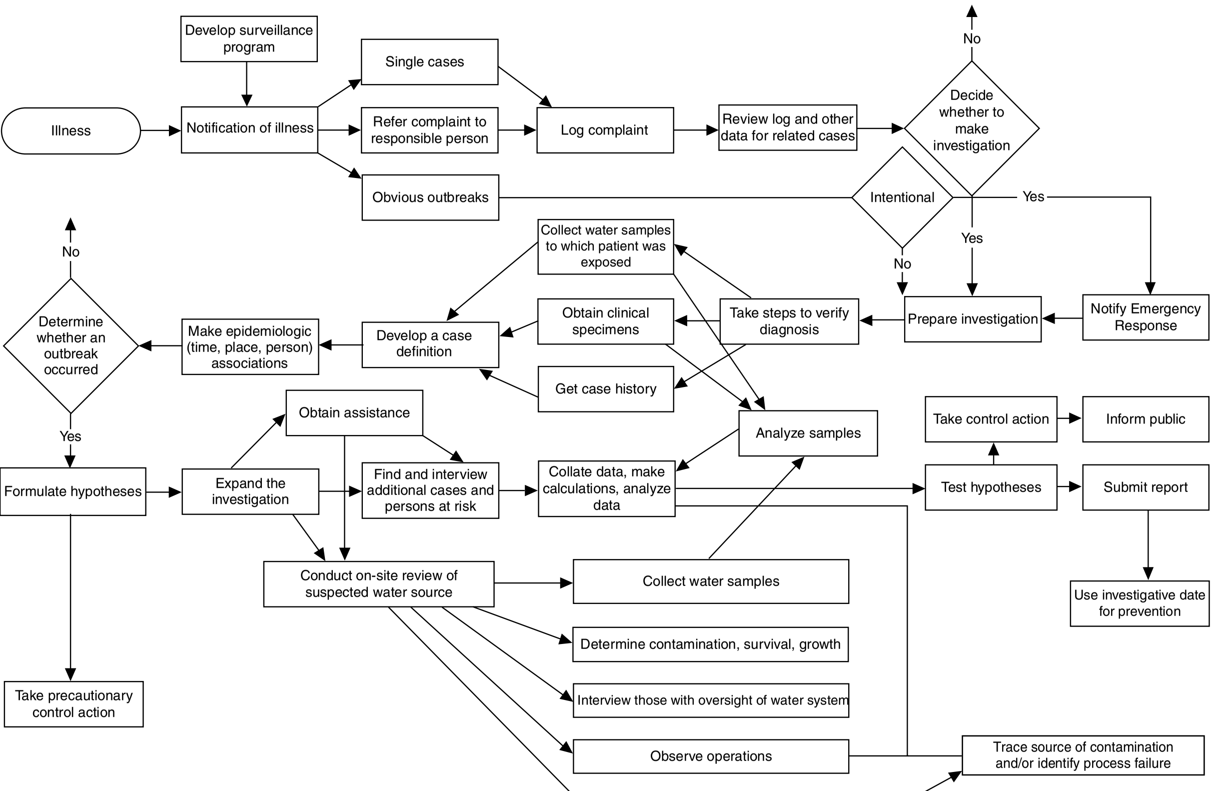

Fig. 1 Sequence of events in investigating a typical outbreak of waterborne illness. An intentional water contamination event may or may not be obvious. It can be recognized at any point during the outbreak investigation. If intentional contamination is suspected follow your notification scheme in emergency response plans (this could include law enforcement, emergency management and other government agencies)

Develop a Waterborne Disease Surveillance and Emergency Operations Program

The primary purpose of a waterborne disease surveillance system is to systematically gather accurate information on the occurrence of water-related illnesses in a community, thus allowing development of a rational approach for the detection, control and prevention of waterborne illness. Other purposes are to (a) determine trends in the incidence of waterborne diseases, (b) characterize the epidemiology of waterborne diseases, (c) gather and disseminate information on waterborne diseases, and (d) develop a basis for evaluating control efforts. It may be useful to coordinate this system with, or integrate it into a foodborne disease surveillance system. However, while the procedures are quite similar from an epidemiologic viewpoint, they may differ with respect to personnel or agencies involved. An effective disease surveillance system is essential for detection of disease caused by either unintentional or intentional contamination of food.

An effective waterborne disease surveillance system consists of:

- Early reports of enteric and other illnesses that may be related to water exposure or consumption
- Coordinated effort between local and state public health partners, water utility and water recreation staff
- Systematic organization and interpretation of data
- Timely investigation of identified outbreaks or clusters of illness
- Dissemination of outbreak reports and surveillance summaries to all appropriate stakeholders

Many types of reporting systems may already exist at the local or state/provincial level, and these should be incorporated into a waterborne disease surveillance program. These include (a) mandatory (or voluntary) laboratory- or physician-based reporting of specific infectious diseases, (b) national-based surveillance systems such as CaliciNet (CDC 2009) or NORS (CDC 2009) in the US, (c) physician office, hospital emergency, and urgent-care clinic medical records, (d) public complaints made to health agencies and/or local water utilities, (e) school illness and absentee records, (f) absentee records of major employers, (g) water treatment records kept by water utilities (e.g., turbidity, disinfection levels, occurrence of coliforms), (h) increased sales of antidiarrheal drugs and anti-nausea medications, and (i) source water quality data kept by environmental agencies (e.g., departments of natural resources and geological survey agencies). Another type of surveillance mechanism that may supplement or enhance existing reporting systems is a daily log of illness and water quality complaints.

Organize the System and Develop Procedures

An effective waterborne illness surveillance system requires close cooperation between key personnel in public and private health agencies, laboratories, water utilities and water recreation staff, and environmental health agencies. When your

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agency contemplates initiation or development of a waterborne illness surveillance program, give top priority to identification of appropriate financial, political, strategic, and administrative support. Then, identify a key person to create, implement, and manage the system.

This person takes responsibility for:

- Reviewing the types of reporting systems that already exist in your agency or in other agencies that could be incorporated into a waterborne illness surveillance system
- Identifying the types of information that cannot be obtained from existing reporting systems but that need to be collected or addressed by the waterborne illness surveillance system
- Identifying ways to merge or integrate the data collected by existing systems with data gathered in the waterborne illness surveillance system
- Identifying collaborating agencies and staff
- Develop a mechanism to communicate and update all stakeholders (may be by blast e-mail or periodic conference calls)
- Providing training in surveillance methods for agency staff and other partners to enhance cooperation
- Assembling materials that will be required during an outbreak investigation
- Evaluating the effectiveness of the system.

Develop procedures to seek and record complaints about waterborne illnesses, water supplies, and water recreational sites. For example, list the telephone number of the waterborne illness investigation unit prominently on local and state public health and water utility websites. To be most effective, have this number monitored 24/7 by staff or an answering service. If possible, the utilization of social media such as Facebook or Twitter should be considered and monitored as many large municipalities (including drinking water utilities) and recreational facilities have an Internet presence. If your agency has social media accounts, consider using this vehicle to further disseminate information regarding waterborne illness clusters or outbreaks. Identify medical care facilities and practitioners and seek their participation. Direct educational activities, such as newsletters and talks at meetings, to stimulate participation in the program. Encourage water treatment utilities and operators of recreational water sites to report suspected complaints of waterborne illness to the appropriate local agencies. Also, encourage private and hospital laboratories to report isolations of parasitic agents (e.g., *Giardia*, *Cryptosporidium*), viruses (e.g., norovirus and hepatitis A virus), bacteria (e.g., *E. coli* (pathogenic), *Salmonella*, *Shigella*, *Vibrio cholerae*), and other agents that may be waterborne. Develop a protocol for notification and coordination with agencies that might cooperate in investigational activities, including 24-h-a-day, 7-days-a-week contacts. A comprehensive contact list should be constructed and updated at least twice a year as individuals may change. Notify and coordinate with state/provincial or district agencies, national agencies that have surveillance and water regulatory responsibilities, and other national and international health agencies, as appropriate. For example, it may be useful to find out the level of participation within a certain jurisdiction in national-level outbreak surveillance programs such as NORS (CDC, 2015) or other national surveillance system.

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Assign Responsibility

Delegate responsibility to a professionally trained person who is familiar with epidemiologic methods and with the principles of water treatment and recreational water protection. This person will (a) direct the surveillance program, (b) take charge if waterborne and enteric outbreaks are suspected, and (c) handle publicity during outbreaks. Delegate responsibility to others who will carry out specific epidemiologic, laboratory and on-site investigations. If an intentional contamination event is suspected, local and national law enforcement agencies will likely become the lead agency responsible for the investigation. With this in mind, it is critical to identify appropriate individuals and include them in communication and any practice drills that may occur. If a relationship has been established prior to any event, the investigation may run more smoothly.

Establish an Investigation Team

Enlist help from a team of epidemiologists, microbiologists, sanitarians/environmental health officers/public health inspectors, engineers, chemists, nurses, physicians, public information specialists, and other (e.g., toxicologists) as needed. Free flow of information and coordination among those participating in waterborne disease surveillance and investigation are essential, particularly when several different agencies are involved. Water-related complaints are equally likely to be directed at health departments or water utilities but also perhaps to different jurisdictions. Therefore, it is essential that these complaints be registered by an agency and that the information is rapidly shared within and perhaps outside of a particular jurisdiction as part of an integrated surveillance system. Whenever possible, share the information with participating parties by rapid means such as e-mail and by calling 24/7 contact phone numbers. If an intentional contamination event is suspected, contact emergency response and law enforcement for their early involvement.

Train Staff

Select staff members who will participate in the waterborne disease surveillance program on the basis of interest and ability. Inform them of the objectives and protocol of the program. Emphasize not only the value of disease surveillance, but also the value of monitoring water quality and treatment performance. If possible, provide printed learning material that can be referenced later. Encourage the use of epidemiologic information and approaches in routine disease surveillance and prevention activities. Develop their skills so that they can carry out their role effectively during an investigation, and teach them how to interpret data collected during investigations. Conduct seminars routinely and during or after investigations to

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update staff and keep agency personnel informed. Train office workers who will receive calls concerning waterborne illnesses to give appropriate instructions. Those who participate in the investigation will learn from the experience and often are in a position to implement improvements after completion of the investigation.

Assemble Materials

Assemble and have readily available kits with forms and equipment as specified in Table A (Equipment useful for investigations). Restock and maintain kits on a schedule recommended by, and in cooperation with, laboratory staff to ensure their stability and sterility. Verify expiration dates, and use kits before this date or discard and reorder. Assemble a reference library on waterborne illnesses, investigation techniques, and control measures from reference books, scientific journal articles, manuals, and reputable Internet sources (e.g., www.cdc.gov, www.who.int/en/, www.hc-sc.gc.ca/index-eng.php, www.gov.uk/topic/health-protection/infectious-diseases); make it available to the staff in an easy-to-access format. (See Further Reading for suggestions).

Emergency Preparation

Organize a multiagency team with representatives from public health agencies, regulatory agencies, and water utilities and with local political officials to review and exercise existing emergency response plans in the event of a large scale waterborne disease outbreak or other disaster likely to result in waterborne illnesses. Local public health agencies have the primary responsibility for the restoration and protection of health of a community following an outbreak event or other emergency.

Emergency operational procedures should include the following:

- An emergency notification list that includes phone numbers and e-mail addresses of key persons/agencies that need to be informed about possible outbreaks and that should receive emergency press releases. Every state/province has an emergency management agency and depending upon the scale of the event, it may be useful to coordinate efforts.
- Clear guidelines for household water consumption following an event. For example, boil-water advisories or instructions to drink only bottled water. Statements should be reviewed to ensure current relevance and updated to reflect the most current knowledge.
- A plan for dissemination of information to the public; select a coordination point to which all news media and outside agencies will be directed, and designate one person or one telephone number as the contact. (More than one contact person can create confusion).
- Alternative drinking water sources to be used in cases of emergency and plans for the distribution of this water, if necessary. These include alternative munici-

pal systems, bottled water supplies, portable filtration and/or disinfection devices and home treatment units. (Special attention should be given to backup supplies for hospitals, nursing homes and other places where lack of safe water would be immediately life-threatening).

- Identification of specialty laboratories at the state/provincial and national level that are capable of performing (and willing to perform) procedures not routinely done at local laboratories (e.g., large volume water sampling and processing for pathogenic parasites and viruses, serotyping, electron microscopic examination of stool samples, molecular and immunodiagnostic tests for pathogens in environmental and clinical samples). One or more of these tests may be necessary to identify the causative agents in an outbreak and confirm their transmission route.
- A plan to exercise procedure with tabletop exercises involving all pertinent partners on a regular basis and implement any necessary adjustments based upon review of after-action findings.

Investigate Outbreaks

Notification of a suspected outbreak is often received by health authorities from a laboratory report or a family physician and can be documented on a log such as Form A (Foodborne, Waterborne, Enteric Illness Complaint Report). Public health investigators will then interview cases and persons at risk who are well (controls) to make epidemiologic associations to find a common source. From here a hypothesis can be formed. Further investigation will involve:

- Collecting clinical samples and water samples
- Conducting an on-site investigation at the facility alleged to be responsible to determine the mode of contamination or process failure, e.g., low disinfectant level
- Characterizing the etiologic agents by laboratory analysis using various typing schemes. DNA profiling or pulsed-field gel electrophoresis (PFGE), of isolates from clinical and water samples to "fingerprint" and link strains of the etiologic agent among cases and to the source

Act on Notification of Illness

Prompt handling and referral of water-related complaints, rapid recognition of the problem, and prevention of further illnesses are the foundations of a successful investigation. Complaints of water problems are as likely to be reported to a water utility as to a health department. Communication is essential between these agencies. This first contact with the public is a vital aspect of an investigation of a

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potential outbreak and needs to begin by public health professionals as quickly as possible, usually within 24 hours, sometimes by putting less urgent activities aside. As indicated earlier, any action respecting a potential deliberate contamination of water will generate a specific approach to further action.

Receive Complaints or Alerts

Upon receiving a complaint or an alert about a water supply or water exposure or illness potentially attributed to these sources, record the information on Form A.

Alerts may be initiated by reports from physicians, laboratories, or from hospital emergency rooms. Alerts may also include an increase in a particular PFGE pattern from clinical isolates. An investigation may be initiated to determine if there is a common water exposure among patients with the PFGE pattern. The pattern may be compared with similar PFGE patterns in PulseNet databases to determine if there are similar occurrences of the pattern in water and clinical isolates nationwide or internationally, e.g., for food that might have been contaminated with water, bottled water. The form provides information upon which to decide whether an incident should be investigated. Form A is not difficult to fill out and can be completed by a public health professional, a trained water utility staff member, or trained office worker.

Assign a sequential number to each complaint. If additional space is needed to record information, use the reverse side or attach additional sheets. Always ask the complainant to provide names of other persons at the event, or using the water supply or recreational water under suspicion, whether or not ill, and names of any other persons who are known to be ill with the same symptoms. Follow up by contacting these additional persons.

Emphasize to the persons making alerts or complaints the need to retain a sample of the suspect water and to save clinical specimens (vomitus and stool) from ill persons using a clean utensil in a clean jar or plastic bag and to seal tightly and label clearly with the name of the person and date, and store in a refrigerator (do not freeze). Also consider family members not ill for case-control studies. Advise complainants to collect a liter (quart) of water immediately, preferably in sterile containers but otherwise in jars that have been boiled or in plastic bags, or if this is not feasible, in other clean containers. Tell the complainant to save any ice cubes or refrigerated water, either in their present containers or in unused plastic bags, in the refrigerator or freezer (if already frozen) where they are normally kept. Instruct the ill person to hold all clinical specimens and water samples until the health agency evaluates the epidemiological evidence and arranges, if necessary, to collect them. If it is determined that the specimen or sample is not necessary, notify the complainant and advise on proper disposal of the material.

Unfortunately, the specific etiologic agent cannot be identified in a large proportion of waterborne outbreaks because water samples and clinical specimens (a) were not collected in an appropriate time-frame (not early enough during illness),

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(b) are too old, (c) are too small in volume, especially for *Giardia* and viruses which require liters, (d) have not been examined for the appropriate agent. Contaminants may be in the water system for only a short time, and concentrations of toxic substances and numbers of microorganisms may decrease significantly because of dilution or disinfection.

If there is a cluster of cases, monitor reports from physicians, complaints about water, or records of laboratory isolation of enteric pathogens that may suggest outbreaks of disease or contributory situations. Collect clinical specimens and water as soon as practicable according to the section *Obtain Clinical Specimens* in this book.

Log Alert and Complaint Data

Extract key information (see* and † entries) from Form A and enter it onto Form B (Foodborne, Waterborne, Enteric Illness and Complaint Log). Record time of onset of the first symptom or sign of illness, number of persons who became ill, predominant symptoms and signs, whether ice or water was ingested, and the name of the water supply or recreational water alleged to have caused the illness, and whether a physician had been consulted, and/or had taken feces or emesis samples, and/or prescribed antibiotics. Also, enter on Form B names of the places or common gatherings (other than home) at which the stricken persons ingested water during the 2 weeks before onset of illness (see Table 1 for an example). Enter a code for the water source (e.g., community, non-community, individual well, bottled, stream/lake, vended, or untreated). Under "history of exposures" column indicate whether the afflicted person had recent domestic or international travel, attended a child care facility, or had recent contact with ill persons or animals. Under "comment" column, enter notations of type of agent isolated, results of specimen tests, places where water was consumed during travel, names and locations of restaurants or other foodservice facilities, and other pertinent information including hospitalization, occupation, or place of employment. At this phase of the investigation it will probably not be known whether the illness is waterborne, foodborne, or person-to-person spread. This log can be kept either in hardcopy or in electronic format. See Table 1 (below) as an example of a log.

Interpretation of Table 1.

Entry 101—Get further details on the patient's symptoms and seek other cases. The report of foreign travel suggests an infection that may have been acquired outside the country. Follow-up of such cases may identify an outbreak of international scope. If so, inform state/provincial and national authorities concerned with surveillance of waterborne disease about the situation.

Entry 102—Possibly food associated; alert food safety officials.

Entry 103—Initiate investigation; the two cases of conjunctivitis suggest the possibility of a common-source outbreak associated with the motel pool.

Entry 104—Initiate investigation; 12 cases indicate an outbreak that has a common time-place association.

Entry 105—This could be related to entry 103, because this person reported swimming in the same pool.

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Table 1 Foodborne, waterborne, enteric illness and complaint log

Complaint[a]			Illnesss			Food		Water			History of exposure[b]	Comments
No.	Date	Type	Onset date	No. ill	Predominant symptoms/signs	Alleged/ suspected	Where eaten within 72 h	Where ingested within 72 h	Where contacted within 2 weeks	Source[c]		
101	8/16	I	8/15	1	Diarrhea			Redguard		C	IT	*Giardia* isolated from stool
102	8/23	I/UE	8/22	1	Diarrhea	Roast beef	Speedy Foods: Joe's Diner	Dixon		C		
103	8/23	I/RW	8/22	2	Conjunctivitis			Plainville	Shadygrove Motel pool yesterday	C		
104	8/23	I	8/22	12	Diarrhea		Dixon day care center	Dixon			CC	
105	8/25	I	8/25	1	Conjunctivitis			Plainville	Shadygrove Motel	C		
106	8/26	I	8/26	1	Diarrhea, fever			Hampton		W		*Escherichia coli* O157 isolated from stool
107	8/26	RW		0				Lake Orly			NC	Water smelled like rotten eggs
108	8/27	I	8/27	1	Facial flushing, dizziness	Tuna fish	Fred's Café	Dixon		C		
109	8/29	I	8/27	2	Bloody diarrhea					S	DT	Drank stream water while hiking
110	8/29	DW	0					Private well		W		Rust and silt in water

(continued)

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Table 1 (continued)

Complaint[a]			Illnesss			Food		Water			History of exposure[b]	Comments
No.	Date	Type	Onset date	No. ill	Predominant symptoms/signs	Alleged/ suspected	Where eaten within 72 h	Where ingested within 72 h	Where contacted within 2 weeks	Source[c]		
111	8/29	I	8/29	1	Diarrhea, abdominal cramping			Hampton		W	A	*Escherichia coli* O157 isolated from stool
112	8/31	I	8/30	1	Baby bluish color			Farm well		W		Ill 2 weeks after moving to farm
113	8/31			0				Redguard		C		Total coliform count 100/100 mL
114	8/31	I	8/28	6	Itching red papules on skin			Dixon		W/L		Swam in Brown's farm pond
115	9/7	CF		0		Baby cereal		Dixon		C		Metal fragments found in Brand X, Lot JK20-111E
116	9/8	DW		0						C		Heavy rains, flooding, turbid water at Winding Bend area of town

[a]Type of complaint: I illness; CF contaminated/adulterated food; UE unsanitary food establishment; DW poor quality drinking water; RW poor quality recreational water; MP complaint related to media publicity; D disasters; O others

[b]Exposure history: DT domestic travel (out of town, within country); IT international travel; CC child care; CI contact with ill person outside household or visitor to household; A An Exposure to ill animal; C contact with ill person within household

[c]Water source: C community; NC non-community; W well; B bottled; S/L stream/lake

Entry 106—This entry and that of entry 111 could have a common source; investigate. quality of water.
Entry 107—This entry and those of 110 and 116 indicate the possibility of substandard water. Either advise callers or refer their complaints to someone who can (e.g., the water utility), but stay alert and check for illness in communities where these situations occurred.
Entry 108—Possibly food associated; alert food safety officials.
Entry 109—Initiate an investigation; the situation suggests a common source outbreak.
Entry 110—See entry 107.
Entry 111—See entry 106. Could also be exposure to an animal.
Entry 112—The syndrome suggests methemoglobinemia. Sample water and test for nitrites/nitrates.
Entry 113—Resample and investigate to find the likely cause of the elevated coliform count.
Entry 114—The syndrome suggests an outbreak of water-contact infection, possibly swimmer's itch.
Entry 115—Possibly food associated; alert food safety officials.
Entry 116—See entry 107.

Review the log each time an entry is made and also each week to identify clusters of cases and/or involvement of a common exposure that might otherwise go undetected. If your agency has district offices or if there are nearby jurisdictions (as in metropolitan areas), periodically send copies of log sheets to a central coordinating office (e.g., weekly or when there are 10–20 entries). Reports of current illness levels should include historical information on illness trends in the community so that new data can be considered in the appropriate context. Report to your supervisor if you suspect any time, place, or person associations and take steps to initiate an investigation.

Refer Complaint to Proper Agency

Refer complaints that fall outside your agency's range of operations to the appropriate authority, such as the Department of Health, Ministry of the Environment, and indicate the action taken in the disposition box on Form A. Develop a working relationship with such authorities so they will reciprocate in situations which may be associated with illness. Often an investigation requires efforts of more than one agency. Cooperation and prompt exchange of information between agencies are vital.

Prepare for the Investigation

Prior to conducting investigations, personnel should know the surveillance protocol, and be trained on how to develop questionnaires, conduct interviews, and use investigation related software. All trained investigative team members should be assigned a role and the person heading the investigative team, should "be made"

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responsible for the investigation, if this was not done when the surveillance protocol was established. Delegate sufficient authority and provide resources to the head investigator so that the investigation tasks can be accomplished effectively and efficiently. Inform all outbreak investigative team members that any findings are to be reported to this delegated authority. A list of all team members and additional contacts such as administrative contacts, sanitarians/environmental health officers/public health inspectors, local and regional contacts, physicians, clinical laboratories, or other persons who may become involved in outbreak investigations should be assembled.

Before beginning the investigation, check the supply of forms and the availability of equipment suggested in Table A (Equipment useful for investigations) and obtain any needed materials or additional equipment. General resource materials describing signs and symptoms, incubation times, and specifics regarding specimen collection and appropriate kits to be used should be maintained and readily available to those processing the initial calls, which may help to formulate the initial hypothesis.

If the alert or complaint suggests a possible outbreak, inform laboratory personnel of the type of outbreak and estimated quantity and arrival time of clinical specimens and water samples collected. This information will give laboratory managers time to prepare laboratory culture media, prepare reagents, and allocate personnel. At a minimum, the laboratory should have six to eight stool culture kits on hand or readily available, since in many cases, stool specimens must be collected within 72 h of onset of illness to isolate and identify certain pathogens (e.g., *Campylobacter* spp., and *Salmonella* spp.). Consult laboratory personnel about proper methods for collecting, preserving, and shipping environmental samples and clinical specimens if such information is needed. Obtain appropriate specimen containers and sample submission (chain of custody forms) from them.

Once the investigation is underway, the proper clinical specimens should be collected as soon as possible before patients recover and become less likely to submit specimens. All suspected waterborne outbreaks should be examined and a determination made regarding the feasibility of conducting a thorough investigation even if the time to collect proper clinical specimens has passed.

Verify Diagnosis

An ill person or family member, physician, hospital staff member, or operator of a water utility or recreational site may report suspected cases of waterborne illness. Whatever the source of the report, verify the diagnosis by taking a thorough case history and, if possible, by reviewing clinical information and laboratory findings. (This analysis can be further substantiated by detecting suspected etiologic agents in water). Verification of the diagnosis is done in consultation with medical professionals.

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Get Case Histories

When a complaint involves illness, complete Forms C1–2 (Case History: Clinical Data and Case History: Food/Water History and Common Sources) either at the time of initial notification, during a personal visit, or during a telephone call to the person reported to be ill. Use this same detailed interview approach with every person who has been identified in the initial complaint or alert, even though some may not have been ill. Be aware that potential cultural and language barriers can make interviews difficult. A different interviewer may be needed to accommodate these barriers. Continue this until sufficient information is obtained to decide whether there is, indeed, an outbreak of waterborne illness. From persons who are at risk of illness but who remained well, also obtain water and 72-hour food histories, inquire about recreational water exposure in past 2 weeks, and information about their activities in common with the ill persons. Information from these persons is as important to make epidemiologic associations as it is from the cases.

When it is apparent that an outbreak has occurred and a specific event has come under suspicion, substitute Forms D1–2 (Case History Summaries: Clinical Data and Case History Summaries: Water/Laboratory Data) for Form C. Form D1 can be used initially in many routine waterborne illness outbreak investigations where it is obvious that a common-source outbreak has occurred or when all of the ill persons consumed water together (e.g., drank from the same public system, consumed ice at an event) or recreated at the same place (e.g., swam in the same lake or used the same hot tubs). This will simplify recording, because most affected persons will give similar information. At this time, notify the district, state, or provincial epidemiologist about the outbreak.

If a specific pathogen (e.g., norovirus, *E. coli* O157:H7, *Cryptosporidium* spp.) has been identified as the etiologic agent, consider developing a form for recording relevant information. Many state/provincial or national public health agencies have standard forms tailored to specific pathogens. Include signs and symptoms of the illness and other clinical information, the etiology of the agent, and usual methods of transmission. Computer programs (e.g., Epi Info™) can aid in the design of such standard forms.

Upon contact with the affected person, identify yourself and your agency and explain the purpose of the visit or call. A professional attitude, appropriate attire, friendly manner, and confidence in discussing epidemiology and control of waterborne illnesses are essential for developing rapport with affected persons or their families and in projecting a good image of the investigating agency. Keep in mind that you are not interviewing someone you inspect or regulate, but that you are providing a service to the affected person. Exhibit genuine concern for persons affected and be sincere when requesting personal and confidential information.

Communicate a sense of the urgency of the investigation, and emphasize that their participation will make a positive contribution for the control and prevention of waterborne illness. Parental consent must be obtained before interviewing children under 18 years of age. In some locations, consent from the affected person's physician may also be required.

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After asking open-ended questions about the person's food exposures and illness history, follow up with more specific questions to fill in the details and better ensure a thorough recall. Base your level of communication on a general impression of the person being interviewed, considering information about age, occupation, education, or socioeconomic status. Tact is essential. Use either Form C or Form D, as appropriate, as a guide. State questions so that the persons who are being interviewed will describe their illnesses and associated events in their own words. Try not to suggest answers by the way you phrase questions.

Fill in Form C1–2 (if appropriate) and take additional notes during the interview. Ask specific questions to clarify the patient's comments. Think questions through before conducting the interview. Realize that people are sometimes sensitive to questions about age, sex, special dietary habits, ethnic group, excreta disposal, and housing conditions. Nevertheless, any or all information of this type can be relevant. Word questions thoughtfully when discussing these characteristics and habits. Such information can often be deduced from observations. If doubt remains, confirm your guesses by asking indirect questions. Information on recent travel, gatherings, or visitors may provide a clue to common sources or events that would otherwise be difficult to pinpoint. Review known allergies, recent immunizations, recent changes in the patient's medical status, and similar information. Remember that the agents associated with waterborne disease can also be spread by other means such as consuming food, person-to-person, visiting child care centers, animal-to-person in petting zoos, through walk-in-spray fountains, and pools for young children.

As persons describe their illnesses, check boxes next to appropriate symptoms or signs on Form C1. Do not ask about all symptoms or signs listed; however, ask about those marked with an asterisk if the ill person does not mention them. If there are questions, explain symptoms to the patient in understandable terms. The symptoms and signs in the first two columns of Form C1 are usually associated with poisoning or intoxication, although some occur during infections. Those in the third, fourth, and fifth columns are usually associated with enteric infections, generalized infections, and localized infections, respectively. Those in the last column are usually associated with disturbance of the central nervous system.

Diseases in any category will sometimes be characterized by a few symptoms and signs listed in the other columns, and not all signs and symptoms occur for any one ailment or for all persons reporting illness. If an illness seems to fall into one of these categories, mention other symptoms in the category and record the patient's response.

Whenever possible, use physician and hospital records to verify signs and symptoms reported by patients. Clinical data may strengthen or dismiss the possibility of waterborne illness. Before contacting a physician or a hospital, become familiar with laws and codes relating to medical records to ensure that you have legal access to these records. Legal release forms may be necessary to obtain some records. Do not distribute names of patients, their other personal identities (e.g., address, phone number), or their clinical information to unauthorized persons.

The entries begin with the day of illness, followed by the previous 2 days. If the illness, however, began early in the day or before any of the listed meals, modify the entries on the form so that the 72-hour history can be completed in the space pro-

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vided on the form. If the incubation period is 3 days to a week in duration, use additional copies of Form C2 and modify day or day before subtitles.

Signs and symptoms will sometimes give clues to the transmission route by indicating the organ systems affected. If the early and predominant symptoms are nausea and vomiting, ask about the most recently ingested water or beverage within the past 6 h. In these situations, suspect high-acid water supplies, carbonated beverages and fruit drinks, because these tend to leach metallic ions from water pipes and containers. If diarrhea, chills, and fever predominate, be suspicious of water and beverages ingested 12–72 hours before onset of illness for salmonellosis, shigellosis, and norovirus related gastroenteritis. If the incubation period averages 1–2 weeks, consider typhoid fever, cryptosporidiosis or giardiasis. Diseases with incubation periods exceeding 2 weeks (e.g., hepatitis A and E, amebic dysentery, or schistosomiasis) can be handled as special cases for which longer histories would be sought. Others, such as chronic lead and arsenic poisoning, have incubation periods of variable durations and onsets so gradual as to be indeterminable. See Table B (Illness acquired by ingestion of contaminated water: A condensed classification by symptoms, incubation periods, and types of agents) for details on specific pathogens, Table C (Illnesses acquired by contact with water: A condensed classification by, symptoms, incubation period, and types of agents), and Table D (Illnesses acquired by inhalation of microorganisms aerosolized from water. A classification by symptoms, incubation period, and type of agent).

Other microorganisms not listed in Tables B, C, and D that can be potentially spread by water include the bacteria *Klebsiella pneumoniae*, *Mycobacterium avium* complex, *Acinetobacter calcoaceticus*, *Elizabethkingia meningoseptica*, *Stenotrophomonas maltophilia*, *Pseudomonas putida*, *Serratia marcescens*, protozoa *Isospora*, *Microsporidium*, algae *Schizothrix calcicola*. These microorganisms are less frequently identified with waterborne illness, but they may become opportunistic pathogens, particularly for highly susceptible and immunosuppressed persons. Further investigation is needed to confirm their role in the spread of waterborne diseases.

Gather information about all sources of water to which the patient(s) may have been exposed 2 weeks before onset of illness. The water supply and the event that precipitated the illness might not be obvious. Persons often have difficulty recalling exposure to all water sources including; ice or water ingested; aerosols and recreational water contact. Therefore, if the person does not remember specific exposures to water, ask about the water consumed in usual or routine daily habits and the amounts ingested; exposure to recreational waters; and unusual exposures or events attended during this interval. This may stimulate recall of away-from-home water consumption or contact that was unusual. Ask about other risk factors for enteric illness, such as contact with young children and child care centers, animal contact, ingestion of raw foods of animal origin, and usual food preference habits.

For persons who have been traveling, ask them where (both cities and rural areas) they have traveled during the incubation period of suspected agents. Determine if they drank water from any taps or pumps in rural areas they visited. Ask whether unheated (or untreated) tap water or beverages containing unheated (or untreated)

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water or ice was ingested at restaurants, in hotels or at events in the places they visited. Also, ask whether they ingested bottled water and, if so, the brand name. Find out whether they drank water from streams, ponds, springs, or other natural water sources. If they did, ask if they observed any abnormal condition of the water such as algal growth, high turbidity or discoloration. Ask if domestic or wild animals had access to the water.

If they have skin or eye infections or generalized infections, ask them to name all swimming pools, water slides, beaches, lakes, ponds, or other chlorinated and non-chlorinated water courses where they swam, waded or bathed during their trip. Also ask them whether they used any hot tubs, spas, whirlpools, or similar devices. This information sometimes provides clues to common sources or to events that otherwise would be difficult to discover. Record the information on Form C1.

In a protracted outbreak, or when investigating an outbreak of a disease with a long incubation period, expect recall to be poor. In this situation, obtain from ill persons and others at risk a listing of their water, ice, and beverage preferences and amounts usually ingested, or their purchases of these items within the range of the incubation period of the suspected disease. As a guide, draw up a list of either water, ice, and beverages that are commonly consumed by the affected group or those waters, ice, and beverages previously identified as vehicles of the suspected disease under investigation. Summarize data from all copies of Forms C1–2 on Form D. Form D allows rapid review of all exposed persons (ill or not ill) and serves as a basis for analyzing the data.

Obtain Clinical Specimens

Diagnosis of most diseases can be confirmed only if etiologic agents are isolated and identified from specimens obtained from ill persons. Get specimens from the ill persons to confirm an etiologic agent.

- In large outbreaks, obtain fecal specimens from at least ten persons who manifest illness typical of the outbreak
- In smaller outbreaks, obtain specimens from as many of those ill and those at risk as practicable, but from at least two, and preferably ten, ill persons
- Try to collect specimens before the patient takes any medication. If medication has already been taken, collect specimens anyway, and find out the kinds and amounts of medicine taken and the time that each dose was taken
- Also get control specimens from persons with similar exposure histories that did not become ill

Obtain clinical specimens at the time of the initial interview during acute illness or as soon as practicable thereafter. Even though this is not always possible, take specimens even after recovery because etiologic agents may remain in low populations or concentrations. If a disease has already been diagnosed, collect specimens as listed in Table B. If a disease has not yet been diagnosed, choose kinds of speci-

mens that are appropriate to the clinical features. Laboratory information obtained from the first patients may be useful to physicians in the treatment of cases detected later. Apart from the fact that people are more likely to cooperate while they are ill, some pathogens or poisonous substances remain in the intestinal tract for only a day or so after onset of illness. If the patient is reluctant to provide a fecal specimen explain that the specimen will be tested to identify the causative agent and compare it to any agent recovered from the water.

If a disease has not yet been diagnosed, choose specimens that are appropriate to the clinical features. Laboratory information obtained from the first patients may be useful to physicians in treating cases detected later. Some pathogens (e.g., *Salmonella*, parasites) may be recovered for weeks after symptoms have abated. If applicable for the disease under investigation, take specimens even after recovery because some etiologic agents may remain in low numbers, and changes in serologic titers can be detected.

Before collecting specimens, review Table E (Guidelines for specimen collection) and, if necessary, get additional instructions from laboratory personnel and seek their advice on how to preserve the stool specimens if you cannot deliver them to the laboratory immediately. Many public health agencies have special fecal specimen kits. Demonstrate to the patient how to use the materials in the kit, how to complete the form in the kit and how to mail it if you are not going to pick it up. If mailing specimens, make sure that you are aware of the regulatory requirements that may apply to the transport of infectious material.

Stool specimen containers for intestinal parasite examination are not suitable for bacterial or viral examinations because they ordinarily contain a preservative, such as formalin or polyvinyl alcohol. If an inappropriate transport medium is used, a specimen can be rendered unsuitable for laboratory examination.

Feces. If the patient has diarrhea or is suspected of having had an enteric disease, obtain a stool specimen (preferred specimen) or a rectal swab. Instruct patients to provide you with their own specimens by one of the following means.

1. If practicable, give the patient a stool specimen container with a wooden or plastic spoon or a tongue depressor. A clean container available in the home (e.g., a jar, or disposable container that can be sealed) and a clean plastic spoon or similar utensil can be used if laboratory containers are not available.
2. Label the specimen container with the patient's name age/date of birth and date of collection.
3. Collect the stool specimen by one of the following methods:

   (a) Put sheets of plastic wrap or aluminum foil under the toilet seat and push them down slightly in the center, but not so far as to touch the water in the bowl. Sheets of paper can be tacked on the rise of a latrine and pushed down to form a depression in which to catch feces. Take care to ensure that toilet cleaning chemicals and other microorganisms in the toilet bowl do not contaminate the fecal specimen. After defecating, use a clean spoon or other utensil to transfer about 10 g of feces into a specimen container or other clean container.

(b) Defecate directly into a large clean dry container or bedpan. Use a clean spoon or other utensil to transfer about 10 g or the size of a walnut of feces into a specimen container or other clean container.
(c) Scrape feces off a diaper with a clean spoon or other utensil to transfer about 10 g of feces into a specimen container or other clean container.

4. Collect fecal swabs by twisting the cotton-wrapped end of the swab into the stool obtained in one of the ways described above. Follow instructions given in Table E. If necessary, use fecal-soiled toilet paper or cloth diaper and twist a swab into the top of feces. Take care to ensure that there is no carryover of toilet paper as they are impregnated with barium salts which are inhibitory to some fecal pathogens.

Dispose of excess fecal material into the toilet and carefully wrap all soiled articles (e.g., by placing them inside two plastic bags) and dispose of in domestic waste. Check that the specimen container is tightly sealed and properly labeled and place into a clean outer plastic bag (special zip lock bags for clinical specimens, if available). Store the specimen in a cool place, preferably at 4°C to await pick-up or despatch. DO NOT FREEZE.

Feces from Rectal Swabs. Collect rectal swabs by carefully inserting the swab approximately 2.5 cm (1 in) beyond the anal sphincter. Gently rotate the swab. Fecal matter should be evident on the swab.

Vomitus. If the person is vomiting or subsequently does so, arrange to collect vomitus. Tell the patient to vomit directly into a sterile specimen container or a plastic bag. Otherwise, transfer some vomitus from a clean receptacle into the container with a clean spoon. Refrigerate, but DO NOT FREEZE, this specimen until it can be picked up or delivered to the laboratory.

Blood. Take blood if a patient has a febrile infection or when infectious agents are suspected (see Tables B, C, and D). Blood specimens are collected for:

- Bacterial culture
- Detection of antibodies to specific agents
- Detection of certain toxins

Before collecting specimens, get additional instructions from laboratory personnel and seek their advice. Blood should be obtained by an appropriately trained and accredited person (check appropriate laws). Collect blood during the acute phase of illness, as soon as the febrile patient is seen (within a week after onset of illness) and, if comparing of serologic titers, again within 6 weeks (usually 2–4 weeks later) during the convalescent phase. Draw 15 mL of blood (from an adult) or 3 mL (from a child) or 1–2 mL (from an infant). If possible, collect the blood from the same patients from which stool specimens were obtained if both specimens are to be examined. Label tubes and vials at every step of serum transfer. DO NOT FREEZE whole blood because the resultant hemolysis interferes with serologic reactions.

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Blood for culture (for pathogens such as invasive *Salmonella* species, *Vibrio vulnificus*)	Inoculate freshly collected blood into culture bottle supplied by the laboratory
Blood for detection of – Antibodies (to pathogens such as *Salmonella* Typhi, hepatitis A virus, *Toxoplasma gondii*) – Toxins	Collect into a sterile syringe or evacuated sterile tube that does not contain anticoagulants. If practicable, centrifuge the blood at 1,000 rpm for 10 min; pour off the serum into small screw-cap vials and store at approximately −18°C. If the serum cannot be separated immediately, rim the clot with a sterile applicator stick and refrigerate approximately 4°C to get maximum clot retraction if the specimen is to be stored unfrozen overnight. If centrifugation cannot be done, store the blood specimens in a refrigerator until a clot has formed, then remove the serum and transfer it with a Pasteur pipette into an empty sterile tube. Send only the serum for analysis

Urine. Instruct patients to collect urine in the following manner. Clean the area immediately around the urethral orifice with a paper pad that has been pre-moistened with 4% tincture of iodine or other appropriate antiseptic. Then begin to urinate into a toilet and collect 30 mL (about 1 oz) of midstream urine into a sterile bottle. Use either a second antiseptic-moistened pad or an alcohol-moistened cotton ball or tissue to clean any drops from the top or side of the bottle.

Other Instructions. Follow applicable instructions given in Table E. Before or immediately after collecting clinical specimens, use waterproof permanent markers to label each container with the patients name, complaint number, case identification number, specimen number, date and time of collection, tests requested, and other appropriate information. Tightly seal all containers.

Clinical Specimen Collection Report for each specimen. Complete Form E (Clinical specimen collection report). The complaint number, case identification (ID) number, and specimen number must be entered on each report so that laboratory results can later be correlated with other data. On Form C1 record the type of specimen collected, and submit both the specimen and a copy of Form E to the laboratory. Send a copy of the laboratory report to the patient's physician or call if urgent.

Pick Up Water/Ice Samples and Containers that the Patient Collected

If the patient/case or other household member collected any water, ice, or beverage as instructed during initial contact, label containers with the complaint/outbreak and sample numbers. Proceed as instructed in Table F (General Instructions for collecting water samples for microbiological analysis) and complete Form F (Water/Ice collection report) and/or Forms G3–G8 as applicable. Record conditions of collection as called for on the forms. If a hypothesis associates the illness with water,

caution these persons not to use the water source unless the water is first boiled and to discard all previously prepared ice and water-containing beverages until notified otherwise.

Develop a Working Case Definition

Develop a working case definition to classify exposed persons as either cases or non-cases. Start with the most specific symptoms (such as diarrhea and vomiting) rather than broader symptoms such as nausea or malaise. For example in an outbreak of gastroenteritis, a case might be defined as a person from whose stool a specific pathogen was isolated. It may be a person who was at risk and developed diarrhea within a specified period of time. Diarrhea will have to be defined, perhaps as three or more loose, watery stools during a 24-hour period. In some cases, a particular pathogen responsible for the outbreak might have been identified from clinical specimens. A case definition, which is developed later in the investigation, might include either a person having specific signs and/or symptoms within a period of time or a person from whom a specific pathogen was isolated. The ultimate case definition has a tremendous impact on the investigator's ability to make illness and exposure associations and to calculate probability of these associations.

Sometimes the first symptom or sign provides a clue to developing a case definition. Information in Tables B, C, D, G, and H can be useful in making case definitions. Compare newly identified cases with the definition to see whether each is part of the outbreak.

Classify cases into categories:

- A **confirmed case** is a person with signs and symptoms that are clinically compatible with the disease under consideration and for which there is either (a) isolation of an etiologic agent from (or otherwise identified in) an appropriate specimen from the patient, or (b) serologic evidence of a fourfold or greater rise in convalescent antibody titer. A confirmed case must also have possible exposure to the etiologic agent within the incubation period of disease. See Table E.

  Criteria for confirmation of etiologic agent responsible for outbreaks of waterborne illnesses for definitions of confirmed cases for specific waterborne diseases:
- A **presumptive case** is a person with signs and symptoms that are clinically compatible with the disease under consideration, and for which there is laboratory evidence of infection (e.g., an elevated antibody titer but less than a fourfold increase), but the etiologic agent was not found in specimens from patients or no specimens were collected. A presumptive case must also have possible exposure to the etiologic agent within the incubation period of disease.
- A **suspected case** is a person with signs and symptoms that are clinically compatible with the disease under consideration and history of possible exposure, but laboratory evidence is absent, inconclusive or incomplete.

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- A **secondary case** is a person who became infected from contact with a primary (outbreak-associated) case or from a vehicle contaminated by a primary case. Onset of illness for secondary cases typically is one or more incubation periods after the outbreak-associated cases.

It is not essential, however, to classify cases into these categories. Do so only if it aids in developing a final case definition or in making comparative analyses of data. Consider doing analyses using case definitions of both confirmed and combined confirmed, presumptive, and highly suspect cases, and compare the results.

Make Epidemiologic Associations

Make a preliminary evaluation of the data collected as soon as possible. If you decide that there is an outbreak, use the information you have to develop a hypothesis about the causal factors.

Determine Whether an Outbreak Has Occurred

An outbreak is an incident in which two or more persons have the same disease, have similar clinical features, or have the same pathogen (thus meeting the case definition), and there is a time, place, or person association among these persons. A waterborne outbreak is traceable to ingestion of contaminated water or ice or to contact with contaminated water.

A single case of either chemical poisoning or a disease that can be definitely related to ingestion of drinking water or contact with water can be considered an incident of waterborne illness and warrants further investigation. Waterborne methemoglobinemia in an infant who resides in a rural area having a high concentration of nitrates in well water is an example of a single case of waterborne illness due to ingestion. A rare diagnosis such as primary amebic meningoencephalitis in a person who swam in a body of freshwater and inadvertently ingested the ameba, *Naegleria fowleri*, through the nose is an example of a single incident related to water contact.

Sometimes it will be obvious from an initial report that an outbreak of waterborne disease has occurred simply because of the number of persons displaying certain signs and symptoms at or near the same time. Many complaints, however, involve illness in only one or a few persons. It is often difficult to decide whether ingestion or contact with a particular water source and onset of illness was associated or coincidental. Certain diseases that are highly communicable (e.g., shigellosis and epidemic viral gastroenteritis) may result in secondary infections from person-to-person spread or from subsequently contaminated food or water.

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However, if complaints are received from several persons who are associated with ingesting water or contact with water at the same place, water is likely to be involved. Routine review of the log pertaining to potential waterborne illnesses for similar complaints can often be useful in detecting time, place or person associations. An investigation may also proceed based upon the suspicion of an intentional contamination of a water source.

Make Time, Place, and/or Person Associations

A time association exists if the time of onset of similar illnesses is within a few hours or days of each other. Place associations exist when persons have ingested water from a particular single source, have swum in, worked in or otherwise been exposed to the same water, have attended the same event, or reside in an area common to all. Person associations indicate a shared personal characteristic, such as being of the same age group, sex, ethnic group, occupation, social group, or religion. Waterborne illnesses transmitted by a community water supply usually afflict persons of both sexes and all ages throughout the community. Non-community water sources, such as bottled water, ice, water from individual wells, or water from areas of recreation should also be considered when making associations. Keep in mind that water can contaminate foods during washing or freshening, and it can contaminate utensils and vessels that are used to handle or store foods. Water may therefore be a source of contamination of another vehicle. Also, water can be ingested as aerosols generated by shower heads, whirlpools, hot tubs, fountains, cooling towers, and irrigation devices. Once some of these associations become obvious, question other persons who could be at risk because of their time, place, or person associations with the ill persons.

Formulate Hypotheses

From time, place, or person associations that have been established or suggested by the investigation, formulate hypotheses to explain (a) the most likely type of illness, (b) the most likely vehicle involved, (c) where and the manner by which the vehicle became contaminated, and (d) other possible causal relationships. The section "Collection and Analysis of Data" describes calculations that can aid in the formation of these hypotheses. Test hypotheses by obtaining additional information to support or reject them. If the hypothesis includes food contamination, the instructions given in the manual, *Procedures to Investigate Foodborne Illness*, might be useful. Guidelines for confirmation of waterborne outbreaks are presented in Table G and Guidelines for confirmation that water is responsible for illness are presented in Table H.

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Possible Precautionary Control Actions

If there is strong evidence to support a hypothesis that the outbreak is waterborne, take precautionary actions. The choice of action is dictated by the (a) suspected causal agent, (b) size of the water source, (c) availability of alternate water sources, and (d) expected use of the water. On the basis of available information, estimate the population at risk and engage any public relations staff with your organization to help inform all persons potentially impacted.

When dealing with a microbiological contaminant or agent, consider issuing a boil-water advisory with water treatment guidelines (e.g., heating water in a covered container to a rolling boil for at least 1 min and keeping it covered until use). Other options that can be explored include chlorinators that can be installed in individual and non-community systems. For community and non-community supplies in which chlorine is already used, increasing the chlorine dosage and opening hydrants and taps to draw the super-chlorinated water through the whole system might be an option. Increasing chlorine is sometimes not effective because the chlorine contact time is too short or super-chlorinated water does not reach some parts of the system. Furthermore, chlorination is ineffective against *Cryptosporidium* oocysts and requires a long contact time to kill other human pathogens like hepatitis A virus and *Giardia*.

For suspected chemical contamination contact a specialist for further assessment and remedial strategies, such as activated charcoal filters. As a last resort, shut off the contaminated system until the source of contamination is found and controlled. Be cautious when you take this drastic measure, because it may do greater harm than good by causing lack of water for hospitals, nursing homes, or for firefighters to extinguish fires. If the water is shut off or the treatment facility or distribution system disrupted (as in the case of floods or other disasters), consider means to distribute water from an alternative source to healthcare facilities and homes.

If an illness could have resulted from water contact, close the offending water source, post warning signs around it, and patrol the area. Where there is a swimming pool, hot tub, spa, fountain, or whirlpool, evaluate the recirculation system and its operation. It may be that increasing disinfectant concentration by super-disinfection could resolve the problem. Where there may be chronic operational problems, evaluate pH, disinfectant concentration, and bacteriological laboratory records. Choose your course of action, including consultation with appropriate professional experts, depending on the contributing factors existing at the time of investigation.

Verify the effectiveness of these actions (e.g., boil-water advisory, super-chlorination, provision of alternate water source) to protect public health by monitoring illness levels in the population to determine if the outbreak terminates. If the outbreak continues unabated, consider the possibility of other transmission routes. Also, verify the effectiveness of repairs to the water system, super-disinfection, and other actions by closely monitoring the quality of the water supply or recreational water to determine if laboratory reports indicate that the water is now safe for consumption or contact.

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Inform the Public

If there is a public health threat, work with any available public relations staff to announce the outbreak in the mass media so that the public who consumed or was otherwise exposed to the implicated water can be alerted to take appropriate action including seeking medical consultation or treatment. Provide only objective factual information about the outbreak. Coordinate among the investigating agencies to assure that a consistent and accurate message is delivered. It is easy for agencies to miscommunicate before and during a water crisis (See Box 1, False Alarm; Box 2, The Walkerton Outbreak; Box 3, The Flint Water Crisis). It is often preferable to have one spokesperson for all agencies. **Do not release preliminary information that has not been confirmed**. The person giving information about an outbreak should be well informed about the etiologic agent being investigated and prepared to deal with questions. If the health hazard warrants a public warning at the hypothesis stage, tell the public why emergency measures are being invoked and that subsequent information may be cause to modify the action. As the investigation proceeds and the etiologic agent is confirmed and contributory factors are identified, consider terminating emergency measures, and give advice on specific control and preventive measures. Attempt to reach all segments of the population at risk; this may require communication in multiple languages. Route all news releases or statements to all persons involved in the investigation. In situations involving large outbreaks or highly virulent or toxigenic etiologic agents, set up an emergency hotline for the public to call to ask questions. This is likely to occur if there is an intentional contamination event where there is high publicity and public concern. Train staff to handle these calls in a consistent manner so that the advice is the same who gives it. Faulty information derived from poorly tested hypotheses can lead to severe political, legal or economic consequences. An example of this occurred in Sydney, Australia, in 1998 when an apparent water contamination event was publicized for the public to take precautionary actions. The false alarm was costly because of rebates to water customers, additional water testing, and for hiring extra staff, as well as a loss of confidence in the facility (see Box 1, False Alarm). They may then be disseminated by the mass media with inappropriate interpretations of the public health significance. Furthermore, this information may be used as an unrealistic base for water programs or water regulations because of either misinterpretations or pressure from misinformed consumer–advocate groups. All involved parties should follow a written protocol for cross-agency communication and release of information to the public. Unreasonable delays are unacceptable.

Expand the Investigation

Test hypotheses by obtaining additional information to either confirm or refute their validity. Do this by case–control or cohort studies, additional laboratory investigations, and on-site investigations (e.g., laboratory reports of water testing).

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Box 1 False Alarm: The Impact of a Poor Water Pathogen Oversight System

Sydney Water (a New South Wales state-owned corporation) supplies 1600 million liters of water each day to 1.5 million properties in Sydney and its outlying areas. The city has a large and complex catchment with nine major dams and several storage reservoirs. About 21,000 km of water main, almost 200 pumping stations and many tunnels deliver water from four main river systems. The water is filtered through eleven treatment plants. Seven are owned by Sydney Water and four are privately owned. These plants provide 90% of Sydney's drinking water and one plant, Prospect, provides up to 80%. In 1998, the quality of Sydney's drinking water came under acute review when *Giardia* and *Cryptosporidium* were found in the city's main water supply at the Warragamba Dam. Initially, low levels of these parasites were first detected in the water supply on 21 July, but these were within the acceptable health limits. In days following, much higher levels were recorded, and on July 27 the first "boil water" alert (in which residents were instructed to boil their tap water before use) was declared for the eastern Central Business District of Sydney. However, by late on July 29 high readings were found in samples at the Prospect Filtration Plant, in a reservoir and at a location further down the system, and a "boil water" alert was issued for the south of Sydney Harbour, and on July 30 a Sydney-wide "boil water" alert was issued affecting most of Sydney's residents. On August 4 the warning was discontinued. However, high levels were again found on August 13 (the second event), although it was believed that most organisms would likely be dead. More positive readings were found on August 14, although at lower levels. Further contamination was identified on August 24 and an extended boil water alert was again declared. This was progressively lifted suburb by suburb until further contamination was reported on September 5 (the third event). A 2-week alert was then instituted, which was finally lifted on September 19. It was determined that the parasitic contamination was caused by low-quality surface water entering the dam. This contaminated source was attributed to moderate rainfall in July, followed by heavy rainfall in August and September which caused intermittent supplies of the raw water to enter the dam. Despite high levels of *Cryptosporidium* (up to >12,000 oocysts) and *Giardia* (up to >7600 cysts) being recorded in July and August, 1998, no increase in human cryptosporidiosis or giardiasis was detected in the exposed population.

The incident was highly publicized and caused major a public alarm because the number of people affected, the on and off boil water alerts, and the fact that the filtration plant had been advertised as one of the best in the world. The economic and political repercussions were extensive. The cost of the crisis to Sydney Water was estimated at A$33 million which included $20 million paid in rebates to customers, $13 million in lost revenue, water testing and staff costs and at least $2.5 million for damages claims. These costs exclude those relating to improvements to the system and infrastructure. The lack of cases of cryptosporidiosis, giardiasis or other water-related health problems led to suggestions that the parasites were either not an infectious type, or not as extensively distributed. An inquiry after the event revealed the publicity as an exaggeration of fact, with Australian Water Technologies, part of Sydney Water, severely overestimating levels of *Cryptosporidium* and *Giardia* present in the water, with the recorded levels exposed to consumers as not harmful to human health. The handling of the crisis by State-owned Sydney Water was heavily criticized, causing the resignation of both the chairman and the managing director, and bringing up issues of private vs. public ownership and scientific uncertainty. The eventual consequence of the State Inquiry was the establishment of the Sydney Catchment Authority in 1999 to assume control of Sydney's catchments and dams, while Sydney Water maintained responsibility for water treatment and distribution and for sewage collection, treatment and disposal.

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Obtain Assistance

If an outbreak investigation requires resources beyond your agency's capacity, request assistance from other health professionals. It is desirable to have a team including, if feasible, an epidemiologist, an engineer, a microbiologist, a sanitarian/ environmental health office/public health inspector, a chemist, a physician and others, to undertake a detailed waterborne illness investigation. Such personnel can usually be provided by local, state/provincial, or national agencies concerned with health, environment, or agriculture, depending on the expertise needed. For events suspected to arise from intentionally contaminated food, contact emergency response or law enforcement agencies.

Find and Interview Additional Cases

Continue to search for and interview both ill persons who have had time, place, or person associations with the identified cases (see the section on "Make Time, Place, and/or Person Associations").

Review recently received complaints in the water-related complaint log (Form B). Contact other nearby health agencies, hospital emergency rooms, elderly care centers, and local physicians to discover other epidemiologically related cases. Call previously contacted persons to see whether they know anyone else who has become ill or had a common association suggested by data in the log. The illness you are investigating may be part of a larger multijurisdictional outbreak, and therefore communicate with adjoining local and state agencies to learn if they are seeing similar illnesses. State or provincial public health agencies can check reportable disease records and state/provincial public health laboratories can start looking for clusters in isolates that they are characterizing. For outbreaks where intentional contamination of water is suspected or confirmed, public health and law enforcement agency officials may conduct the investigation jointly.

If it becomes apparent that an outbreak is associated with a specific water supply (source) or recreational water or event, use Form D1 for recording information. At this stage of the investigation, interviews can be expedited by reviewing the event itself to stimulate each person's recall. Ask about specific symptoms and signs that are known to be common to the syndrome, as well as, time of ingestion or contact with water and onset of illness. Mention each source of water to which the person may have been exposed, and ask each person (whether a case and well persons at risk) which of the water sources had been ingested or contacted.

The number of persons to be interviewed depends on the number exposed and the proportion of them who are probably affected; if fewer than 100 persons were at risk, try to interview all of them; if several hundred are involved, interview a

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representative sample. Be sure to obtain clinical specimens from these cases and well persons at risk (controls). It is more difficult to obtain positive results if symptoms from persons have ceased. There may be situations where self-administrated questionnaires are sent to cases and persons at risk. Use either Form C or Form D or modified versions for this purpose. After questionnaires have been completed, summarize the data on Form D. Also, identify and interview secondary cases if they become apparent.

Because no two waterborne disease outbreaks are identical, the order of the expanded investigation may not always follow the outlined sequence of procedures. Some investigative steps can usually be done simultaneously by different investigators. Additional procedures may also be required. The principles and techniques described will suffice for most investigations. Modify forms, if necessary, to accommodate the type and amount of information to be collected.

Sources and Modes of Contamination and Ways by Which the Contaminants Survived Treatment

Make on-site observations. Prove or refute hypotheses developed during the epidemiological portion of the investigation. Focus on sources and modes of contamination and ways contaminants could survive and pass through water treatment. As applicable, conduct an on-site investigation of source (lakes, streams, areas around groundwater, etc.), treatment facilities, distribution lines, cross connections, water reservoirs, places of recreational water contact and/or sites at which aerosols were generated. Such an epidemiologically focused investigation is quite different from sanitary surveys done during routine evaluations of water source sites, treatment plants or recreational water facilities.

Not all drinking water (even municipal and bottled water) is disinfected; so, it is important to identify whether the water source is treated and if so, how. Some treatments (filtration, reverse osmosis, membrane treatments, riverbank filtration, and others) may not be complemented with a disinfection step. Sanitary survey information can provide information about potential sources of contamination in the area of a usually pristine water source. Microbiological records of a water supplier, particularly if any total coliform positive samples were found by the system in the last 6 months, may help identify a contamination pathway. If significant matters relating to water quality are observed or otherwise identified during the investigation, note them and communicate them to those responsible for the water system and to the proper authorities. Do not lose the focus and objectivity of the investigation by confusing matters of quality and aesthetics with factors related to contamination by, and survival of, infectious and toxic agents. Use the HACCP-system, also known as systems analysis, way of thinking in your investigation.

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Plan On-Site investigation

Contact the person with the highest responsibility for the operation and maintenance for the implicated water source, water treatment facility, and/or distribution lines. Identify the types of records that ought to be reviewed during the investigation and their likely source. Do not forget that the responsible authorities also can have records (about water quality, if there has been a change of municipal water supply, industrial water pollution, wastewater pollution). They can be good sources of information about recent pipe breaks and other water system issues that could be related. In many cases they will be aware of the potential for contamination upstream of source water intakes. If applicable, obtain water distribution maps and recent water quality reports from appropriate departments. If you are not familiar with the community in which the investigation is to be done, obtain maps of the area to locate streams, lakes, water treatment facilities, and other community features that might have a bearing on the investigation. Check if there are water protection areas and their rules. Get plans and specifications on design of treatment facilities from consulting engineers or state agencies that approve these facilities. Contact weather bureaus, airports, radio/television stations, or newspapers for information on heavy rainfall, flooding, extremely low temperatures, droughts, or other unusual weather conditions that preceded the outbreak, if this information is unknown to investigators. Contact police or fire departments about traffic accidents, which can be the source of the outbreak. Review all background data pertaining to the suspect water. As information is gathered, record it on applicable parts of Form G.

Discuss with laboratory personnel that a field investigation will be made, and get their suggestions regarding samples and specimens that should be collected (see Tables E and F). Confer with them about special analyses, media, and sampling procedures; make arrangements for rapid transport of samples to the laboratory. The samples must maybe be transported at the right temperature. Pick up appropriate forms and sample collection equipment (preferably preassembled in a kit—see Table A). The laboratory can probably help assemble this kit.

Identify Contributory Factors of Outbreaks

During the investigation, identify factors that contributed to contamination and survival of the etiologic agents and perhaps also to their growth or amplification or another cause of the outbreak. Identified factors and situations that have contributed to waterborne disease outbreaks include those listed in Table 2.

Focus the investigation on the potential situations listed in Table 2, as applicable. Remember that other possibilities can occur. Describe circumstances that contributed to contamination and that permitted the etiologic agent to survive so that it reached drinking, agricultural, industrial, or recreational water. Also describe circumstances that allowed pathogenic bacteria or algae to multiply in the water. Write your findings down on the back of Form G1 (Illustration of contamination flow) or on a separate sheet. Continually update the listing in Table 2 with newly available data.

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Table 2 Factors that have contributed to waterborne disease outbreaks according to various water sources and the following systems

Source/system	Factors
Surface water	Ingestion of untreated surface water
	Contamination of watershed by human or animal feces
	Use of contaminated surface water for supplementary water source
	Water from sewage treatment facilities
	Overflow of sewage or outfalls near water intake
	Heavy rains and/or flooding
	Contamination from algal blooming
	Dead animals in stream or reservoir
	Live animals and birds in stream, reservoir or watershed
	Poorer quality of water supply for economic reasons
	Accidental industrial pollution of water
	Traffic accidents with transportation of chemicals
	Fire drill sites—fire foam
Groundwater	Overflow or seepage of sewage into well or spring
	Surface runoff into well or spring
	Contamination through limestone or fissured rock
	Heavy rains and/or flooding
	Contamination by pesticides or other chemicals
	Seepage from abandoned well
	Contamination of raw-water transmission line or suction pipe
	Improper well construction and lack of maintenance
	Surface water percolation
	Migrating landfill leachates
	Contamination from grazing animals and from their manure
	Pests (e.g., rodents, snakes) can come into well
Inadequate treatment of water or other problems in facilities	No disinfection or too much disinfection
	Inadequate concentration or contact time of disinfectant
	Interruption of disinfection
	Leakage of sewage water to the drinking water (e.g., from floor drains)
	Inadvertent by-pass of treatment process
	UV-light treatment not functioning (improper cleaning and maintenance of lamps/bulbs)
	No functioning of alarm system
	Inadequate filtration
	Inadequate prefiltration treatment
	Excessive fluoridation
	Excessive dosage of process chemicals
Storage/transportation deficiencies	Unprotected storage tanks, reservoirs, pumping stations, reservoirs, hydrants or tanks

(continued)

Table 2 (continued)

Source/system	Factors
	Contamination of cistern or individual storage facility by surface water runoff, sewage seepage or nearby animal clustering (e.g., feed lots)
	Leakage of sewage water to the drinking water
	Improper or no disinfection of new storage facility
	Lack of maintenance
	Microbial growth in water reservoirs
	Microbial after growth in pipes and tanks
	Unsuitable material in contact with water
Distribution/plumbing deficiencies	Back siphonage
	Cross connections
	Illegal connections
	Corrosion inhibitors not added when the water supply is known to have industrial chemicals
	Contamination of mains during construction or repair
	Water main and sewer in same trench or inadequately separated or inadequately overpressure
	Improper or no disinfection of mains or plumbing
	Unaccounted water loss
	Unauthorized tap-ins
	Frequent line breaks
	Lead pipes not replaced, especially where the water has a low pH
Other factors from ingestion of water or ice	Use of water not intended for drinking
	Contaminated buckets and other containers
	Drinking water bottles used for chemicals (unlabeled)
	Contaminated drinking fountains
	Contaminated taps
	Deliberate contamination/vandalism
	Contaminated ice
	Hand scooping of ice
Water contact	Puncture injuries or wounds
	Swimming or wading in parasite-infested waters
	Swimming water with contaminations from animals
	Snails in water
	Algal blooms in swimming water
	Sewage contamination of swimming water
	Improper pH adjustment
	Improper chlorination or other disinfectants
	Excessive process chemicals
	Improper filtration
	Rough pool wall construction
Aerosolized water	Stagnation of water
	Water temperatures conducive to growth of pathogen

(continued)

Table 2 (continued)

Source/system	Factors
	Dead ends in water distribution lines
	Generation of aerosols
	Poorly maintained air conditioning units
	Poorly maintained humidifier for fruits and vegetables in grocery stores
	Poorly operated and/or maintained water systems
	Contaminated cooling towers during sustained heat spells that tax air-conditioning systems
	Excessive exposure to showers, running faucets, waterfalls, irrigation and misting systems

Meet Managers

Introduce yourself (who you are, where you come from, who ordered you there) to the owner, resident, or persons in charge and state your purpose, when you arrive at the place of the suspected contaminated water source. Emphasize that your visit is to confirm or eliminate suspicion that this water was a source of illness. Tell him or her that a complete epidemiologic study is in progress and that other possible sources (such as food) will be investigated as well as operations of this site. Explain that your investigation is not to fix blame but to identify the cause of the outbreak. Emphasize that the findings can yield benefits related to the ability to identify needed improvements, to educate staff and to provide public support. Try to create an atmosphere of cooperation. Maintain an open mind and try to answer all questions. If you can not answer a question, tell the person that you will come back with an answer. Come back to the person within 1 or 2 weeks even if you do not have any new information. Give the person your phone number and e-mail address and tell the person to contact you if the person has more information later.

Privately interview key persons responsible for operating or repairing water facilities. Do not forget to interview persons from other work shifts. Identify persons who were working there at the likely time of contamination and have since left and interview them as well. Ask questions to determine the flow of water and operations from intake through distribution through plumbing systems. Ask about any changes in operation, unusual events in the watershed or repairs to the water facilities. Ask if you can check records, both in paper form and on the computer (monitoring system), analyses of results, and/or incident reports.

Plant personnel may not describe water treatment or installations exactly as they existed at the time that a mishap occurred. They may fear criticism or punitive action as a result of their possible role in the causation of the outbreak. Their descriptions should be plausible and should account for possible sources and modes of contamination and indicate possibilities for survival of pathogens. If a description does not contain all the information desired, reword questions and continue the

inquiry. Confirm accounts by private interviews with others knowledgeable of water treatment or operation of the facility. Be alert for inconsistencies among the accounts told by different persons.

Seek resolution of discrepancies in accounts by watching actual procedures as they are being carried out, by taking appropriate samples, or by conducting experiments. A communicative working relationship between the plant management and the investigator influences plant workers' attitudes toward the investigative team. Consider the position, feelings, and concerns of the manager and staff; defensive reactions are normal on their part.

Diagram each phase of the water system or situation under study on Form G1 (Illustration of contamination flow). Insert special symbols and notes for all sites that might be involved in introducing contamination to the water or where contaminants might have survived treatment. Record other information gathered on the appropriate parts of Forms G2–8.

Gather and Review Records

Review and collate appropriate information on quality control and operational records from the water utility and from responsible agencies. As applicable, obtain information on quality of untreated surface or groundwater from a local, state/provincial, or national pollution control or geological survey agency. Also, seek water distribution maps, well logs, descriptions of geological conditions and indices of groundwater quality from them. For surface water supplies, obtain information on upstream discharges and unusual events that may have affected raw water quality.

Get data on finished water quality in the distribution system from a local, state/provincial water surveillance or regulatory agency. Water suppliers also frequently have records of raw and finished water quality. Review data on quality control tests (e.g., pH, chlorine residual, chlorine demand, bacteriological and chemical tests, turbidity, jar test data, incident reports) that are available. Obtain data on cross connection control programs and sewer repairs from the water supplier or other local agencies (e.g., building inspectors, sewage departments). Review files for data concerning potential sources of contamination for individual or semipublic water supplies (e.g., diagrams of septic tank systems, sewer line locations, well logs, small individual wastewater plants, accidental industrial pollution of water, traffic accidents involving chemicals, salting of roads or sawmills).

Check if they have any HACCP-systems or water safety plan and, if so, how they monitor their CCPs (critical control points) and if they are implementing control measures. Ask them about HACCP, to see if they understand the system and if results are documented. Check if the HACCP-system is validated (should be documented) and that they are conducting internal audits.

Get information on all aspects of normal operations as well as unusual events or conditions to determine whether such events were coincidental with the time of

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suspected contamination as determined from the epidemic curve. Also, consider the time it takes for a contaminant in the raw water or treatment plant to reach households in the affected community. Ask responsible persons for this information.

Compare data on heterotrophic plate and total coliform counts of raw and finished water leaving the treatment facility and of water in distribution lines. Also, compare data on chlorine residuals within the plant with that in distribution and check, if they have, that the UV-light is functioning. Review other test data (e.g., turbidity and chemical analyses) that may indicate a problem situation. Identify locations and dates of sample collection. Take photos, if it is allowed, of things you suspect are not right. Go back more than one incubation period of the disease under investigation. Record this information on Form G2 (Record review of on-site investigations, and test results prior to and during outbreak). Photocopy appropriate records for confirmation and subsequent review and attach them to the record review form. Be alert for evidence of falsification of records. While reviewing records, watch for evidence of the following:

- Potential contamination of groundwater systems because of proximity to septic tank systems, latrines, animals manure or landfills, industrial contamination of the water supply, small sewage plants, especially old and nearly forgotten ones, and recent heavy rain
- High heterotrophic plate or coliform counts, or counts that exceeded the average (median) or typical count
- Sudden changes in water quality or operating practices that suggest the possibility of contamination or treatment failure
- High turbidity, unusual odor, color, or taste, or high coliform counts in raw water, which can indicate potential overloading of the normal treatment process
- High levels of ammonium, nitrate and nitrite, which can indicate organic and inorganic contaminants
- Low chlorine residuals in treated water or higher-than-normal amounts of chlorine used, which can indicate either a high chlorine demand or a sudden high level of contamination
- A sudden drop in amount of disinfectant used, possibly indicating failure or interruption of a disinfection process. No functioning alarm system
- A sudden change in the amount of a chemical (e.g., alum or ferric sulfate) used, suggesting equipment disfunction or inadequate coagulation or flocculation and thus poor filtration
- Lack of treatment chemicals if a more corrosive water supply is used (see Box 3, The Flint Water Crisis)
- Pump failures, draining of distribution lines or reservoirs, or massive pumping to fight fires, which can produce low pressures that can cause contamination through cross connections or back siphonage
- Repairs to water mains, wells or pumps where contamination could have been introduced

Record this information on Form G2 or other appropriate form in the G series.

Conduct On-Site Investigations

As applicable, investigate the water source, treatment facility, distribution and plumbing systems, sites where water was contacted, and sites at which microorganisms amplified and aerosols disseminated. Use forms in the G series as guides while observing facilities, gathering data, making measurements and collecting samples. Google or Bing maps or other similar resources' views of the watershed can be very helpful in identifying potential sources of contamination that you will need to investigate further. These maps can also facilitate your own map and diagram making on Form G1.

Investigate the Water Source

The water source may be surface or ground or in some cases a combination of the two. Verify this by observations at the site and by talking to the property owner or persons responsible for operation or maintenance of water supply or recreational facilities, as applicable. Examination of "weather events" such as heavy rainfall may indicate a potential for surface water contamination (See Box 2, The Walkerton Outbreak).

When a surface water is either suspected or implicated as the source of a contaminated supply, get information about the watershed concerning possible sites of contamination of the suspected etiologic agent. This includes, but is not limited to (a) land use, (b) sewage effluent from treatment plants and septic tanks, (c) industrial plants that may be discharging toxic waste, (d) mining wastes, (e) landfill leachates, (f) slaughterhouse discharge wastes, (g) animal feed lots, (h) both domestic and wild animals that use the source water for drinking, (i) sludge disposal from sewage treatment plants or septic tanks (e.g., land spreading or lagoons), (j) storm water discharge. If this information is not available from records or persons familiar with the site, visit the site and observe possible sources of contamination and pollution (e.g., while traveling by foot, vehicle, boat, or helicopter, as applicable). Record this information on Form G3 (Source and mode of contamination of surface water). Diagram the surface water and sites of contamination on Form G1. Note type and location of sources of contamination and their distances from the water.

Visit groundwater sources. Using Form G4 (Source and mode of contamination of groundwaters) as a guide, question owner or operator and inspect groundwater installations to ascertain character of the land and surface and subsurface soil and water. When a well or improved spring is under consideration as the source of the contaminated supply, observe its location relative to possible sites of contamination and to whether its construction allows contaminants to reach the water. Determine locations of all sewage outflows or disposal sites (e.g., septic tanks and absorption lines, cesspools, privies, and other sewage disposal facilities), gradients, and distances from the well or spring. Determine the type of soil at the site. If the soil is limestone or fissured rock or if there is a high ground or perched water table, pollution may travel many miles. In this case, the search for sources of contamination may have to be expanded for a considerable distance from the well or spring. Ascertain whether there were heavy rains, heavy snow melts, or sudden discharge

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Box 2 The Walkerton Outbreak

In May, 2000, many people in Walkerton, a small Ontario, Canada, community of about 5,000 people, began to simultaneously experience bloody diarrhea and other gastrointestinal infections. On May 8–12, torrential rain had unknowingly contaminated the town's water system, but operators failed to check residual levels for a period of several days, allowing unchlorinated water to enter the distribution system. However, the privately-owned Walkerton Public Utilities Commission insisted there was no problem with the water despite other laboratory tests showing evidence of *E. coli* contamination. Illnesses began about May 18, with the first death occurring on May 22 and the seventh and last on May 30. By May 21, however, many more cases had been diagnosed, the infectious agent determined to be *E. coli* O157:H7, and contaminated well water was confirmed as the source of the *E. coli*; all this allowed the region's Medical Officer of Health to issue a boil water advisory, warning residents not to drink the tap water. Two days later, laboratory results identified the presence of *Campylobacter* and *E. coli* O151:H7 and DNA testing showed that the contaminating source was a cattle farm a short distance from a well used for the water supply. By the time the outbreak was over, >2300 were ill and 7 had died. The people who died directly from drinking the *E. coli*-contaminated water might have been saved if the Walkerton Public Utilities Commission had admitted to contaminated water sooner. Those in charge of the water utilities at the Commission had no formal training in their positions, retaining their jobs through three decades of on-the-job experience. They were later found to fail to use adequate doses of chlorine, fail to monitor chlorine residuals daily, make false entries about residuals in daily operating records, and misstate the locations at which microbiological samples were taken. Regulations state that water suppliers are required to treat groundwater with chlorine to sufficiently neutralize contaminants and sustain a chlorine residual of 0.5 mg/L of water after 15 min of contact. Had utility operators adhered to the protocol, the disaster most likely would have been averted. The operators knew that these practices were unacceptable and contrary to Ministry of Environment guidelines and directives; they eventually admitted falsifying reports and were sentenced to short jail terms. The Ontario government was also blamed for not regulating water quality and not enforcing the guidelines that had been in place. The water testing had been privatized in October 1996. An enquiry found that the water supply, drawn from groundwater, became contaminated with the *E. coli* O157:H7 strain from manure from cattle on a farm washing into a shallow water supply well after heavy rainfall. The risk of contamination from farm runoff into the adjacent water well had been known since 1978. Key recommendations from the enquiry included source water protection as part of a comprehensive multi-barrier approach, the training and certification of operators, a quality management system for water suppliers, and more competent enforcement, which were incorporated into Ontario new legislation. The bottom line of the enquiry was that officials and municipal water facilities operators and managers across North America need to recognize public waters are a most valued but vulnerable public resource. Investment in keeping them safe and secure needs to be a community top priority.

from dams that could have resulted in flooding within the duration of the incubation period of the disease under investigation.

Obtain information on the depth of the well in reference to the ground water table from the owner or by referring to any available well logs on public file or from local drillers. Observe well construction and get information about depth of casing, depth and method of grouting, and whether there is an underground discharge. Observe whether there is an impervious well platform and whether the pump or casing seal was subjected to flooding. Illustrate the situation by showing location of the well in

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reference to possible sites of contamination on Form G1. Note distances between the well and contamination sites and elevations. Determine whether any pumps were out of order or had been repaired during the interval of concern. If priming of the pump was done, find out the source of the water used. Record this information on Form G4. Test hypotheses of modes of contamination by conducting a dye test and/or sampling the water. (See appropriate sections of this manual for directions.)

Collect samples of water from these sites and submit them for analysis of the suspected etiologic agent or for any physical, bacterial, or chemical tests that will provide evidence of contamination or movement of the contaminants. (See Procedures for collecting water samples) Record these results on Forms G3 or G4 and I (Laboratory Results Summary). When appropriate, confirm hypotheses by a dye or other tracer test. (See section on this subject).

Investigate the Water Treatment Facility or Individual Treatment Devices

Determine the means by which the etiologic agent survived treatment or was otherwise not eliminated or inactivated. Consider the treatment process as a series of barriers placed between contaminants and consumption of the treated water. The operation of each barrier should be optimized. Review available data for each step in the treatment process. Records of well-maintained and properly calibrated continuous monitoring equipment will be especially valuable. Look for failures in the barriers, which could include (a) lack of disinfection, (b) inadequate concentration of disinfectant or contact time, (c) interruption of disinfection, (d) inadequate filtration, (e) lack of corrosion inhibitors, which may follow inadequate pre-filtration treatments. In 2015 in Flint, Michigan excessive levels of lead were found in drinking water from corrosion of water distribution pipes (see Box 3, The Flint Water Crisis). Corrosion inhibitor had not been added. Also, look for possible introduction of contaminants within the treatment process, such as in treatment chemicals.

Box 3 The Flint Water Crisis

This event is considered a disaster, still unfolding, initiated from a political decision to save money, and ending up with acute and chronic illnesses and deaths to residents of a Michigan city, as well as high system remediation and health-related costs to the taxpayer. On April 24, 2014, Flint, Genesee County, Michigan, switched its water supply from Detroit's system to the Flint River as a cost-saving measure for the struggling, majority-black city on the recommendation of the state-appointed emergency manager. Flint agreed to separate from the Detroit Water and Sewerage Department and go with the Karegnondi Water Authority, including the decision to pump Flint River water. This was to be an interim measure until a new pipeline from Lake Huron was constructed in 2016 to serve the region. Soon after the switch, residents begin to complain about the water's color, taste and odor, and to report rashes and concerns about bacteria. In August and September 2014 city officials issued boil-water advisories after coliform bacteria were detected in tap water. In October 2014, the Michigan Department of Environmental Quality (MDEQ) blamed the cold weather, aging pipes and a population decline. In the same month a General Motors plant in Flint

(continued)

stopped using municipal water, saying it was rusting car parts. On January 4, 2015, the city announced that Flint's water contained a high level of trihalomethanes, a byproduct from increased disinfection by the city. Though this is in violation of the Safe Drinking Water Act, officials told residents with normal immune systems that they have nothing to worry about. In January 2015, Detroit's water system offered to reconnect to Flint, waiving a $4 million connection fee but the offer was declined by the emergency manager. By February, State officials continued to play down any water problems saying that the water was not an imminent "threat to public health." On February 18, 104 parts per billion (ppb) of lead were detected in drinking water at a Flint home and the federal Environmental Protection Agency (EPA) was notified. The EPA does not require action until levels reach 15 parts per billion, but science indicates that there is no safe level for lead in potable water. Officials from EPA and MDEQ discussed the lead level in the sample, and EPA found that the State was testing the water in a way that could profoundly understate the lead levels. On March 3, 2015, a second testing detected 397 ppb of lead in Flint drinking water. A consultant group hired by Flint, reported that the city's water met state and federal standards, and it did not specifically report on any lead levels. In May, tests revealed high lead levels in two more homes in Flint. In July, an EPA administrator told Flint's mayor that "it would be premature to draw any conclusions" based on a leaked internal EPA memo regarding lead. However, in September, Flint was asked to stop using the Flint water supply or consider corrosion control for it, because it was causing lead to leach from the water pipes and children had high levels in their blood. State regulators insisted the water was safe. Nevertheless, on October 1, the Governor of Michigan ordered the distribution of filters, the testing of water in schools, and the expansion of water and blood testing after a briefing on the lead problems with the MDEQ and federal officials. At the same time, Flint city officials urged residents to stop drinking water. On October 16, Flint reconnected to Detroit's water system, and residents were advised not to use unfiltered tap water for drinking, cooking or bathing. On October 19, the Director of MDEQ reported that his staff had used inappropriate federal protocol for corrosion control, and soon after, the Governor announced that an independent advisory task force would review water use and testing in Flint. On December 9 Flint added additional corrosion controls, and soon after an emergency was declared. At the end of December, the task force found that the MDEQ was accountable for its lack of appropriate action, and the Director resigned. On January 16, 2016, the Governor asked the National Guard to distribute bottled water and filters in Flint, and President Obama declared a state of emergency in the city and surrounding county, allowing the Federal Emergency Management Agency to provide up to $5 million in aid.

Three days earlier the crisis expanded to include Legionnaires' disease, because of a spike in cases, including ten deaths, after the city started using river water. On January 21, the Michigan Department of Health and Human Services stated it did not have enough information to conclude that the increase in cases was related to the ongoing Flint water crisis, although the. head of Michigan's Communicable Disease Division had said three months earlier that the number of *Legionella* cases at that time "likely represents the tip of the iceberg." As of February 2016, the number of reported cases was close to 100. A Flint hospital official was surprised that Michigan and local health agencies did not inform the public about the Legionnaires' outbreak in Genesee County in 2014–15 until January 13; the hospital earlier had spent more than $300,000 on a water treatment system and bought bottled water for patients. The source of *Legionella* is not known but it was likely in the Flint River, and possibly extensive flushing of Flint's colored water, which had undesirable odors and tastes, by residents may have caused chlorine residual in the pipes to be washed away, leaving the pipes susceptible to growth of the *Legionella*; in addition, aerosols from the extensive flushing from turned-on faucets might have led to close contact between the residents and the pathogen. The investigation of the cause of the illnesses continues with criminal charges laid against Michigan departmental employees.

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Observe treatment processes from the water inlet to the finished water discharge. Diagram on Form G1 the treatment process; insert notations of hazardous situations that were observed. Collect samples of water at the inlet, after each phase of treatment that may have functioned suboptimally or failed, and at the outlet. Test the samples for pathogens that cause a syndrome characteristic of that being investigated, for indicator organisms and for physical and chemical characteristics of the water, as appropriate to the situation.

Evaluate effectiveness of the disinfection process and resulting residuals. Determine the type of disinfectant (e.g., gaseous chlorine, hypochlorite, chlorine dioxide, chloramine, ozone, ultraviolet irradiation) used and whether the disinfection treatment was adequate for the volume of water treated. Determine, by talking to water treatment plant employees and reviewing records of the plant or regulatory agency, whether there were any interruptions of disinfection during the two weeks prior to the first onset date. Determine contact time between the point of addition of the disinfectant and the first point of use. Measure the chlorine residual, pH and temperature of the water just before it leaves the plant. Observe the condition, operation, and maintenance of disinfectant dispensing equipment. Review plant records to identify any sudden changes in disinfectant demand that causes temporary depletion of disinfectant residuals and allows survival of pathogens. Review maintenance records for disinfectant dispensing equipment and quality assurance records for online analyzers. Record this information on Form G5a (Disinfection failures that allowed survival of pathogens or toxic substances).

Calculate disinfectant rate applied and usage (see formulae in Form 5a). For example, to calculate disinfectant rate, if the flow rate is 1,000,000 gal/day and the dosage is 15 lb/day:

$$
\begin{aligned}
&15\,\text{lb/day} \div 1{,}000{,}000\,\text{gal/day} = 0.000015\,\text{lb/gal} \\
&0.000015\,\text{lb/gal} \times 454{,}000\,\text{mg/lb} \times 0.264\,\text{gal/L} = \\
&1.8\,\text{mg/L disinfectant rate applied}
\end{aligned}
$$

The destruction of pathogens is dependent on (a) type and condition of microorganisms present, (b) type of disinfectant used, (c) concentration of available chlorine or other disinfectant, (d) contact time, (e) water temperature, (f) pH, (g) degree of mixing, (h) presence of interfering substances (which may be related to turbidity). Utilize treatment records that provide small scale time resolution, such as online monitoring data, to determine whether the process was stable during the time period in question. Daily averages may provide evidence of massive failures, but will not provide information about whether consistent treatment was being provided.

In general, the relative effectiveness of microorganisms' resistance to free chlorine, from high resistance to low resistance, is as follows:

- Protozoan oocysts (i.e., *Cryptosporidium*)
- Protozoan cysts (i.e., *Giardia*, *Entamoeba histolytica*)
- Viruses (hepatitis A virus, poliovirus)
- Vegetative bacterial cells (*Shigella*, *Escherichia coli*)

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Protozoan oocysts are highly resistant to chemical disinfectants, but not to physical means such as UV light or ozone (gas). Microorganisms within each group and strains among the same species differ somewhat in resistance. The state of injury induced by environmental impacts and selection of resistant strains influence survival. Aggregation of microorganisms and/or close association with debris shield them to various degrees from lethal effects of disinfectants and attachment to surfaces such as pipe walls to form biofilms that protect organisms from inactivation by disinfectants.

A measurement of microbiological inactivation by disinfectants is the *CT* value (CT_{calc}), which is the product of the free residual disinfectant concentration (*C*) in mg/L that is determined before or at the first user (customer) and the corresponding disinfectant contact time (*T*) in minutes (i.e., $C \times T$). Refer to Table I ($CT_{99.9}$ values for inactivation of *Giardia* cysts at different concentrations of disinfectants, temperatures, and pH values) and Table I (*CT* values for inactivation of viruses at pH 6–9, at different temperatures with different disinfectants for comparing disinfectant efficiencies). Make residual measurements during peak hourly flow. For comparisons of *CT* values between the indicated pH, temperature, and concentration values, use linear interpolation. (For example, for free chlorine, 10°C, concentration 1 mg/L, pH 7.5 = [166 − 112 = 54; 54/2 = 27; 112 + 27] = 139). If no interpolation is done, use the $CT_{99.9}$ value at the higher temperature, at the higher pH and higher concentration.

A simple *CT* calculation, for example, using a disinfectant concentration (*C*) at the basin effluent of 1.3 mg/L and a detention time (*T*) of 22 min, is as follows:

$$C \times T = 1.3\,\text{mg} / \text{L} \times 22\,\text{min} = 28.6\,\text{mg}\,\text{min} / \text{L}$$

Use this calculation for comparing to values in Table I or J. The calculated *CT* value should be higher than the value stated in the table for specific conditions of disinfection, temperature, pH, and concentration (residual). In this situation, if the temperature of the water was 15°C, the pH 7 and the concentration of chlorine 1 mg/L, a *CT* value of 75 would be needed for a 99.9 reduction of *Giardia* cysts. The *CT* value of 28.6 would have been inadequate to meet the criteria and could explain the survival of the pathogen under investigation.

Microbial inactivation efficiencies vary considerably among different disinfectants and are influenced by the characteristics of the water and water temperature. Tables I and J show that, in general, ozone is more effective than chlorine dioxide, which is more effective than free chlorine, which is more effective than chloramines. Also, in general, longer contact time increases the degree of inactivation, and higher water temperatures as well as lower pH values increase rates of inactivation. Rapid mixing of the disinfectant with water increases disinfection efficiencies, whereas dissolved organic matter reacts with and consumes the disinfectant and forms products that have weak or no disinfection activity. Certain inorganic compounds and particulate matter also react with disinfectants.

The *CT* value must be determined sequentially whenever a disinfectant is added to water. Contact time (*T*) is the duration in minutes for water to move from the point of application of the disinfectant or the previous point of residual disinfectant

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measurement to the point where residual disinfectant concentration (*C*) is measured. It is measured from the first point of disinfectant application and from all subsequent applications until or before the water reaches the first user. Determine contact time in pipelines by dividing internal volume of the pipe by the maximal hourly flow rate through the pipe. Determine the flow rate from (a) plant records, (b) continuous monitoring device readings, (c) measurements at hourly intervals, or (d) if this sort of information is unavailable, measurements at expected high flow periods. Use tracer studies to determine contact time within mixing basins and storage reservoirs. These values represent only 90% effectiveness because of short circuiting. Chlorine, fluoride, and rhodamine WT (but not B) are commonly used tracer chemicals. Contact time is usually measured by a step-dose method, but a slug-dose method is used where chemical feed equipment is not available at the designated point of addition or where such equipment does not have the capacity to provide the necessary concentration. (See appropriate EPA literature for procedures, and consider getting engineering expertise if these matters are too complex.)

Estimate whether pathogens had been inactivated. To do this, divide the CT_{calc} value by a value ($CT_{x\%}$) resulting in a certain percentage inactivation (e.g., 99.9% [3-log] or $CT_{99.9}$ for *Giardia* cysts and 99.99% [4-log] or $CT_{99.99}$ for viruses). This gives an inactivation ratio. See Table I for $CT_{99.9}$ values for *Giardia* and Table J for $CT_{99.99}$ values for viruses.

Following is a sample calculation for data in Table I when water temperature is 20°C, pH in a clearwell (reservoir for storing filtered water) is 7.0, time (*T*) (either calculated or measured by dye test) is 38 min, and the disinfectant used is chlorine: The desired *CT* value for 99.9% inactivation of *Giardia* for pH 7 at 20°C is between 52 and 68 depending on concentration of disinfectant. In this case, the disinfectant measured at the clearwell outlet is 2.0 mg/L. Therefore,

$$CT \text{ is } 38\,\text{min} \times 2.0\,\text{mg/L} = 76\,(\text{mg min/L}).$$

The result, 76, is larger than the value, 62, required in the table; hence, these disinfection concentration (*C*) and time (*T*) conditions should result in a 99.9% or greater inactivation of *Giardia* cysts. For free chlorine, a 3-log inactivation of *Giardia* cysts provides greater than a 4-log inactivation of viruses.

The following example, using the data in Table I, demonstrates a means to calculate the increased disinfectant dosage needed for a plant during the transition from summer to winter, when the water temperature fell from 15 to 5°C, chlorine dioxide was the disinfectant used and the *T* value (calculated or measured) is 12 min.

Using Table I, the required *CT* at 15°C for a 3-log inactivation of *Giardia* cysts by chlorine dioxide is 19. Therefore,

$$19\,\text{mg min/L} \div 12\,\text{min} = 1.58\,\text{mg/L}.$$

The *CT* value for 5°C for this disinfectant for the same inactivation is 26. Therefore,

$$26\,\text{mg min/L} \div 12\,\text{min} = 2.17\,\text{mg/L}.$$

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Table 3 Sum of calculated CT values for free chlorine

Location	Disinfection (mg/L)	Contact (min)	$C \times T$ (CT_{calc})	pH	Water temperature (°C)	$CT_{99.9}$ (Table I)	$CT_{calc}/CT_{99.9}$
Basin 1	1.3	30	39	7	15	76	0.513
Basin 2	1.0	25	25	8	15	108	0.231
Basin 3	0.8	60	48	8	15	105	0.457
SUM							1.201

In this situation, the plant should have increased the chlorine dioxide concentration from 1.58 mg/L to 2.17 mg/L to maintain the same efficiency of disinfection. If this had not been done, it may explain the survival of pathogens in the water supply.

The sum of these ratios gives the total inactivation ratio, which should equal 1 or more to provide effective disinfection. Make calculations and record information on Form G5a. The following example shows the way this is done. Chlorine is added to three basins. Chlorine concentration, contact time, temperature and pH are measured at these locations and recorded as shown in Table 3. Data from Table I is combined to do the calculation.

The resulting sum exceeds 1.0. This ensures that the plant met the recommended or required *CT*.

Regulations may require that community and non-community public water systems that use surface water or water under direct influence of surface water meet a criterion (e.g., minimum of 99.9% [3-log] removal and/or inactivation of *Giardia* cysts and a minimum of 99.99% [4-log] removal and/or inactivation of viruses of fecal origin that are infectious to humans). Removal and/or inactivation of microorganisms may be accomplished by either filtration plus disinfection or disinfection alone, depending on the water source. Water systems using chlorine with *CT* values that attain minimal level or inactivation of *Giardia* cysts will result in inactivation of 99.99% (4-log) of viruses.

Evaluate the prefiltration processes (e.g., coagulation, flocculation and sedimentation). Coagulation is a process that uses coagulant chemicals and mixing, by which colloidal and suspended materials are destabilized and aggregated into flocs. Flocculation is the process that enhances agglutination of smaller floc particles into larger ones by stirring. Sedimentation is the process by which solids are removed by gravity separation before filtration. Observe whether these processes reduce turbidity. Calculate detention (transit) time within the settling tank and seek information about frequency and method of cleaning the tank. For example, if an 8-ft-deep sedimentation basin has a volume of 1 million gal, and the plant flow rate is 20 million gal/day, detention time in the basin is: (in your country you may want to calculate rates based on metric measurements)

$$1{,}000{,}000\,\text{gal} \div 20{,}000{,}000\,\text{gal/day} = 0.05\,\text{days}\,(\text{or } 1.2\,\text{h, or } 72\,\text{min.})$$

$$\text{Then, depth / time is:}$$

$$8\,\text{ft} \div 72\,\text{min} = 0.11\,\text{ft/min.}$$

Several different types of filtration may be used in water treatment facilities. These are conventional, direct (both conventional and direct are referred to as "rapid" filtration), slow sand filtration, and diatomaceous earth filtration. Conventional filtration consists of a series of processes including coagulation, flocculation, sedimentation, and filtration. Direct filtration consists of a series of processes including coagulation and filtration but excluding sedimentation. Slow sand filtration is a process involving passage of raw water through a bed of sand at low velocity (usually less than 0.4 m/h), utilizing both physical and biological means to remove particles and microorganisms. In diatomaceous earth filtration, water is passed through a precoat cake of diatomaceous earth filter medium while additional filter medium is continuously added to the feed water to maintain the permeability of the filter cake.

If done properly, each filtration method results in substantial particulate removal. When rapid sand filters have a head loss of about 7–10 ft, they require back washing. Filters are backwashed by reversing the flow of the filtered water back through the filter at a rate between 15 and 30 gal/min/ft^2 of sand-bed area. Sometimes water jets at the surface aid in loosening and removing deposited material on the sand. Observe an actual backwash and look for indications of short-circuiting or areas of the filter material that seem agglomerated or resist being cleaned by the flowing water. If backwash water is not discharged to waste, evaluate where it is released. Slow sand filters eventually become clogged. When this occurs, a scraper or flat shovel is used to remove the top layer of clogged sand, and new sand (equivalent to the depth removed by scraping) replaces the old.

Test the effectiveness of filtration for each filter unit by observing capacity and filtering area relative to volume and turbidity of the filtered water. Also, review turbidity, headloss, and filter rate record. Look for anomalies, especially in the few hours after a filter is returned to service, and before the filter is backwashed. Review criteria that cause a backwash to be initiated, and establish if these criteria were followed during the time preceeding the outbreak. Determine the source of backwash water and the frequency of back washing of filters from records and head gauge readings. Check whether the water used to back wash or clean filters came from an untreated source and determine the fate of the backwash water. In the case of illness due to chemical substances, evaluate types of chemicals used and condition, operation and maintenance of chemical feeding equipment. Consider sampling backwash water for pathogens under investigation. Review plant records for results of monitoring and be alert for changes that suggest treatment failure. Record this information on Form G5b (Source of contamination and treatment failures that allowed survival of pathogens or toxic substances.)

Data in Table K (Estimated removal of *Giardia* and viruses by various methods of filtration), give a summary of expected minimal removal of *Giardia* and viruses in well operated filtration systems. Values can be subtracted from *CT* values required for disinfection.

Although contamination is likely to be associated with raw incoming surface water, look for bypass connections where raw or partly treated water can get into treated water. Also look for common walls that separate treated and untreated water.

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Consider the possibility that a contaminant was introduced in any of the treatment chemicals themselves, or as an act of sabotage. Determine whether any flooding has occurred during the interval of concern. Check absentee records for possible enteric illness of the water treatment plant staff. Such illness may reflect either sources of contamination or victims. Record this information on Form G5b.

At domestic locations, evaluate treatment devices (such as chlorinators, filtration units, softening equipment) as described above, but modified to fit the situation under investigation. Record observations and measurements on Forms G5a and G5b, as applicable.

Evaluate the Water Distribution and Plumbing Systems

The water distribution system can be complex, with multiple entrance points for treated water and different pressure zones in which water can enter but not leave. Water flows in the direction in which it is being "requested," so can flow in different directions in the same pipes from one hour to the next. Contaminated water can enter a potable water supply from a non-potable water supply when the two are directly connected. Such interconnections are referred to as cross connections. To evaluate such situations, trace lines of the treated supply from the point of treatment or entrance into a building to points of use and associated plumbing. Look for any interconnections of other water supplies, such as wells, waste lines or holding tanks for water intended for fire control. If cross connections are found, look to see whether backflow prevention devices are inserted between the lines and, if so, whether they are functioning properly. Also, look to see whether there is an air gap between the water inlet and vessel or tank. Evaluate the arrangement and operation of check valves on connections between the two water systems. Review inspection report for backflow prevention devices.

Contaminated water can also enter a treated supply by siphonage from a contaminated vessel or sewerage to the potable water line having negative pressure. This is referred to as back siphonage. Examine all water vessels to see whether they contain submerged inlets or hoses connected to water faucets, and if so, whether properly functioning vacuum breakers are in place. Without proper air gaps or properly functioning vacuum breakers, there is a possibility of siphonage of water from plumbing fixtures in upper stories to lower stories when line pressure is negative. This may occur when faucets on lower floors are opened after the water supply valve has been turned off for repairs or when the supply line has had a sudden loss of pressure, as can happen with nearby heavy use of water (e.g., to fight fires or irrigate) or when pressure lines are broken. Measure water pressure on upper stories of buildings to determine whether negative pressure occurs. (Pressure losses may be transient and of very short duration.)

Interview building managers and residents about whether there were (a) any repairs of water service during the past month, (b) fires that occurred nearby, or (c) other situations that could have caused negative pressure in the water line. Also, if appropriate, review fire and utility department records for information about these

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situations. Get dates of line repairs to see whether they correlate with the time of incubation periods of early cases. Measure chlorine residual (of chlorinated water systems) and take samples for microbiological tests at several strategic locations in the distribution systems. Perform calculation on comparison of disinfectant residual. If a toxic chemical poisoning is under investigation, talk to home owner, building manager or maintenance staff about whether pesticides or other toxic compounds were sprayed with equipment connected to a hose or a sprinkler system. Furthermore, interview building managers and residents about whether there are persons residing there who either are or recently were ill with diarrhea. They may represent sources of the etiologic agent or may identify victims. Interview those identified about the onset of their illness and symptoms and examine their plumbing systems. Record information obtained during the investigation of distribution and plumbing systems, and record related calculations on Form G6 (Source and mode of contamination during distribution and at point-of-use).

Investigate Water Contact Sites

Evaluate implicated waters used for swimming, water skiing, bathing, clothes washing by hand, or agricultural activities, in a manner similar to that described under the section on investigation of surface water source. If the potential site of contact was natural surface water, determine whether the water is likely to be infested by parasites and look for the presence of snails (Swimmer's itch). For swimming pools, measure the water's pH, chlorine residual, water temperature, and turbidity, if applicable. Also, review pool records for previous information on these characteristics. High turbidity in pools, hot tubs, and spas is a sign of either poor filtration or inadequate disinfection. Evaluate whether the resulting water would adequately protect those who swam or waded in it or had any physical contact with it. Evaluate filter and chlorination equipment as described for water treatment. Backwash filters and collect a sample to get an indication of microorganisms present on the filter (thus obtaining historical information). This approach has been useful for identification of *Pseudomonas aeruginosa*. Look for the presence of slime on tub, whirlpool, slide and pool surfaces, and collect some of this material for analysis for *P. aeruginosa*. If the answer is not obvious, ask ill persons whether they had puncture injuries or wounds or scrapes while immersed in water. Record this information on appropriate parts of Form G7 (Contamination source and survival of pathogens or toxic substances for recreational waters). Collect samples of the water (see section on "Collect Water Samples"), and test them for pathogens and/or indicator organisms, as applicable.

Investigate Sites at Which Respiratory-Acquired Waterborne Agents Amplified and Were Disseminated by Aerosols

The agents listed in Table D can multiply in water and if such water is aerosolized, they can be transmitted to human beings via the respiratory route. Highly susceptible persons (e.g., the elderly, smokers, immunosuppressed individuals) are the usual victims.

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Look for possible sites where water may have been or is being disseminated as aerosols. Consider (a) air conditioning cooling towers and evaporative towers, (b) hot water systems (heaters and tanks), (c) shower heads, (d) faucets with aerators, (e) mist machines used to freshen fruits and vegetables in markets, (f) humidifiers, (g) nebulizers/respiratory therapy equipment, (h) whirlpools and spas, (i) dental drills and cleaners, (j) cooling water apparatus for grinders, (k) splash from hoses, (l) water pressure line breaks, (m) decorative water features, (n) outside misters, (o) other aerosol-producing devices. Sample water from all suspect sites for *Legionella* or other waterborne agents that may cause illness when inhaled.

It is not possible to recognize by visual inspection the potential for water to be contaminated with legionellae. Warm temperatures, especially those between 27°C (80°F) and 46°C (115°F), are conducive to growth of legionellae. Additionally, stagnant water allows time for legionellae to multiply, especially in dead-end lines, reservoirs and hot water tanks, and in water trapped in shower heads and faucet aerators. If it is deemed appropriate or necessary to sample for detection of *Legionella* in the environment, collect water samples from suspect sources. It is important to use a lab with proven expertise in isolating and characterizing *Legionella*, such as those labs in the U.S. certified under the Environmental Legionella Isolation Techniques Evaluation (ELITE) Program. The Centers for Disease Control (CDC) have a convenient form for recording case histories (http://www.cdc.gov/legionella/downloads/case-report-form.pdf).

It is not appropriate to sample air for detection of *Legionella* hazards. It may, however, be appropriate to use micromanometers or smoke to trace direction of air flow to determine route of dissemination. Micromanometers measure pressure differences, and flow can be assumed to travel from high to low pressure areas. Smoke moves from areas of higher pressure to areas of lower pressure and is extremely sensitive to air currents. Observe direction and spread of smoke movement. Record this information on Form G8 (Contamination source and sites of amplification and aerosolization of pathogens).

Collect Water Samples

Prior to the collection of samples, investigators should consult with the testing laboratory that will be used, to receive specific laboratory sampling instructions and sampling kits. Sampling Protocols for potable and non-potable sources are dependent on the specific etiological agent and the related analytical procedures performed by the testing laboratory.

Collect samples promptly to test for possible etiologic agents and for microorganisms indicative of fecal contamination. Contaminants in water are in a dynamic state; their presence and quantity differ with time and place. See Table F (General instructions for collecting drinking water samples) for guidance on collecting and shipping samples for viral, bacterial, and parasitic analyses.

Samples for bacteriological tests can be collected in one of three ways: (a) by letting a stream of water flow into a container or by submersing a container into a

volume of water, (b) by passing a large volume of water through a filter, (c) by putting Moore swabs (see Table A for description) or similar absorbent materials in surface water or drains for a few days (see Table F).

Use bottles that have been cleaned, rinsed, and sterilized, or use sterile plastic bags to collect and store samples for bacteriological examination. For a chlorinated water supply, or when in doubt about the presence of residual chlorine, use bottles containing 100 mg/L sodium thiosulfate to combine with any free chlorine in the sample and prevent lethal effects of chlorine on microorganisms in the sample. This compound will not interfere if used for non-chlorinated water.

When collecting water samples, first try to get "historical" samples that might give an indication of the condition of the water at the time it was ingested by those who became ill. Obtain historical samples from water in bottles in refrigerators, toilet tanks, hot water tanks (for chemical analyses only), fire truck reservoirs, storage tanks, and taps at seldom-used and dead-end locations, and from ice in refrigerators and commercial ice plants. Direct the laboratory to test historical samples for pathogenic organisms or toxic chemicals, as well as indicator organisms, because these samples have a chance of still containing the etiologic agent, whereas samples collected during the investigation several days or weeks after the event may be of water that has been flushed free of contamination or has been significantly diluted.

Take samples from 8 to 10 points throughout the distribution system. Sample dead-end locations if they are found. Do not neglect to obtain raw water samples even though treatment is provided. This is important, as it suggests possible sources of contamination and reflects the effectiveness of treatment. Compare these test results with records of results on previous samples of raw or treated water.

Before drawing a sample from a water tap, make sure the tap is connected to the supply to be tested. Do not collect samples (other than for *Legionella*) from hose connections, sprays, or swivel faucets; uncouple these connections or choose different outlets. It is unnecessary to flame outlets, as this does not improve the quality of the sample. First, ensure your hands have been thoroughly washed then take a line sample by allowing the water to run to waste for 5–10 min. Adjust the flow of water so that the thiosulfate will not wash out of the bottle or bag (do not overfill—most laboratory bottles indicate a maximum fill line). Keep sample containers closed until the moment they are to be filled. Hold the bottle near the base, fill to the "fill line" or within an inch of the top without rinsing, and immediately replace the stopper or cap and secure the hood, if attached. If a Whirl-pak™-type plastic bag is used instead of a bottle, hold the base, rip off the perforated top, open the bag by pulling the side tabs apart, grasp the end wires, and place the bag under the flowing water. Remove the bag before it is completely filled and squeeze most of the air out; fold over the top of the bag several times and secure by twisting the end wires. Take a source or a distribution line sample by opening the tap fully and letting the water run to waste for sufficient time to empty the service line (or if in doubt, for 5 min) and proceed as above.

Collect samples from open shallow wells and step wells by dropping a clean wide mouth container on a string or rope into the well. Allow the container to sink below the water surface and then pull it out of the well. Pour contents into a sample jar or bag.

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Collect samples from rivers, streams, lakes, reservoirs, springs, toilet tanks, and non-pressurized storage tanks by holding a 200 mL sample bottle near the bottom and plunging it neck down to a depth of 15 cm (6 in) below the surface; turn it right side up, and allow it to fill. Don a plastic disposable glove when small vessels used for drinking are sampled in this manner. When collecting these samples, move the bottle in a sweeping, continuous, arc-shaped motion, counter to stream flow or in a direction away from the hand. Collect samples at locations approximately one-quarter, one-half, and three-quarters the width of the stream or water course. Special apparatus can be used for sampling at various depths. Samples can then be taken by positioning large bottles on a rod or pole at the desired depth and location before pulling their stoppers with a wire, string or thin rod. Samples of bottom sediments are sometimes useful for the detection of certain pathogens. Collect surface scum or regions containing dense particulate colored material when seeking cyanobacteria (blue-green algae). Collect slime, if present, when seeking *Pseudomonas*. If large amounts of water are needed, seek assistance and obtain specialized sampling equipment from agencies responsible for water quality.

If possible, avoid wading when sampling bodies of water because wading often stirs up bottom sediments. If this is the only way to get a sample, however, wade against any current (e.g., upstream in creek or river) and keep moving forward until sample taking is completed. Piers or similar structures, or the front end of a drifting or slow moving boat, make good sampling stations.

Concentration of bacteria by the use of swabs, filters, or by absorption, is particularly important when waterborne pathogens are sought. To concentrate bacterial pathogens from flowing water (e.g., streams, lakes, sewer lines, or drains), suspend Moore swabs (or non-medicated sanitary napkins or non-medicated tampons if Moore swabs are unavailable) for 3–5 days. These can be held in place by wire just below the surface or at other depths. If rodents are about, put Moore swabs in wire baskets. After the sampling period, either put swabs or pads into a plastic bag and pack in ice, or put the swabs or pads directly into an enrichment broth for the pathogen sought. Take or send these to the laboratory promptly.

Concentration of microorganisms can be increased by filtration with a variety of filters (e.g., membrane filters, cartridge filters, or other filter media). When membrane filters are used for pathogenic bacteria recovery, pass at least 1 L of water (relatively free of turbidity) through a sterile 0.45 μm membrane filter. For viral analysis, use virus-absorbing electropositive cartridge filter to concentrate 400 L or more water (see Table F). Keep filters cool (but not frozen) and ship to a reference laboratory for further processing. For *Giardia* cysts and *Cryptosporidium* detection, collect samples by passing at least 400 L water through a cartridge filter (see Table F).

For inorganic chemical analyses, use 1 L polyethylene containers. These should be new, or acid-washed if previously used. Collect the water without flushing the lines, preferably in the early morning before water is used. For trace metal analyses, preserve one sample with 2 mL of high-grade nitric acid to a pH of 1 or less. This is particularly important whenever it is suspected that metals may have leached from water pipes or vessels. For organic chemical analysis, use 4 L glass containers with teflon-lined caps. Clean and rinse the containers with a good quality laboratory

solvent and heat at 400°C for 20 min. Rinse the cap thoroughly with distilled water. Fill the container so that there is a minimum of air space. For physical analyses, collect at least 2 L, or other amounts requested by the laboratory.

Collect ice aseptically in sterile plastic bags or jars. Use sterile tongs to collect cubes; sterile spoons for collecting chipped or crushed ice; and sterile chisel, hammer, or pick to chip block ice. Put block ice or large chips into plastic bags.

If *Legionella* is sought, sample water at sites of any source that may have been aerosolized and send to a lab with proven expertise in *Legionella* isolation and characterization, such those in the CDC ELITE Program. This includes cooling towers, evaporative condensers, water heaters and holding tanks, humidifiers, nebulizers, decorative fountains and whirlpool baths (see section on investigating sites where aerosols are disseminated for a more complete listing). Turn off fans of condensers before sampling; if this is not possible, wear a respirator. Use 250 mL to 1 L polyethylene bottles that have had sodium thiosulfate added if the water to be tested has been chlorinated. For each sample, don disposable plastic gloves and collect the sample by inverting the bottle and moving it in a continuous arc away from the hand. Measure and record water temperature. Handle samples as described in Table F. Rub swab over faucet aerators and shower heads if these are considered as sources of aerosols. Break stick and allow tip to fall in a tube containing 3–5 mL sterile water (not saline).

Investigators are often requested to test air to demonstrate the presence of *Legionella* in aerosols. Although legionellosis is an airborne disease, legionellae are susceptible to low humidity and become non-viable on drying. Therefore, air sampling is an ineffective and inefficient way of determining whether a *Legionella* hazard exists, and it can thus be misleading.

Label each container with sample number, date, time of collection, and your name or initials. Complete the Water/Ice Sample Collection Report, Form F, for the first sample. List additional samples with sample numbers and other pertinent information on the back of the form. In those situations where the laboratory needs additional information, attach the appropriate G series forms. Send the original Form F and list with samples to the laboratory; retain a copy for your files. Inform the laboratory of the type and number of samples and specimens; also, consult with the laboratory on methods to preserve and transport samples, if necessary, and on time of their arrival.

If legal proceedings are anticipated, deliver sample personally to the analyst, or seal the sample container in such a way that it cannot be opened without breaking the seal. Note on Form F the method by which the bottle was sealed. Maintain a chain-of-custody log to document the handling of the sample, and have the log signed and dated each time it changes hands. Consult with state/provincial regulatory agency on complying with legal requirements for chain-of-custody procedures. Recipient should record on the form whether the sample was sealed when the laboratory received it.

If analysis cannot be done on the day of collection, chill water samples rapidly and hold them at temperatures at or below 4°C (39°F), but Do NOT FREEZE, because populations of bacteria such as *Escherichia coli* and of parasites decrease during frozen storage. Hold ice samples frozen; if this is not possible, keep the temperature below 4°C h (39°F).

How to Transport

Investigators should consult with the testing laboratory that will be used to receive specific laboratory sample packaging, labeling, and transportation instructions as protocols are dependent on specific transportation regulations (IAFTA, TDGR) within each jurisdiction.

Ensure each sample is uniquely identified and labeled (as per the receiving laboratories requirements). Many laboratories include barcode labels along with the sample containers within the sample collection kits. Ensure that the correct label is affixed onto the correct sample container and that this information is transferred to the shipping manifest accurately (chain of custody form). Specimens should be packed and the packages labeled according to applicable regulations governing transport of hazardous materials.

Generally, the transport of samples of water and ice intended for laboratory analyses are packed and shipped in a manner to ensure the sample does not change from the time of sampling to the time received by the testing laboratory and shipped using the most expeditious means (e.g., personal delivery or overnight mail). Typically samples of water or ice are packed with refrigerant (ice packs, dry ice, etc.) in insulated and sealed containers (see Table F).

Receipt of laboratory analysis. Record results of laboratory test samples on Form I.

How to Take On-Site Measurements

Several measurements are routinely called for during on-site investigations. Brief instructions are given for those that are commonly done; nevertheless, follow manufacturer's instructions if these are available.

Measure free, combined and total residual chlorine and other disinfectants. Color comparison kits are available for testing for free, combined and total residual chlorine. The diethyl-*p*-phenylenediamine (DPD) test is an example (see Table A). Check instrument calibration regularly. Use dry reagents, because the liquid forms are unstable. Chlorine comparators can be used to test for bromine by multiplying the result by the factor 2.25 and to test for iodine by multiplying the result by the factor 3.6.

Measure temperature. Measure water temperature by immersing the sensing end of either thermocouples, transistors, or thermometers into the water. Sometimes measurements need to be made at various depths; use thermocouples with wire leads of sufficient length for this purpose. Calibrate temperature measuring devices periodically.

Measure pH. Calibrate the pH meter as recommended by the manufacturer with at least two standard buffers (e.g., pH 7.0 or 10.0) and compensate for temperature, if the meter does not do it automatically, before each series of tests. Remove a sample

of water to be tested and immerse the pH electrode into the sample; record the reading. pH can also be measured by color comparators that employ color indicator solutions or discs. (Ranges of pH color indicator solutions are bromophenol blue, 3.0–4.6; bromocresol green, 4.0–5.6; methyl red, 4.4–6.0; bromocresol purple, 5.0–6.6; bromothymol blue, 6.0–7.6; phenol red, 6.8–8.4; cresol red, 7.2–8.8; thymol blue, 8.0–9.6; and phenolphthalein, 8.6–10.2.) In this case, water containing more than 1 mg/L chlorine in any form must be dechlorinated with sodium thiosulfate before the pH indicator solution is added to prevent decolorization of the indicator. Always report temperature at which the pH is measured.

Measure turbidity. Nephelometric Turbidity Unit (NTU) is the usual standard unit, but other turbidity measurements (such as particle counts) are used. The NTU requires a nephelometer, which measures the amount of light scattered predominantly at right angles and absorbed by suspended particles (e.g., clay, silt, finely divided organic matter, inorganic matter, soluble colored organic compounds, and microscopic organisms) in the water sample. Calibrate turbidimeters with a standard reference suspension. Make turbidity measures on the day samples are taken. Vigorously shake samples, wait until all air bubbles have disappeared, and then pour sample into turbidimeter tube. Read directly from scale on instrument or from an appropriate calibration scale.

Measure air flow. Pump chemical smoke into the air at the exit of the device suspected of releasing aerosols. Observe the direction and spread of the smoke. Otherwise, measure pressure differentials with a micromanometer.

Measure other attributes of water. Follow instructions given by manufacturers or in standard reference books (see Further Reading).

Trace and Confirm Source of Contamination

Use fluorescein dye, lithium or other tracers in appropriate soils to determine the means by which contamination from sewage, industrial wastes, or other sites of pollution reached the water supply. Fluorescein dye is particularly helpful in evaluating flow of contamination through fissured rock, limestone, gravel, and certain other soils. This dye is not readily absorbed or discolored by passage through these soils or sand, as are many other dyes, but it is discolored by peaty formations or highly acid ($pH < 5.5$) soils.

Make a concentrated fluorescein dye solution by mixing 300 g of fluorescein powder into a liter of water. Usually, 2/3 to 3 L of this solution are sufficient for the test for up to 60,000 L of water. Fluorescein dye is also available in liquid and tablet form. One tablet will dye approximately 480 L (~120 US gal).

Pour the calculated amount of fluorescein solution or put a sufficient number of fluorescein tablets into a receptacle at a point of potential pollution. Usually this point will be located within 100 yards and at a higher elevation than the water

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source under study. Cesspools, latrines, distribution boxes, sink holes, borings, septic tanks, drains, manholes, toilets and plumbing fixtures are typical places to introduce the dye. If dye is poured into a plumbing fixture or dry hole or boring, add water to wash it down. The amount of dye to use varies with the distance the dye must travel, the expected time of the journey, the size of the aquifer or water channel, and the nature of the soil.

Take samples of the water when the dye is introduced into the test hole or fixture and then hourly for up to 12 h to detect arrival and departure of fluorescein. If no dye is observed, repeat the test with twice the amount of dye. Whenever possible, use a fluorescent light or fluorometer to analyze water samples for evidence of fluorescein. A fluorometer can be set up and calibrated, and a continuous recording can be made. This meter can detect fluorescein in concentrations of μg/L (ppb). Fluorescein dye will temporarily color water, which discourages use of the water until the dye is sufficiently degraded or diluted. Alternate tracers can be used if specific ion meters are available.

The dye stains all it touches. Methanol is a good solvent for the dye, and hypochlorite solutions decolorize it; both can aid in removing stains. Abrasive soaps are useful for cleaning stained skin; fluorescein-stained clothing should be washed separately.

Appearance of dye in a water supply is conclusive evidence of seepage from the site where the dye was introduced. Failure to detect dye, however, is not conclusive evidence that seepage did not or would not occur if more dye had been added or if weather conditions or subsurface flow had been different at the time of the test than during the outbreak event.

Illustrate source and direction of contaminated water flow as indicated by the dye test on Form G1. Take photographs of sources of contamination and evidence of staining of the ground at the site or dye-stained color of the water. In situations where a single source of contamination is obvious or where multiple sources are readily apparent, dye studies serve little purpose.

Water not Intended for Drinking as a Source of Illness

Drinking water, however, is not the only source of water that may contribute to outbreaks. Other sources of water that can contribute to outbreaks include water not intended for drinking, recreational water and water used in agriculture during harvesting and packaging.

Legionnaires disease is the pneumonia caused by the inhalation of contaminated water aerosol containing the bacteria *Legionella*, with *Legionella pneumophila* being responsible for 85% of all infections. It is also a common cause of healthcare associated pneumonia. *Legionella* can replicate within free-living amebae in water, allowing it to resist low levels of chlorine used in water distribution systems. Risk of infection is more common in warm and humid weather, when water droplets are able to drift further due to higher absolute humidity.

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Fifty percent of all *Legionella* outbreaks have been traced to cooling towers with *L. pneumophila* serogroup 1 responsible for all cooling tower outbreaks. All aerosol generating devices, however, can be potential sources of *Legionella*. Some other sources of aerosolization that may have contributed to or be associated with outbreaks include: whirlpool displays, building's air conditioning systems, water spray fountains, public bath houses, vegetable misting systems in grocery stores, evaporative condensers, showerheads, humidifiers, air scrubbers, car washes, ornamental and decorative fountains, potting soil, respiratory therapy equipment, dental units, road asphalt paving machines, car windshield washer fluid and car air-conditioning systems.

In the investigation of a *Legionella* outbreak, (See Box 3, The Flint Water Crisis, which describes a likely *Legionella* outbreak from a commercial water source) due to the varied sources, there is a need to use a broad investigative questionnaire and the collection of environmental data. Environmental factors such as dry bulb temperature, relative humidity and wind rose data can provide information regarding drift evaporation, deposition (settling) and the size of the affected zone. Although aerosol drift can carry *Legionella* up to 6 mi (10 km), the risk of infection is usually highest within 1600 ft (500 m) of the source. There are also air dispersion models that can be used to determine drift zone and the use of Human Activity Mapping in the identification of potential sources.

Recreational Water

In general, *E. coli* and norovirus are the most common pathogens responsible for recreational waterborne outbreaks associated with non-treated water such as beaches and lakes. *Cryptosporidium*, which is resistant to chlorination, is the most common pathogen resulting in outbreaks in treated water venues such as swimming pools and water spray parks. It should be noted that *E. coli*, the indicator of choice of recreational water samples, is not indicative for the presence of norovirus and *Giardia*, *Cryptosporidium. E. coli* can also be "naturalized" and have been found to survive and multiply in beach sand. Beach water sampling results therefore may provide false positive or false negative results and may not be the best indicator for the presence or absence of pathogens.

Recreational waterborne outbreaks are not just traced to the ingestion of contaminated water (Table C. Illnesses acquired by contact with water: A condensed classification by, symptoms, incubation period, and types of agents). Hot Tub Rash, or *Pseudomonas* Dermatitis/Folliculitis commonly occurs in public hot tubs or spas such as those found in hotels. The rash is often a result of skin infection from the bacteria *Pseudomonas aeruginosa* colonizing in the hair follicles after exposure to contaminated water. *Pseudomonas aeruginosa* is an opportunistic pathogen that can survive within the biofilm on the tub surface or within the piping system. Outbreaks can occur when there is a heavy bather load resulting in an increase in chlorine demand, which in turn reduces the effectiveness of the disinfectant to control the population of *Pseudomonas*.

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Blue-green algae or cyanobacteria bloom can occur in warm, slow-moving or still water. When conditions are favorable, mostly during hot summer weather, cyanobacteria populations may increase dramatically, resulting in a "bloom" as they rise to the surfaces of lakes and ponds. They resemble thick pea soup and are often blue-green in color. Although blooms can occur naturally, water bodies which have been enriched with plant nutrients from municipal, industrial, and agricultural sources are particularly susceptible. Some cyanobacterial species may contain various toxins, some are known to attack the liver (hepatotoxins) or the nervous system (neurotoxins); others simply irritate the skin. Health effects from cyanotoxin exposure may include dermatologic, gastrointestinal, respiratory and neurologic signs and symptoms (Table B. Illness acquired by ingestion of contaminated water: A condensed classification by symptoms, incubation periods, and types of agents).

Irrigation and Processing Water

Water can also be an indirect cause of foodborne outbreaks by providing a media for the survival, transportation and the introduction of pathogens into food products. Water used during production, including irrigation, pesticides and fertilizers application and washing, frost protection, harvesting, has long been recognized by food safety scientists as one of plausible and probable sources of the contamination of fresh fruits and vegetables. There have been many outbreaks from produce traced to pathogens being introduced by contaminated irrigation water. Although harvested products are sometimes washed with chlorine solution, pathogens may still survive the process through internalization. *E. coli* O157:H7 may migrate to internal locations in plant tissue and be protected from the action of sanitizing agents by virtue of its inaccessibility. Experiments have also demonstrated that *E. coli* O157:H7 can enter the lettuce plant through the root system and migrate throughout the edible portion of the plant. However, this claim has been refuted by others. *Salmonella* and *E. coli* can also adhere to the surface of plants, and enter through stomata, stem and bud scars and breaks in the plant surface caused by harvesting and processing. Water containing bacteria can be drawn into the produce if it is immersed in or sprayed with water that is colder than the produce itself. *E. coli* O157:H7 may also use its flagella to penetrate the plant cell walls and attached to the inside of the plant. Once attached, it may be able to grow and colonize the surface of the plant. The concerns are not just with bacteria. The present of norovirus in the hydroponic water can result in internalization via roots and dissemination to the shoots and leaves of the hydroponically grown lettuce.

Irrigation water may be contaminated from runoff from nearby domesticated animals and their lagoons, feedlots, ranches into rivers; from feral/domestic animals with direct access to creeks, ditches, rivers, ponds; from sewage flows into waterways and contaminated wells. In some parts of the world sewage contaminated water is preferred for irrigation despite a potential risk of transporting enteric pathogens, since it carries nutrients (N and P) for the plants.

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There is sufficient information to conclude that the application method of irrigation water to fresh produce can have an effect on the microbiological risks associated with the crop. In general, keeping water away from the edible parts of ready-to-eat crops that are consumed without cooking can result in a lowered risk of a foodborne outbreak.

The least to more risky methods for irrigations for microbial contamination are:

Subsurface irrigation (buried soak hoses) < drip irrigation < indoor flood irrigation (hydroponics) < outdoors flood irrigation (water-filled furrows) < overhead irrigation (sprinklers).

Collection and Analysis of Data

The Epidemiological Approach

An outbreak of illness arising from exposure to water demands immediate epidemiological investigation to assess the situation, gather, evaluate, and analyze all relevant information, with the goal of (1) halting further spread of this illness, and (2) predicting, preventing, and/or attenuating future outbreaks. This twofold mandate of epidemiology is usually described as "*surveillance and containment*."

At the commencement of an investigation, the *unknowns* usually outnumber the *known* facts. There is no substitute for prompt, thorough, and careful collection of interview data from ill and well persons who ingested or contacted the suspect water, attended a common event, or who were part of a group of persons where illness occurred. Careful analysis of these data, particularly with reference to common patterns of "time," "place," and the characteristics of the persons involved, can often eliminate many vehicles, agents, and pathways quite early in the investigation, and focus on the remaining possible vehicles, routes, and agents. Later, laboratory results may confirm the agent, the specific pathology, the route taken by the infection or toxic agent, and indicate what is needed to stop the spread, but early epidemiology can often be invaluable in predicting the outcome and taking preventive steps to contain the problem before the lab results are available. Lessons can be learned from most outbreak investigations and are invaluable for increasing our understanding of these pathologies, and preventing their future occurrence.

Determining an Outbreak

An *outbreak* is defined as either *an unusually large occurrence of an expected illness* at that time of year in that place, or *the occurrence of a type of illness that does not usually appear* at that season and location. The "time" factor should be studied

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immediately by plotting the onset time of each case on a time-based grid, to create the *epidemic curve*. Although any number of cases can be involved, the minimum number for an "*outbreak*" to be declared is *two associated cases*, with special exceptions such as *Naegleria fowleri* where, because of the severity and the possibility that cases may have been missed, *a single case* constitutes an "outbreak." Although the epidemic curve is usually measured in hours or days, protracted exposure to agents in water may mean apparent sporadic cases linked to a common source over months or years.

The "Case Definition" and Its Importance for the Analysis

If an "outbreak" is suspected by a sudden increase of cases, determining who is to be categorized as a "case" is not necessarily a simple process. Many people notoriously fail to report enteric illness for many reasons: embarrassment, lack of time, no clear idea which agency should be notified, mild self-treatable symptoms, or simply because they prefer not to make a fuss. They may therefore be incorrectly classified at least initially as "*non-ill.*" Consider also that 4–6% of the general population will have experienced some form of "upset stomach" in the last 24 hours, regardless of exposure to the suspect item, and they may be incorrectly classified, at least initially, as "*ill.*" To reduce the "*false negatives*" and "*false positives*" that are expected with self-reporting, the investigator needs to establish a working case-definition.

A careful case definition categorizes people as "*case*" or "*control*" with the best accuracy possible within the time constraints and resources available. A case definition could be considered "*too sensitive*" if it classifies as a "*case*" a person who experiences: "… *at least one episode of stomach cramps, nausea, vomiting, or diarrhea in the last 48 hours.*" This would confuse subsequent analysis, and produce more false positives. Similarly, a case definition could be considered "*too specific*" if it classifies as "*not-ill*" a person who had experienced only three episodes of diarrhea or vomiting, because they failed to satisfy a case definition requiring "…*at least four episodes of vomiting or diarrhea in the last 48 hours*." Should this last individual, having been declared as not fitting the case definition, be taken into the "*not-ill*" group, the error and subsequent analysis is confounded even further.

A reasonable case definition therefore attempts to reduce both types of errors, and will depend upon the early indications of what the etiology may be. In the instance of a suspected salmonellosis, a case could be defined as "*A person who was in good health before attending the event on Monday May 3rd, and who experienced two or more of the following symptoms anytime up to midnight, Sunday May 9th.: nausea, vomiting, stomach-cramps, diarrhea, headache, or fever.*" Note that a case definition should include a *place* of exposure if known, a *timeframe* during which symptoms may have been experienced (salmonellosis has a range from 6 to 72 h. usually 24–30 h), and the additional footnote that the individual was not already symptomatic before the suspected "exposure."

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Table 4 Symptom profile

	Number of cases	Percentage reporting each symptom
Diarrhea	195	95
Abdominal cramps	182	89
Nausea	52	25
Vomiting	42	20
Fever	6	3
Headache	2	1
Total cases	205	

The Symptom Profile

Calculate the percentage of ill persons who manifest each symptom by dividing the number of persons reporting the given symptom by the number of cases (205 for the example, Table 4) and multiplying the quotient by 100. The distribution of symptoms can be used to identify the most likely pathogen, and aids in requests to the laboratory for microbiological assays of samples and specimens. Other symptoms (e.g., prostration, lethargy, weakness) may be included if deemed appropriate or helpful, but the six symptoms in Table 4 should always be included. Headache, for instance, is associated with many viral infections (e.g., norovirus, rotavirus), but much less so with bacterial infections. Fever is usually associated with an invasive bacterial infection (such as salmonellosis or campylobacteriosis), and is not usually seen in outbreaks of simple enteritis (such as with cholera).

This information helps to determine whether the outbreak was caused by an agent that produced intoxication, an enteric infection, or generalized illness. In the example given, a predominantly diarrheal syndrome without much fever or headache tends to eliminate some of the viral infections (norovirus or rotavirus) or the host-adaptive/invasive serotypes of *Salmonella* (e.g., *S.* Dublin or *S.* Choleraesuis). Median onset time calculations may further reduce possible candidate etiologies. In historical investigations, or where no laboratory confirmation is possible, the symptom profile and onset times can sometimes predict the etiology of the outbreak within reasonable certainty.

The Epidemic Curve

Plotting the Cases

An *epidemic curve* (also called an *onset curve* or *onset distribution*) is a graphic illustration called a histogram that shows the distribution of the time of onset of first symptoms for all cases that are associated with the disease outbreak. Paper printed with square "grid" lines will allow the investigator while on site to represent each

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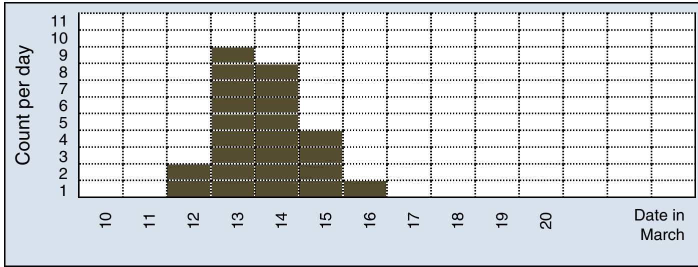

Fig. 2A Onset histogram for 24 cases of acute enteritis from March 12 to March 16, illustrating a point-source without propagation

case as a single "block." The horizontal axis is the sequence of intervals of time and date. The unit of time that defines the width of each interval depends on the characteristics of the illness under investigation. For example, intervals of days or weeks are appropriate for diseases with long incubation periods, such as cryptosporidiosis or hepatitis A. Intervals of a day or half-day are appropriate for outbreaks of enterohemorrhagic *E. coli* strains or shigellosis, while single-hour, 4-h, or 6-h intervals will be more suitable for illnesses with shorter incubation periods, such as chemical poisonings. The vertical axis is always the actual count, or "*frequency*" of cases (blocks) stacked at each interval. It is often necessary to redraw the onset curve as more accurate information becomes available.

If the illness is known, a rule of thumb is that the time interval used for each "block" on the *x*-axis should be no more than ¼ the incubation period of the disease under investigation. If the illness is not known, select an interval where the data produces a bell-shaped curve; not too flat and not too tall. Construct this graph using time-of-onset data from Forms C or D, employing an appropriate time scale.

Means, Medians, and Modes

Once all the onset times for the cases have been plotted on the histogram, determine the *range* as the interval between the shortest and longest incubation periods. In Fig. 2A, the range is the 5 day period from the 12th to 16th March. The *median* onset time is preferred to the *mean* because the latter is vulnerable to a few or even a single very small or very large value. The *median* on the other hand, is the *mid-value of a list of all individual onset times*, including duplicate entries, that are ordered in a series, from shortest to longest. If the series comprises an even number of values, the median is the mean of the two middle values. Most standard reference texts on communicable diseases give onset times as median values.

The *mode* is simply the *interval having the largest number of observations*. A distribution with a single "peak" is called a *uni-modal distribution*, while an outbreak with two peaks is called "*bi-modal*." Subsequent modal peaks following the first may indicate either a "secondary wave" of cases or the exposure of *other* people at a *later* time.

Interpreting the Epidemic Curve

The shape of the epidemic curve helps to determine whether the initial cases originated from a single ***point-source*** exposure (such as water or food available for only part of a day), or from repeated exposures for a longer time, or even more gradual person-to-person spread. A *point-source epidemic curve* is characterized by a sharp rise to a peak, followed by a fall that is almost as steep (Fig. 2A). An "explosive" outbreak of this type is common where a municipal water supply is the vehicle, affecting large numbers of people in a very short period of time, but without secondary cases occurring, or any evidence of onward spread within the community.

Propagated outbreaks are those in which the initial victims ("*primary cases*") manage to spread the agent to other people ("*secondary cases*") such as family members, patients, clients, or other contacts in crowded places through aerosols, personal contact, or contaminated water/food/utensils/surfaces, etc. Propagation following a point-source exposure is demonstrated by a second increase in reported cases following the decline of the first cluster. Sometimes this takes the form of a second "modal peak" separated by approximately one incubation period, but this distinction is soon lost. Figure 2A shows no evidence of propagation; Fig. 2B suggests that propagation *may* have taken place, although care must be taken to consider other explanations.

In addition to (1) true propagation, where the secondary wave can be expected to appear one incubation period after the first, secondary waves may be also explained by (2) exposure to the *same point source* (e.g., food or water supply) at *different*, *but specific times* by other people; this might be a repeated offering of contaminated food or water at two or more mealtimes; (3) a second pathogen (perhaps from the same unhygienic food or water source) which may have a different symptom profile and a *different (incubation) time*.

Slow propagation from the *beginning of an outbreak* with neither an obvious point-source, nor any distinctive "waves" separated by an incubation period as in Fig. 2C, usually indicates one-at-a-time person-to-person spread through close-contact, poor personal hygiene, aerosol (e.g., influenza, or SARS), or sexual transmission (e.g., HIV/AIDS). It can also be explained by (non-propagated) continuing

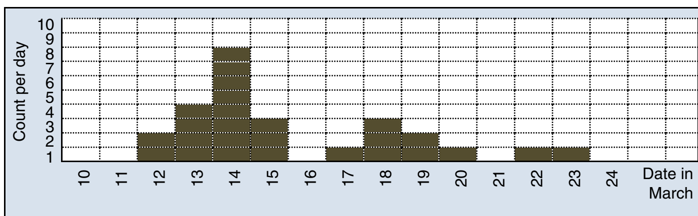

Fig. 2B Onset histogram for outbreak of enterohemorrhagic *E. coli* (EHEC) enteritis, March 12 to March 23. Shown are 16 primary cases, 7 secondary cases, and 2 tertiary cases. Point-source with propagation

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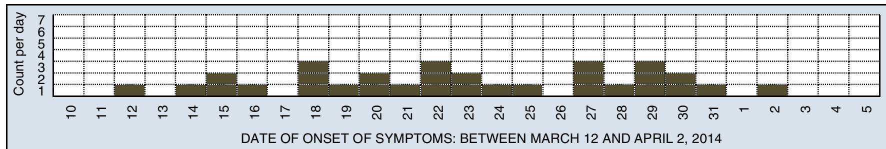

Fig. 2C Onset histogram for 30 cases of shigellosis, March 12 to April 2, illustrating slow spread through a community through either propagation (person-to-person spread via poor hygiene), or exposure by many people to a small well at different times (a non-propagated route)

exposure, for example drinking of contaminated surface water following a conflict, natural disaster or other breakdown of infrastructure. As such it is commonly associated with waterborne cholera, shigellosis, typhoid fever, or *E. coli* infection, and characterized by scattered cases which continue until the chain of infection is cut. Slow, constant and/or intermittent exposure to persons over time to pathogenic microorganisms can also result from sewage run-off after a series of heavy rainfalls.

Estimating the Incubation Period Where the Exposure Point Is not Known

In addition to revealing whether the outbreak was due to a single point-source, or had been spread steadily through the community by propagation in some way, another important objective in constructing the epidemic curve is to estimate the incubation period of the illness if it is not already known. With waterborne illness especially, the time of exposure may be further obscured because people usually drink water several times a day. Hence, the incubation period cannot always be determined for each case, but the actual time of onset is usually available.

The incubation period is the interval between exposure to food or water that is contaminated (with enough pathogens or with a sufficient concentration of toxic substances to cause illness), and the appearance of the first sign or symptom of the illness. Each etiology is characterized by a typical incubation period (Tables B, C, D, and G). Individual onset times will vary due to immune factors, co-morbidities, the dose ingested, and other ingested materials, but the investigator can often make a rough estimate of the average incubation time by examining the aggregation of all onset times as an epidemic curve.

The *modal peak* of a single "cluster" or distribution is the time interval in which most cases commence symptoms. In Fig. 2A this occurs on March 13, and in Fig. 2D that occurs at the double interval Feb 10–11th. Where two *separate* modal peaks (a "*bi-modal distribution*") suggests secondary cases ("propagation"), then the distance between the first two modes is a good estimate of the incubation period. Figure 2B shows about 4 days between primary and secondary modal peaks, suggesting that the initial exposure is likely to have been 4 days before the first mode. In Fig. 2B this would be sometime on or near the 10th of the month.

If the exposure point is *known* but the agent is not, then that estimation of the *median incubation period* will allow many etiologic agents to be excluded due to

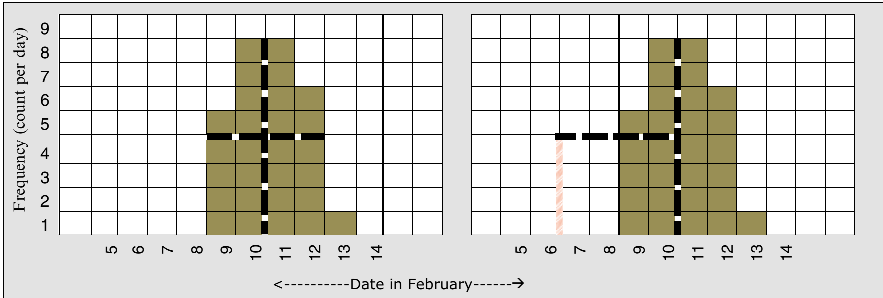

Fig. 2D Cases ($n=28$) commencing during 1-day intervals in February, 2013. If the curve rises rapidly to a peak and drops sharply, (1) draw a vertical line from the modal peak to the x axis; (2) mark the point half-way up that height; (3) draw a horizontal line to show the width of the peak at that point; (4) slide the horizontal line to the left such that the right end touches the vertical line exactly. (5) A perpendicular dropped from the left of the horizontal line will be the best estimate of the exposure time on the horizontal axis. In this case it was the evening of February 6th

incubation periods that are clearly outside the range of times observed. The list of possible candidates can be further reduced by examining the symptom profile and other characteristics of the illness and suspect food or water vehicle. As time passes, the onset curve also provides an ongoing measure of the potential for propagation, and the incidence rate. All this information can be useful in deciding whether the illness in question is an infection or intoxication and thereby determining which laboratory tests should be requested (Tables B, C, D, and G). Note that not all water or foodborne illnesses listed in a standard reference such as the "*Control of Communicable Diseases Manual*" (APHA 2014), are *directly* communicable *person-to-person*; many require a suitable substrate (food or drink) and adequate time/temperature combinations to attain sufficient numbers or the production of enough toxin to induce a pathological condition.

An exposure time can sometimes be estimated from a clear, point-source, single-exposure onset distribution (Fig. 2D). It has no solid basis in statistics, but has sometimes been found to be useful in practice.

The typical incubation periods for most foodborne and waterborne illnesses are readily available for comparison (e.g., *Control of Communicable Diseases Manual*, APHA/CDC, 2014), and in this manual in Tables B, C, D, and G.

Calculate Incidence, Attack, Exposure Rates for Groups Affected

Overall Attack (Incidence) Rates

An incidence rate is the number of *new* cases of a specified disease reported during a given time period in relation to the size of the population being studied, multiplied by a constant, usually 100, to give percentages. Thus 14 new cases of *E. coli* O157:H7 infection among the 140 residents of a children's summer camp in July is an incidence rate of $(14/140) \times 100$ or $(0.10) \times 100 = 10.0\%$ for that month. If several people

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have left and their state of wellness is *not known*, their impact should be expressed in the form of the *possible range of values* around the known incidence rate within the two extremes whereby they may *all* be well or they may *all* be ill. Thus, where six children who were at the camp had departed around the time of the outbreak and their health is unknown, the range could be from a possible (14/146) × 100 (or 9.6%) if all of the six had been well, up to (20/146) × 100 (or 13.7%) if all had been ill. Note that the "missing" six are added to the denominator only when we speculate that none were ill, whereas they are added to the numerator AND denominator if we speculate that they might all have been ill. In this example, the *overall incidence rate* would be reported as "*10%, with a possible range from 9.6 to 13.7%.*"

Factor-Specific Attack (Incidence) Rates Where Possible

Depending upon the situation, it is often necessary to identify exposures which may be related to the illness, and to calculate an incidence rate for each such exposure. For example, in the summer camp illustration (above) it might be useful to enquire if gender, age, location, or some other attribute or activity increased the risk of becoming ill. This should not be interpreted automatically as implying that a given exposure would be associated with the outcome in any situation. By hypothesizing that gender was linked to the risk of illness, for example, does not imply that males are more vulnerable to the illness (the outcome) than females, but it can indicate that gender may have been *related to the exposure*, which in turn increased the risk. As an illustration, suppose that boys at the camp had been swimming, while the girls had gone on a nature walk. The boys may subsequently show increased incidence rate for *E. coli* O157 infection, not because they are more susceptible, but because of their activity. Every proportion or percentage statement should be made with clear reference to the appropriate denominator used.

Incidence rates of waterborne illnesses are usually similar for both sexes at any given age group in the population, but differences in activities or dietary habits or susceptibility due to age or underlying health status can change the risk. The very young, the elderly and the immunocompromised can be at more susceptible, while in some instances, previously exposed populations may have developed a measure of immunity to an infection that may still cause more serious illness among visitors.

A further complication arises where the "at-risk" population (perhaps residents at an institution, summer camp, or on a cruise ship) have generally consumed *all* the food and water for the extended period. Careful interviewing of affected persons often uncovers one or more persons who entered the subject community shortly before becoming ill or who visited the community for a short time and became ill after leaving it.

Attack (Incidence) Rates by Place of Residence

Example: The south-west part of the county is served by three semi-private water systems. Thirty cases of waterborne illness are being investigated in the area. When the numbers of cases are displayed for each water system, no clear grouping or

clustering is evident, although the ***Delta*** supply appears associated with about 50% more cases than the other two (Table 5A).

However, when the analysis introduces the total population of persons who depend upon each water system (as denominators), a different scenario emerges. The incidence rates (expressed here as percentages) now allow a meaningful comparison (Table 5B). We can see that persons using the ***Bravo*** system have roughly *five times the risk of illness* compared to people who are served by the other two systems. The use of the denominator is vital for most calculations. Caution: Numerous other factors may also explain the outbreak and these should be carefully examined. For example, the households using the ***Bravo*** supply may be closer to an unhygienic corner store, drink from a cross-connected public water fountain, or their children may swim in a more polluted pond than the other communities. Potential sources such as these should be eliminated before the water supply is announced as the source of the illness.

Sometimes a *spot map* may be useful in showing the location of the residence of each case, while on a larger scale, the rates of illness can be shown using city blocks, census tracts, townships, or other subdivisions. Different colors or symbols to indicate cases with different time of onset periods (such as weeks) may help to support a hypothesis as to where contamination was introduced, inasmuch as the earliest cases tend to cluster around the point where contamination first occurred. The weakness of this procedure is that if the exposure had been at a restaurant, workplace, or school, plotting the relationship to the location of the home would not be useful.

Preparing to Calculate Associations Between Exposure(s) and Illness

The investigation of waterborne or foodborne disease outbreaks invariably commences *after* both exposure and illness have happened. This is the classical "***case-control***" study, where a group of ill people ("*cases*") and a group of non-ill people

Table 5A Comparison by numbers of cases (no denominator)

System	Alpha	Bravo	Delta	Total
Cases	9	8	13	30

Table 5B Comparison by rates (using denominator)

System	Alpha	Bravo	Delta	Total
Cases	9	8	13	30
Population supplied	360	64	496	920
Attack rate	2.50%	12.5%	2.62%	3.26%

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Table 6 Exposure and outcome data arranged in 2×2 table

	Ill (cases)	Not ill (controls)	Total
Exposed to *X*	25	8	33
Not exposed to *X*	12	22	34
Total	37	30	67

("*controls*") are compared in terms of their exposures.[1] To measure the association between exposure and illness, the data are typically displayed in a 2×2 contingency table. Table 6 compares 37 cases and 30 controls in terms of their *exposure* to a suspected factor "*X*." The table is ready for analysis using odds ratio, as well as the chi-square or the Fisher's exact tests where appropriate. One 2×2 table will be used for each possible exposure (e.g., each beverage, food, or other material).

As many cases as can be identified and contacted, and as many non-ill people (controls) as can be found, should be interviewed as quickly as possible about their exposures to each suspect item. Fading memories, the chance of obtaining still-available samples of implicated food or water, and the opportunity to obtain fecal specimens before the patient is started on the ubiquitous broad-spectrum antibiotics are all reasons for rapid response.

Case and control numbers do not have to be the same; the calculations compare ratios so equal numbers in each group are not needed. Generally a 1:1 to 1:2 ratio of cases to controls is perfectly adequate.

Where the Source Is Still Viable, Alert the Public of Potential Risks as You Become Aware of Them

As interview data from cases and controls are accumulated, leading to formation of hypotheses about the source of the illness, human resources should be deployed in two additional essential tasks: (1) tracking down and confirming the hypothesized source of the illness, and (2) promptly issuing warnings to all affected groups about the possible risks from any source that is still accessible, *with assurances that further bulletins will be issued as soon as confirmation is received*. This *precautionary principle* is a vital component of risk management in modern public health. Waiting for *absolute* confirmation before releasing warnings and advisories should not be an option in the twenty-first century. The principle holds that while false alarms can be quickly forgiven, further illness should be avoided at the highest priority. Failure to heed this step has contributed to needless suffering and severe damage to reputation, trust and credibility.

[1] A case-control approach is necessary because *unlike* the data in Table 5B, we rarely have full information about *all* the attendees, and therefore the true *incidence/attack rate* is not available. Very rarely, when *all* cases and controls are available for interview, we would have the *true incidence rates for ill and for not-ill persons* and this would allow a "*retrospective cohort study*" to be carried out. Under such circumstances, and using Table 6 as an illustration, we could state that of 33 persons exposed to item *X*, 25 persons (75.8%) had become ill compared to 12 ill of 34 not exposed (35.3%).

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Use of Exposure Rates Rather Than Attack Rates

Where incubation times are longer than a day, there is increasing likelihood that only a small proportion of the non-ill people will be available for interview, and on many occasions, not even all the ill persons can be contacted. The point here is that the investigator is usually working with sub-sets of the true cases and controls. The 30 controls in Table 6 and possibly even the 37 cases may have been drawn from larger groups, and therefore we cannot state the ***incidence rate***, for example, as: "*…25 of 33 exposed were ill*," because the "33" had been artificially assembled, and may not resemble the true incidence at all. We CAN, however, use ***exposure rates***, for example: "*…of 37 Ill persons, 25 (67.6%) had been exposed to X*," and, "*…of 30 who were not-ill, only 8 (26.7%) had been exposed to X*." The overwhelming majority of waterborne or foodborne illness investigations are run as "case-control" studies (or to be more accurate, "case-comparison" studies, as very little true "controlling" is accomplished during the selection of the comparison group).

A *broadcasted* invitation to all who *might* have been exposed to come forward, typically results in few non-ill persons volunteering information, because non-affected individuals believe they have little if anything to contribute. This reduces validity even further, and more active recruitment is often necessary to convince them that their information is just as essential for the investigation as are the contributions from the less-fortunate attendees.

Odds Ratio as a Measure of Risk

Let us examine a waterborne illness suspected as being due to the consumption of water bottled from a certain spring. You have found 60 people who meet the case definition of illness, and another 29 non-ill people in same neighborhood who report no symptoms at all, and who will be your controls. In Table 7 we display the data and ask the question: *"is drinking this water related to the risk of illness*?" Whenever a 2×2 table appears, the first step is to calculate the odds ratio (OR).

An odds ratio tells us if there is a relationship (where OR ≠ 1), and the *strength* of the relationship (the OR value itself). It also clearly indicates the *direction* of the relationship: *was <u>drinking</u> or <u>not-drinking</u> the dangerous activity*? This is easily

Table 7 Odds ratio

	Ill (cases)	Not-ill (controls)	Total
Drank spring water	56 a	b 14	70
Did not drink water	4 c	d 15	19
Total	60	29	89

Label the four cells a, b, c, d as shown.
The odds ratio is calculated by cross-multiplying

$$\frac{(a \times d)}{(b \times c)} \text{ or } \frac{(56)\times(15)}{(14)\times(4)} = \frac{840}{56} = 15.0\ldots(\text{the odds ratio})$$

This is interpreted as *"An ill person was 15 times as likely to have drunk spring water compared to a person who was not ill."*

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determined by finding the dominant pair from (a×d) or (b×c). In the example above, (a×d) is greater, so cell "a" links the row "*drank*" with the column "*ill*," while cell "d" links "*not drink*" with "*not-ill*." This assumption is not as obvious as it may seem; the cause of the illness may have been whatever "other" thing was drunk by those who avoided spring water!

It is important to clarify that the odds ratio yields *the strength of the association*, not the statistical significance. Most OR values (where many exposures are being assessed) will be close to 1.0 (= "*no association*"), while an OR *clearly exceeding 1.0* signifies a positive association between this exposure and illness, such that this exposure *increased the risk of illness*.

An OR < 1.0 is *protective*, meaning that exposure to this factor *reduced the risk of illness* compared to the other group. For example, an OR of 0.25 means that the exposed group had only *one-quarter* the risk of illness compared to the non-exposed group. The *non-exposed* group therefore has a *greater* risk (by a factor of 4). While this *protective* effect can be due to true therapeutic protection (e.g., exposure to antibiotics when you have an infection), it is frequently explained as "statistical" protection. As an example, consider an outbreak where everyone consumed only one of two possible types of bottled water. One source, A, contains a pathogen, and B does not. If the ill people were found to be five times more likely to have consumed type A (odds ratio = 5.0) then the not-ill would have five times the rate of consuming water B, and only one-fifth of the rate of choosing water A (OR = 0.20 or 20%). This can also be read as the *risk of illness for the non-exposed group*, or as the *risk of staying well* by the *exposed* group. An easier way to interpret an OR less than 1 is to place 1 *over* the OR to reveal a value greater than 1, but clearly labeled "protective."

Weakness and Strength of the Odds Ratio

The OR is a *ratio* between numbers, and therefore not sensitive to the actual numbers of people in individual cells, an important consideration when the numbers of subjects are relatively small. This is illustrated by the common question: "*How large does an odds ratio have to be before it is considered evidence of an association*?" A popular response is "*at least 2.0*," but this must be considered with extreme caution. For instance, with very large studies, an OR of 1.12 (barely more than 1.0) can be shown to be very highly significant statistically ($P = 0.001$), whereas in a small-*n* study, an OR of even 5.0 may not achieve statistical significance.

The odds ratio is certainly a useful measurement, and should always be used when a 2×2 table is encountered. It will quickly advise you (1) that there is an association, (2) the strength of that association, and (3) the direction of the association, none of which are specifically measured by a test of statistical significance. Unfortunately, it is not reliable with small cell sizes, and is unable to answer the question: "*How likely is it that these numbers could happen just due to chance*?" For this, we need to test the statistical significance.

The best advice is to use the OR (or relative risk where appropriate) *together with* a test of statistical significance. Most online statistic calculators or laptop

versions of SAS, SPSS, EpiInfo, etc. will give a selection of useful statistics (odds ratio, relative risk, several versions of chi-square, and Fisher's exact test, both one-tailed and two-tailed.)

Testing Statistical Significance

Basic Concepts

In keeping with all scientific enquiry, we begin by advancing the notion (the "*null hypothesis*") that there is *no association* between the exposure and the illness, and attempt to *support* that notion. If insufficient evidence is found to support the null hypothesis, we *reject* it and cautiously consider that an ***association*** may exist between the two variables. This can be described as a *statistically significant* association. Two methods of testing are presented: the ***chi-square test*** (written χ^2 and pronounced "ky"-square) for most 2×2 (or larger) tables, and the ***Fisher's Exact Test*** (only for 2×2 tables) when chi-square is not valid due to the numbers in the cells being too small (the following sections give advice about this decision).

The Chi-Squared Test (χ^2)

The *original data* value in each cell we call the *observed*, or "*O*" value, and these are compared with the numbers that you would *expect* ("*E*" values) if there were *NO relationship at all*; that is, if the variables were *not* related, and the data were arranged purely by chance (as stated by the null hypothesis). The chi-square test measures the difference between the *O* and *E* values. If they are close, we have to accept that there may be no real relationship; if far apart, we can reject the null hypothesis and cautiously declare that exposure and illness were probably related. Numerous online statistical calculators can be used to yield ORs, RRs, and chi-square values.[2] If you prefer to do the calculation by hand, construct a 2×2 table as shown, with "observed" data, marginal totals, and the grand total. The expected "*E*" values are found from:

$$E = \frac{(\text{row total}) \times (\text{column total})}{\text{grand total}}$$

For cell "a": $E = \frac{70 \times 60}{89} = \frac{4200}{89} = 47.2$ The remaining "*E*" values are shown in parentheses.

[2] Epi-Info is a highly recommended suite of epidemiological and statistical programs, supported by the US CDC and WHO, and freely available for download in numerous languages. Full $2 \times N$ table analysis is included.

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Table 8 Chi-Square analysis for 2×2 tables

	Ill (cases)	Not ill (controls)	Total
Drank spring water	56 (47.2) a	14 (22.8) b	70
Did not drink water	4 (12.8) c	15 (6.2) d	19
Total	60	29	89

To make sure the chi-square analysis is appropriate for your table, you must be sure that all "*E*" numbers[3] are more than 5. The quickest way is to first calculate for the cell with the *smallest E* value; (this will be the cell *with the smaller column total* and *the smaller row total*.) In Table 8, the smallest *E* value will be cell "d," and this is calculated as (19×29)/89=6.2. As this is >5, all other *E* values will be greater than this, so chi-sq. is valid. (Note that the smallest *E* value did not coincide with the smallest *O* value).

Chi-square (χ^2) is the SUM of $\frac{(O-E)^2}{E}$ for all four cells.

For cell "a" $\frac{(O-E)^2}{E} = \frac{(56-47.2)^2}{47.2} = \frac{(8.8)^2}{47.2} = 1.64$

For all four cells the sum (χ^2) is: 1.64+3.40+6.05+12.49=23.58

An online statistical calculator will give you this same chi-square (χ^2) value. To verify by hand whether the *O* vs. *E* difference is statistically significant, compare your chi-sq. value (for a 2×2 table only) with 3.841. If your calculated value ***exceeds*** 3.841, then this is *unlikely to be due to chance*, and thus you can begin to believe that this exposure *did* influence the risk of illness, and you can reject the null hypothesis.

Statistical results usually include a probability (*P*) statement. This is the probability that the null hypothesis ("no association") is correct. The 3.841 value is the minimum needed for statistical significance, where the *P* is less than 5% ($P<0.05$). Recall that the *P* is the *probability that NO real association exists between exposure and illness*. By convention, if ***P*>0.05** (more than 5%) then the relationship is declared ***not statistically significant***. Where ***P*=0.05** or **<0.05**, then the relationship is ***statistically significant***. The smaller the *P* value, ($P<0.01$, $P<0.001$, etc.) the more confidence you have that a relationship really exists. Other critical values exist for assessing calculated chi-sq. values, from larger tables than 2×2, and at more extreme levels of significance. A further chi-square calculation is shown as an appendix.

[3] The requirement is that not more than 20% of the cells should have an *E* value less than 5. In a 2×2 table, one cell (25%) already exceeds this. For larger tables (2×3, 3×3, etc.) the rule will allow one or more *E* values <5. Note that this applies to the *E* value, NOT the original *O* value in the cell.

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Cell Size Limitation

Where a table greater than 2×2 is found to have more than 20% of the cells with an *E* value less than 5, chi-square is not valid. The solution is to *collapse either columns or rows* to allow the *E* values to increase. For example in Table 9A, two cells out of six (33%) have *E* values less than 5, but if "*high dose*" is merged with "*medium dose*" the resulting increase in observed (*O*) cell sizes is also reflected in greater *E* values, while the table becomes 2×2 (Table 9B). Some outcome information has been lost, but the chi-square analysis can proceed. If, after trying to collapse cells and/or rows, a 2×2 table is reached still with an *E* value <5, the Fisher's test is indicated.

Fisher's Exact Test: (Another Example is Shown as Form J2)

This procedure is reserved only for 2×2 tables where one or more expected (*E*) values is less than 5, making the chi-square test not valid. Our example is taken from an investigation into an outbreak of shigellosis presumed to be due to water from a well (Table 10). The odds ratio has been calculated as (8×6)/(4×2)=6.0, *meaning ill persons were six times as likely to have drunk well water compared to non-ill*

Table 9 Collapsing rows or columns to obtain *E* values valid for chi-square analysis

(A) Before collapsing: chi-sq. not valid (two *E* values <5)

	Symptoms	No symptoms	Totals
High dose	16 (9.67)	9 (15.33)	25
Medium dose	2 (2.71)*	5 (4.29)*	7
Control (no dose)	11 (16.63)	32 (26.37)	43
Totals	29	46	75

(B) After collapsing rows: chi-sq. now valid

	Symptoms	No symptoms	Totals
"Any" dose	18 (12.37)	14 (19.63)	32
Control (no dose)	11 (16.63)	32 (26.37)	43
Totals	29	46	75

*Expected values <5
Expected values shown in parentheses

Table 10 Fisher's exact test: Original data

	Ill	Not-ill	Total
Drank well water	8 a	4 b	12 (a+b)
Did not drink well water	2 c	6 d	8 (c+d)
Total	10 (a+c)	10 (b+d)	20 (a+b+c+d)

To test this we calculate a probability value (*P*) directly using

$$P = \frac{(a+c)!\times(b+d)!\times(a+b)!\times(c+d)!}{a!\times b!\times c!\times d!\times(a+b+c+d)!}$$

The "!" denotes a factorial, meaning that number multiplied by the next smallest number, and so on down to 1.
(e.g.: 6!=720)

$$P_1 = \frac{10!\times10!\times12!\times8!}{8!\times4!\times2!\times6!\times20!} = 0.075018$$

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Table 11 Fisher's exact test: Adjusted data

	Ill	Not-ill	Total
Drank well water	~~8~~ 9 a	b ~~4~~ 3	12 (a+b)
Did not drink well water	c ~~2~~ 1	d ~~6~~ 7	8 (c+d)
Total	10 (a+c)	10 (b+d)	20 (a+b+c+d)

The "dominant" a and d are each increased by 1, while b and c are both decreased by 1, keeping marginal totals unchanged.
Recalculating:

$$P_2 = \frac{10! \times 10! \times 12! \times 8!}{9! \times 3! \times 1! \times 7! \times 20!} = 0.009526$$

Table 12 Fisher's exact test: Final data

	Ill	Not-ill	Total
Drank well water	~~9~~ 10 a	b ~~3~~ 2	12 (a+b)
Did not drink well water	c ~~1~~ 0	d ~~7~~ 8	8 (c+d)
Total	10 (a+c)	10 (b+d)	20 (a+b+c+d)

Recalculating:

$$P_3 = \frac{10! \times 10! \times 12! \times 8!}{10! \times 2! \times 0! \times 8! \times 20!} = 0.000357$$

$$P_{\text{Total}} = P_1 + P_2 + P_3$$

$$P_{\text{Total}} = 0.0750 + 0.0095 + 0.0004 = 0.0849$$

persons. An attempt to use chi-square is prevented by at least one E value less than of 5. (Cells c and d both show E values as $(8 \times 10)/20 = 4.0$). The starting null hypothesis is "*that no relationship exists*."

This is not quite the end of the calculation however. The goal is to calculate the probability of the *original data occurring plus all more extreme probabilities*. The original data have to be adjusted by increasing the "dominant" pair of cells by +1 and the others by −1, while leaving the margin totals the same (Table 11).

Because no zero has yet appeared in the matrix of cells, we continue to increase the "dominant" pair by +1 and obtain a zero. The next calculation is the last. (By convention, 1! and 0! = 1) (Table 12).

Interpretation: No reference table is required. The total calculated probability (0.085) is exactly the probability that the null hypothesis ("*that there was no relationship*"), is correct: 8.5%.[4] By convention, for a result to be significant statistically, that probability (P) must be less than 5% (<0.05), so in this instance we are *not* able to reject the null hypothesis and must conclude that the relationship *could* have occurred by chance alone more than 5% of the time. The odds ratio of 6.0 is explained as the number of times more likely it was for a shigellosis victim to have drunk well water than for a non-ill person. This increased risk would normally be impressive, but because of the small number of persons in the study, it has been

[4] The first probability (P1 = 0.075) was already in excess of 0.05, so it was already not significant, and further additions would increase this value still further. The calculations could therefore have stopped after the first probability, with the statement "$P > 0.05$, not-significant." The calculations here are carried out in full to illustrate the process of working toward a full and final probability.

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found not to pass the test of statistical significance. A basic write up of the results might read:

"*A relationship exists between drinking well water and developing shigellosis. A shigellosis patient is six times more likely to have drunk water from the well compared to a non-ill person. This relationship is not statistically significant, however, and could have occurred by chance alone more than 5% of the time. The null hypothesis of no-relationship cannot be rejected.*" *[1 df, $P > 0.05$, OR: 6.0, not statistically significant.]*

Summary Tables

With the odds ratio (OR) calculated for all the suspected exposures, and the chi-square test or Fisher's exact test calculated for the strongest of these, all the results can be displayed in a composite table.

Earlier protocols for the investigation of waterborne and foodborne diseases encouraged the use of the "***factor-specific attack rate table***" (for example the "*food-specific attack rate table*"), but where only a "convenience sample" of controls and cases are available, we are unable to derive valid incidence/attack rates. Investigators are discouraged from using it as it may produce misleading results. The ***exposure-rate table for cases and controls*** is preferred in all case-control studies, and compares the rates of exposure to each factor between both the ill and non-ill people.

Table 13 displays six exposure factors from a hypothetical outbreak involving water contamination. Exposure rates are calculated from both cases and controls. The "spring-water" data that we used for the odds ratio calculation example in Table 8 appears as the first exposure in Table 11. The column headed "Differences in exposure rates" subtracts the exposure rate among the non-ill from the exposure rate among the ill. [Use: Exp. rate (cases) minus Exp. rate (controls), keeping the signs correct]. You are looking for a large positive difference to indicate the most likely culprit. The spring water shows the largest positive difference at +45%. The odds ratio of 15.0 supports this, again the largest value, indicating *that ill persons were 15 times more likely to have drunk the spring water compared to non-ill persons in this group*. Hence both the large positive difference in exposure rates and the large odds ratio point to the spring water being the likely source of the illness, and it is certainly the strongest association between illness and any of the exposures shown. The chi-square value has also been added (23.6), as well as the associated P value. Taken together, the evidence clearly points to this factor as the culprit.

In those less-common circumstances in which ALL the ill and non-ill persons can be contacted for interview, the table can be rearranged to show *attack rates (incidence rates)* for each of the suspect factors (Table 14). Here, the column of "differences" shows the *attack rate (exposed) minus the attack rate (non-exposed),* ($I_E - I_N$), and again a large positive difference will point to the culprit. This measure is called the *attributable risk* and for the spring water example we obtain +59%, the largest value of all the risk factors. Also, because of the availability of valid attack rates (incidence rates), the *true relative risk* (RR) is available, and can be substituted

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Table 13 Exposure-rate table for cases and controls (for use when data are a sample of both cases and controls)

	Cases (ill)				Controls (not ill)							
Factor	Exp (a)	Not exp (c)	Total ($a+c$)	Exp rate ($a/(a+c)$) (%)	Exp (b)	Not exp (d)	Total ($b+d$)	Exp rate ($b/(b+d)$ (%)	Diff. in exp rates (%)	Odds ratio[a]	Chi-Sq	P value[b]
Spring water	56	4	60	93	14	15	29	48	+45	15.0	23.6	<0.001
Soft drink	50	10	60	83	28	1	29	97	−14	0.18	3.15	0.08 ns
Water cress	48	12	60	80	23	6	29	73	+7	1.04		
Washed lettuce	35	25	60	58	16	13	29	55	+3	1.14		
Washed berries	51	9	60	85	24	5	29	83	+2	1.18		
Tomatoes	50	10	60	83	24	5	29	83	0	1.04		

[a]See Form J1 for an illustration of the odds ratio calculation

[b]The P (probability) value is a statement of statistical significance. It indicates the probability that there is NO relationship between the factor and the illness. So as the number becomes very small (as shown here) we can be increasingly satisfied that a real relationship does exist. See the Statistical Significance section for the correct way to calculate this

ns = not significant

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Table 14 Attack-rate table (for use when data are available from ALL cases and ALL controls)

	For those EXPOSED (consumed item)				For those NOT EXPOSED (did NOT consume item)							
Factor	Ill (*a*)	Not ill (*b*)	Total (*a*+*b*)	Attack rate (*a*/(*a*+*b*)) (%)	Ill (*c*)	Not ill (*d*)	Total (*c*+*d*)	Attack rate (*c*/(*c*+*d*)) (%)	Diff. in attack rates (%)	Relative risk[a]	Chi-sq.	*P* value [b]
Spring water	56	14	70	80	4	15	19	21	+59	3.80	23.6	<0.001
Soft drink	50	28	78	64	10	1	11	91	−27	0.71	3.15	0.08 ns
Water cress	48	23	71	68	12	6	18	67	+1	1.01		
Washed lettuce	35	16	51	69	25	13	38	66	+3	1.04		
Washed berries	51	24	75	68	9	5	14	64	+4	1.06		
Tomatoes	50	24	74	68	10	5	15	67	+1	1.01		

[a]The (“true”) relative risk (also called the risk ratio) is only used when we have the true incidence data. It is calculated as the attack rate (exposed) over the attack rate (non-exposed), or I_E/I_N

[b]The *P* (probability) value is a statement of statistical significance. It indicates the probability that there is NO relationship between the factor and the illness. So as the number becomes very small (as shown here) we can be increasingly satisfied that a real relationship does exist. See the Statistical Significance section for the correct way to calculate this

ns = not significant

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for the odds ratio. The data in Table 14 shows the same data as Table 13 rearranged for easy comparison. Values in the column of "differences" are not the same as for Table 13 and of course the relative risks are not the same as the odds ratios in Table 13, but both of these results still point clearly to the suspect exposure.

In both analyses, spring water is clearly the factor most strongly associated with illness. It is important to note that in both tables a high rate (exposure- or attack-) on the left side taken by itself is meaningless until it is compared with the rate from the right side of the table. This again underscores the importance of gathering complete data from the non-ill as well as the ill.

An interesting phenomenon is visible in the second factor listed (soft drink). The OR is listed as 0.18, which is "protective," meaning that this factor is strongly associated with NOT being ill. It is the equivalent of OR equal to 5.55 (1/0.18), and the chi-square is seen as quite large, although not enough for statistical significance. This is sometimes seen where TWO factors are "in competition" with each other; if everyone had drunk one item, and the spring water was the contaminated source, then those drinking the *other* item would be strongly "protected" because they did *not* drink the spring water, and this shows clearly. All other factors have OR values very close to 1.0.

Most attack rate tables record some persons who did not ingest the suspect vehicle but who nevertheless became ill. Plausible explanations are that (a) some people forget which beverages or foods they ingested; (b) some might have become ill from other causes; or (c) some may have exhibited symptoms with a psychosomatic rather than a physiological origin. It is also not unusual for the table to include some persons who ingested contaminated water or food but did not become ill. Plausible explanations are that (a) organisms or toxins are not always evenly distributed in water or food and consequently some persons ingest small doses or perhaps none at all; (b) some persons eat or drink larger quantities than others; (c) some are more resistant to illness than others, and (d) some will not admit that they became ill, or fail to report it.

Whichever table is used, the combined totals for cases (ill) and controls (well) are fixed and should not change for each exposure unless there are "missing" responses from interviewees.

While some procedure manuals include *confidence limits* around both RR and OR, this may be omitted here as the use of the chi-square test or Fishers Exact test yield the statistical significance for both tables.

Other Associations

Quantity-of-Water Ingested

Illness caused by ingestion of waterborne toxicants and some pathogenic organisms can be dose-related in that the risk of developing symptoms, and their severity varies with the quantity ingested. Where the suspect water is no longer available (for example, the well may have been quickly super-chlorinated to break the chain of infection

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before samples were taken), attack rates can be based on the amount of water usually drunk per day by each person. This is easily extended to other non-treated sources of water such as ice cubes, water-reconstituted fruit juices, and flavored crystals. A comparison of attack rates at various water intake levels may provide valuable evidence that water is, or is not, the vehicle responsible for the outbreak. For an example, see Table 15. Here, the entire group was 210 people and we have interviewed them all, so we are justified in calculating the attack/incidence rates:

In this example, the attack rate increased as the consumption of water increased, which suggests that the illness was directly related to water and the agent it contained. This is a trend established from the group as a whole, and an individual's experience may vary with factors such as (a) preferences of water ingestion, (b) intermittent contamination, (c) unequal distribution of the contaminant, or (d) varying susceptibility of individuals. These data can be compared with rates from persons who ingested no water, but only hot tea, hot coffee, soups, and/or other safe sources of liquids. If unheated water was indeed the vehicle, and the agent was a living biological agent, these persons should have attack rates showing no increase in risk of illness. (Outbreaks from a toxic agent may be unaffected by chlorination, boiling, and some types of filtering.)

The data can be displayed in a contingency table as follows for analysis using chi-square procedure (Table 16).

Table 15 Number of glasses of water and water/ice-containing beverages usually ingested per day by interviewees

	Ill	Not ill	Total	Attack rate (%)
5 or more	15	30	45	33
3 or 4	23	59	82	28
1 or 2	9	48	57	16
<1/day	2	24	26	8
Total	49	161	210	

Table 16 Data from Table 13 arranged for Chi-Square analysis

# glasses water/day	Ill	Not-ill	Total
5 or more	a 15 (10.5)	b 30 (34.5)	45
3–4	c 23 (19.1)	d 59 (62.9)	82
1–2	e 9 (13.3)	f 48 (43.7)	57
<1/day	g 2 (6.1)	h 24 (19.9)	26
Total	49	161	210

Follow the procedure as for a 2×2 table. The original data are considered the "observed" (*O*) values, and we calculate the expected (*E*) values using: $\frac{(\text{row total})\times(\text{column total})}{\text{grand total}}$

The expected numbers have been placed in parentheses. All *E* values are 5 or more, although this table could allow one *E* value that was less than 5[a]

The degrees of freedom (df) are calculated as $(\text{No. of rows}-1)\times(\text{No. of columns}-1)$, or $(4-1)\times(2-1)=3$

Chi-square is obtained by calculating …

$$\frac{(O-E)^2}{E}$$

… for each cell and adding the eight values obtained.

[a] For Chi-square to remain valid, not more than 20% of the cells can have an 'E' value less than 5. For 2×2 tables, a single cell is 25% of the total so such a table may have *no* cells with an *E* value less than 5. Tables 2×3 or 2×4 can still employ Chi-square with the *E* value of *one* cell less than 5

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For this example, chi-square equals 8.90 and if calculated by computer or online, P will be shown as $P=0.031$. Reference to Form J1 confirms that for a 2×4 table (3 df), the calculated chi-square (8.90) exceeds the critical value for statistical significance at the 0.05 level (7.82), allowing us to claim statistical significance at $P<0.05$. Odds ratios are normally associated only with 2×2 tables, but here, the OR can usefully be calculated on selective cells or groups of cells as long as you clearly explain the selection process. For instance, persons who were ill were 2.8 times more likely to have drunk three or more glasses of water per day compared to those who were well. For this calculation we *collapse cells* into a 2×2 table and cross-multiply: $(a+c) \times (f+h)/(b+d) \times (e+g) = (38) \times (72)/(89) \times (11) = 2736/979 = 2.79$. Alternatively, because we have all people involved, we can compare the attack rates (AR) for each intake level, and observe the increasing attack rate as the intake increases: For five or more glasses/day, AR: 33%, for 3–4/day, AR: 28%, for 1–2/day, AR: 16%, and for <1/day, AR: 8%. We might summarize as follows:

"*There was a relationship observed between the quantity of water consumed each day and the risk of illness. The incidence rate increased with the quantity consumed from 8% for <1 glasses/day to 33% for five or more glasses/day. This relationship is statistically significant. The null hypothesis of no association can be rejected.*" *[Chi-square: 8.90, 3 df, P < 0.05]*

Other Water-Related Exposures

Water as a vehicle can deliver pathogenic organisms in many ways beyond simply drinking a glass of water, or using a drinking fountain. Investigators should be sure to ask about the preparation of ice-cubes, the mixing of fruit flavored crystal drinks, reconstituting concentrated orange juice, brushing and rinsing teeth, and washing hands, utensils, or containers. Swimming or playing in muddy pools or even swimming pools have caused waterborne poliomyelitis, and naegleriasis, while swimming in saltwater inlets have allowed inadvertent infections from *Vibrio parahaemolyticus* and *V. vulnificus*. Unwashed plastic jugs containing poster paint residue have caused rapid illness when drink crystals are reconstituted in them, while refillable plastic containers and bottles have a long history of contamination from biological and chemical agents. In the late 1970s, an increase of viral ear, nose, and throat infections among people who were using parkland next to a river was hypothesized to have been due to people waterskiing on the river and creating an aerosol. The river was the receiving body for effluent from a water treatment plant upstream.

Interpret Results from Water Samples

Record all laboratory results on Form I, *Laboratory Results Summary*. Compare epidemiological and statistical results with on-site observations, laboratory results and the information summarized on Form I. The agent responsible for the outbreak

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can be determined by (a) isolating and identifying pathogenic microorganisms from patients, (b) identifying the same strain and/or PFGE pattern or genetic sequence of pathogen in specimens from several patients, (c) finding toxic substances or substances indicative of pathological responses in specimens, or (d) demonstrating increased antibody titer in sera from patients whose clinical features are consistent with those known to be produced by the agent.

When implicating the water as a likely (or presumptive) vehicle of transmission, ideally identification of a pathogen in samples of suspect water will correspond to the one found in clinical specimens from ill persons or that produces an illness that is compatible with the incubation period and clinical features of the ill who were exposed to the water. For organisms that are common in the gastrointestinal tract or that have multiple strains, compare strains isolated from ill persons with strains isolated from the suspected water. Additionally, specific microbial markers (e.g., serotype, phage type, immunoblotting, plasmid analysis, antibiotic resistance patterns, restriction endonuclease analysis, nucleotide sequence analysis) or chemical markers identified by chromatography or spectrophotometry can be used for this purpose. For confirmation of water-related transmission, the same pathogen strains should be found in both the ill persons and the epidemiologically implicated water. However, due to the period of time that may have passed after the outbreak was actually reported, and to methodological issues, such as the need for concentrating pathogens in water samples, it is often unlikely that the outbreak-associated pathogen will be found in the water samples.

Laboratories frequently test water samples for indicator organisms, such as fecal coliforms, *Escherichia coli*, or enterococci, rather than pathogens. The finding of these bacteria in high densities in the water may indicate contamination (from a fecal source) and implicate the water was a possible vehicle. However, the finding of increased indicators in water samples alone is insufficient evidence to confirm the water as the source of an outbreak.

The probable source of contamination or the situation that allowed contamination to reach and survive in a water supply (e.g., water supply not disinfected or inadequately disinfected, inadequately filtered, or upstream to sewage or agricultural discharges; cross connection between sewerage and drinking water pipes; well improperly constructed; nearby septic tank system; or livestock in water supply) can often be identified, but the etiologic agent in the water may never be found. Success in finding the etiologic agent is most likely where (a) the incubation period of the illness is short, (b) the agent is stable in water and the system is static, or (c) large amounts of the agent are being continually added to the water supply. Try to recover and identify the specific agent whenever a water supply is suspected to be the vehicle of transmission, even if finding the etiologic agent is likely to be difficult and not considered practical for routine monitoring of water supplies. If water samples do not reveal a likely causal agent, clinical data as well as time, place, and person associations can cast strong suspicion on a water supply, particularly if indicator organisms are found in the water. Tests other than those for pathogens, however, are frequently used to evaluate water supplies on a routine basis.

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Interpret Physical and Organoleptic Tests

Organoleptic tests attempt to evaluate the total effect of all compounds present in water that can be measured by the senses of taste, smell, or sight. Results cannot be expressed in terms of specific compounds present, and the measured qualities are usually a result of a mixture of compounds. These tests are often empirical and arbitrary, but changes in the physical qualities of water (such as pH, turbidity, color, odor, or taste) can indicate abnormalities of the water. Outbreaks have occurred, however, when turbidity readings have met present standards and when water appeared and tasted good.

Interpret Chemical Tests

Chemical examination of water is useful for (a) detecting pollution (especially from industrial wastes and pesticides), (b) determining effectiveness of treatment processes, (c) evaluating the previous history of the water, (d) determining hardness, and (e) detecting the presence of specific toxins. Results are usually expressed in milligrams per liter (mg/L = ppm, parts per million), or micrograms per liter (μg/L = ppb, parts per billion). Historically, acute water-related outbreaks seldom involve chemical substances, so chemical tests are not requested routinely unless either (a) circumstances indicate possible chemical contamination or (b) clinical symptoms suggest chemical poisoning.

Flowing water in a distribution system can be monitored to determine chlorine residual. Free available residual chlorine refers to that portion of the total residual chlorine remaining in chlorinated water at the end of a specific contact period that will react chemically and biologically as hypochlorous acid or hypochlorite ion. The reaction is influenced by pH and temperature. Total or combined residual chlorine refers to chlorine that has reacted with ammonia or other substances and is not available for further reactions, as well as the free available chlorine. A chlorine demand exists in a chlorinated water until a free available residual is produced. A free available chlorine residual, e.g., 1 mg/L (1 ppm) or higher, maintained throughout the distribution system of a community supply is an indicator of safety from enteric bacteria but not necessarily from pseudomonads, viruses or parasites. Outbreaks have occurred when chlorine residue levels have met present standards.

Interpret Microbiological Indicator Tests

Analyses for microbial indicator organisms provide information on the microbiological quality of water and guidance as to its safety for consumption or contact. Indicator organisms are easier to test for than pathogenic organisms, and some serve as a surrogate measure of fecal contamination in water. The absence of indicator

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organisms in the water, however, does not guarantee water safety; numerous outbreaks of water-related disease have occurred from water in which no indicator organisms were detected. Evaluation of the safety of water should be based upon a combination of results of (a) an on-site study to identify sources and modes of contamination and means by which contaminants survived treatment and (b) appropriate laboratory analyses. Microbiological results should be compatible with observed sources of contamination and/or treatment failures found during the investigation.

Heterotrophic Plate Count (HPC)

Although all natural waters contain bacteria, the number and kind vary greatly in different places and under different climatic and environmental conditions. The number of bacteria isolated and reported, however, often represents only a fraction of the total number present, for several reasons. Colonies seen on agar plates develop from either single organisms or clusters or chains of organisms. Heterotrophic bacteria represent only those that can use organic matter and grow at the selected temperature (30–35°C) within 48–72 hours under aerobic/microaerophilic conditions in/on a defined medium when the standard test (spread plate, membrane filter or pour plate) is used. The HPC may also be done using different media under different incubation times/conditions. (Higher counts are usually found when the longer incubation periods are used.) Also, certain microorganisms are unable to grow aerobically either in or on the medium used. Because of these variables, the terms Total Plate Count (TPC), Standard Plate Count (SPC) and Aerobic Plate Counts (APC) should not be used.

HPCs serve as an index of changing sanitary conditions. In general, counts of good-quality well water are fewer than 200–500 colonies per mL. Densities in surface water are higher, but quite variable, depending on water temperature, sources of pollution, amount of organic matter present, and soil that washes into the water. The sources of pathogens, toxic substances, or fecal contamination may not increase the HPC of a surface water sample as much as washings from soil. Nevertheless, marked changes in the number or kind of microorganisms should be viewed with concern, at least until the reason for the change is discovered. Heterotrophic plate counts greater than 1000/mL and some specific antagonistic species may interfere with the growth or recovery of pathogenic or indicator organisms. Some heterotrophic species are opportunistic pathogens that may pose a health threat to immunocompromised persons.

Total Coliforms

The coliform group of bacteria comprises those from non-fecal environmental sources, and those from animal and human intestines, including *Escherichia coli*. The environmental species of non-fecal bacteria are found in soil, on fruits, leaves, and grains, and in run-off water, especially after heavy rains. Some of these species

are capable of surviving in water longer than *E. coli*. Furthermore, some coliform strains and can multiply on decaying vegetation in water, in biofilms in pipelines, or on pump packings, washers, and similar materials. Therefore, finding coliforms may not be indicative of fecal contamination, although most water utilities have standards for coliforms in water. Fecal coliforms are present in large densities in all human and animal feces, normally much higher than pathogens which are typically only present in infected persons and normally at lower levels. As such, high populations of fecal coliforms can indicate recent sewage pollution of water, but are not always indicative of pathogens present, particularly viruses and parasites. None of the coliform group, however persists as long as most viral or protozoan pathogens in water, and indicator bacteria described below (fecal streptococci and *Clostridium perfringens*).

Typical chlorination or ozonation of water inactivates coliform bacteria. Presence of the coliform group or even a high population of coliform bacteria is not proof that a treated water supply contains pathogens. However, coliforms can provide a warning that either the water treatment was inadequate or contamination occurred after treatment, and that some pathogens may be present. As mentioned above, under some conditions, pathogens may be present where there are few or no coliforms. Furthermore, unlike coliforms, many parasites and viruses are resistant to normal levels of disinfectants. Coliforms have little or no correlation with the presence of parasitic protozoa or pathogenic viruses.

The standard test for the coliform group may be carried out by a membrane filtration technique, a multiple-tube fermentation technique (presumptive test, confirmed test, or completed test), or a presence-absence test. Results of the membrane filtration technique are reported as colony forming units (CFU) per 100 mL of water. Results of the multiple-tube fermentation technique are reported as the most probable number (MPN) per 100 mL of water. This is a statistical estimation of the total number present, but the actual number can fall within a considerable range. Counts derived from these two methods are not necessarily the same, but they have the same sanitary significance. False-negative or false-positive results can also occur with the membrane filtration technique because of interfering background growth of nonfecal microorganisms.

Results of the presumptive test of the multiple-tube fermentation technique can be misleading, because other microorganisms frequently found in water also produce gas in laboratory media, and may thereby give false-positive results. Also, especially in waters containing a large number of microorganisms, some coliforms present may produce gas slowly, leading to false-negative results. The presence of coliform bacteria is corroborated by means of the second phase of the multiple-tube fermentation technique, known as the confirmed test. Positive results are usually considered confirmation of the presence of coliforms. A third phase of this test, known as a completed test, further ensures the correct identification of coliform bacteria.

A simple modification of the coliform test is to analyze for the presence or absence of coliforms in a 100-mL drinking water sample. The "presence-absence (P/A) coliform test" allows for simple examination of a larger number of samples.

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When a positive sample is detected, it is advisable to measure coliform densities in repeat samples by one of the other methods to determine the magnitude of the contamination.

Thermotolerant coliform (fecal coliform). Coliform bacteria will frequently grow at a relatively high temperature, 44.5°C, unlike species or strains normally encountered in the environment, which usually have an optimal temperature near 30°C. This thermotolerant characteristic has been used in an attempt to separate coliform bacteria into those of so-called fecal and non-fecal origin. This test may provide better indication of fecal contamination than the coliform test, but it is however, unreliable. Positive results are not proof that either organisms of fecal origin or pathogens are present. The number of thermotolerant coliforms is considerably lower than the number of total coliforms in contaminated water; therefore, the test is less sensitive for testing treated drinking water. Furthermore, *Escherichia coli* O157:H7, which has been implicated as causing water-related illness, does not grow well at 44.5°C.

Escherichia coli. *E. coli* is common in feces of human beings, other mammals, and birds. It can also be found to grow naturally in the environment, specifically in tropical waters. Comprised of the larger coliform group, its detection in water is a more definitive indicator of fecal contamination, compared to total or fecal coliforms. However, a positive test result does not identify if the fecal source is human or nonhuman. Rather, the finding of *E. coli* in water serves as an indicator that fecal matter reached the water and provides a warning, but not proof, that pathogenic organisms may also be present. It should be noted that some strains of *E. coli* are pathogenic (see Table B).

Simple commercial P/A and quantitative tests have been developed to detect the presence of total coliforms and *E. coli* in 24 hours by observing color changes and fluorescence of the media under daylight and UV light. Such tests may be useful for field evaluation of microbiological water quality.

Enterococci (Fecal streptococci). Another group of organisms, collectively known as fecal streptococci, is also used as an indicator of fecal contamination. Enterococci (*Enterococcus faecalis*, *Enterococcus faecium*) are particularly used for testing recreational waters. Like coliforms, enterococci are normal inhabitants of the intestinal tract of human beings and other animals. In human feces, they occur in considerably lower numbers than *E. coli*. Some members of the group, such as *E. faecalis*, subsp. liquefaciens, however, have been associated with vegetation, insects, and certain types of soils. Enterococci generally survive longer than coliforms in fresh water, and therefore the source of contamination may be distant in either time or place from the site where samples were obtained. Their resistance is, however, less than that of *Clostridium perfringens*, enteric viruses, and parasites.

Like *E. coli*, simple commercial P/A and quantitative tests have been developed to detect the presence of enterococci in 24 hours by observing color changes under UV light, which may be useful for field evaluation of microbiological water quality.

***Clostridium perfringens* (sulfite reducing clostridia)**. *C. perfringens* is also of fecal origin, but it occurs in feces in much lower densities than *E. coli* and can also

be found in soils. Being a spore-former, it can survive for long durations in soil and water, and persist when all other bacteria of fecal origin have disappeared. Therefore, it is a useful indicator of remote or intermittent contamination in wells that are not frequently examined by the coliform test; but, it is not, by itself, evidence of recent contamination. Chlorine, in the concentration typically used in water treatment, does not inactivate all spores; and thus *C. perfringens* is not valuable in assessing the efficiency of chlorination for bacterial vegetative cells. Its long persistence and its resistance to chlorine make this organism a potential indicator for viral and parasitic organisms that have similar resistance and disinfectant susceptibility.

Coliphage. Coliphages, which are viruses that infect *E. coli*, are simpler to detect and enumerate, compared to other viruses, and are generally associated with fecal contamination. They have been considered as possible indicators of treatment effectiveness for human enteric viruses. Coliphages are categorized into two groups: the somatic phages, which enter *E. coli* via the cell wall and the male-specific phages, which enter *E. coli* through the sex pili. The somatic and male-specific phages are common in sewage and the feces of human beings and other animals, but in lower densities than the common fecal indicator bacteria, fecal coliforms, *E. coli*, and enterococci. Some strains appear to be more resistant to chemical disinfection than water-related pathogens or indicator bacteria.

Local Standards

Be aware of local standards for water distribution systems, private water systems, and recreational water. Although drinking water standards, such as the total number of coliforms allowed in a water sample, vary from jurisdiction-to-jurisdiction, it is generally agreed that any fecal contamination (e.g. fecal coliforms, *Escherichia coli*) render the water unacceptable for human consumption and may close down recreational bathing waters.

Interpret Tests for Pathogens

There are numerous pathogens that can be transmitted by water, many of which are also able to cause respiratory symptoms, in addition to the classical gastrointestinal symptoms. For a comprehensive summary of waterborne pathogens see "American Waterworks Association Manual of Water Supply Practices, M48 Waterborne Pathogens, 2nd edition (2006)."

For several reasons, analyses for pathogens are not usually conducted during routine water testing, or are only conducted by specialized laboratories. First, tests for pathogens are pathogen-specific, expensive, and often difficult to perform because they may require specialized trained personnel. Secondly, the etiologic agent of the outbreak is often unknown at the time of analysis; hence, many analyses would have to be done blindly. Thirdly, pathogens are not always recovered because they are heterogeneously dispersed and diluted in the environment, and their numbers decline in water over time. As a result, they may be absent or present

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in low densities by the time samples are collected following an outbreak. Fourthly, recovery efficiencies are often poor because microorganisms are stressed by disinfectants or the method is sensitive to interferences from the source waters environment, hence not easily recovered by routine methods. Additionally, recovery efficiencies for viruses and protozoa may be poor because of the interferences of substances within the sample matrix with method reagents (concentrating 1800 L of water down to 200 μL will also concentrate inhibitory chemicals and substances). Finally, the time required for isolation and identification is often long, and the number of samples is usually too small to allow the investigator to have much statistical confidence in the results when pathogens are not found.

Negative results should be reported as "Not Detected" because they do not ensure that the water sampled was not the source of the pathogen. Procedures used for many bacterial pathogens are qualitative because enrichment procedures are used. Quantitative procedures (e.g., MPN) require considerable work and are less reliable than those used for coliforms because small populations may be present, and these may be unevenly distributed. Despite these difficulties, pathogens that cause a syndrome similar to the one being investigated should be sought. See Tables B, C, and D, for descriptions of the disease syndrome associated with the pathogens described in the following material. Finding the same pathogen in specimens from patients and in water samples confirms water as a vehicle.

Submit Report

Summarize investigative data in a narrative report. Describe in this report situations that led to contamination of the water and survival of etiologic agents up to the time of consumption. Include all events that contributed to the outbreak to guide control and preventive measures. Compare your data with the listings in Table G (Guidelines for confirmation of waterborne outbreaks) and Table H (Guidelines for confirmation of water responsible for illness), and criteria for confirmation of vehicle responsible for waterborne illness before assigning the etiologic agent and the vehicle. Outbreak confirmation is based on (a) time, place, person associations, (b) recovery of etiologic agents from clinical specimens from cases and samples of water, and (c) identification of sources and modes of contamination and means by which pathogens or toxic substances survived treatment. All three of these, however, might not be found in any one investigation.

Complete Form K (Waterborne illness summary report). Attach the narrative and the epidemic curve. Also attach Form D2 (Case history summaries: Water/Laboratory data), all applicable parts of Forms G, Forms H, Form I, and other data that will provide supplemental information to reviewers.

Send this report through administrative channels to the appropriate agency responsible for waterborne disease surveillance. Make the final report as complete as possible, so that the agency can accurately interpret the results and develop a meaningful waterborne disease data bank. In the interest of continuing cooperation,

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give all participants in the investigation due credit and send each a copy of the report. Also, send copies of the report through administrative channels to agencies (a) that have jurisdiction over the implicated water, (b) that initiated the alert, and (c) that participated in the investigation.

Those concerned with water sources, treatment and recreation, as well as with public health, should make every effort to ensure the complete investigation and reporting of waterborne diseases. Without reliable, complete information, trends in waterborne disease incidence and causal factors of the disease are difficult to determine. Good surveillance is essential for detecting and evaluating new waterborne disease hazards.

Use Outbreak Data for Prevention

The primary purposes of a waterborne disease investigation are to identify the cause, establish control measures, and take actions to prevent future illness. Prudence may require some action before all the hypotheses regarding the water supply involved and the source of contamination are confirmed. Frequently the local health authority will issue a Boil Water Advisory if a microorganism is suspected to have contaminated the water. Refer to "Possible Precautionary Control Actions" section for a discussion of these precautionary control measures. If these measures have not already been considered, consider them now. Once control measures have been implemented, continue to monitor for disease to evaluate whether the measures were effective. In a waterborne event in Sydney, Australia (see Box 1) Sydney Water severely overestimated levels of *Cryptosporidium* and *Giardia* present in the water raising public alarm. Boil water advisories were announced and rescinded several times. However, it is better to announce boil-water advisories than to have thousands ill, as has happened in the past, such as the *Cryptosporidium* outbreak in Milwaukee in 1993.

Deficiencies in treatment must be corrected and defective parts of distribution systems must be repaired, beginning with those that either contributed to or had a high potential for contributing to the outbreak. The effectiveness of these efforts will be directly related to the thoroughness of the investigation. Document the source and the manner of contamination and survival of the etiologic agent through the water treatment process. Provide clear documentation of contributory factors, so that preventive measures taken will be specific to the problem.

If previous sanitary surveys have revealed, or if subsequent ones reveal, that conditions which contributed to the outbreak are widespread, initiate a training and education program. These programs can be developed for water treatment plant or recreational water operators and employees, engineers, homeowners, or other appropriate groups. Impress upon them the importance of proper construction and operation of facilities and proper protection, treatment, storage, and distribution of water. Follow up with periodic inspections and surveys and verify by sampling, as appropriate, to determine whether faulty conditions have been corrected or allowed

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to be reintroduced. Legal action may be necessary to ensure compliance with official standards and accepted sanitary practices.

Formulate solutions to problems found during outbreak investigations, and incorporate these into regulations for drinking, agricultural, industrial, domestic, and recreational waters. Inform the public, through mass media and other means available to your agency, of hazardous conditions that can affect their water supply, but do so only after hypotheses are confirmed. The public must be told of any potential or actual harm that may result from ingesting or contacting contaminated water and must also be informed of measures that they can take and that official agencies are taking to correct these conditions. The water supply and recreational water facilities must be verified periodically to determine whether critical processes are being monitored and operated within limits of appropriate public health standards (See Box 2, The Walkerton Outbreak).

Most waterborne illnesses are preventable, but prevention requires that those in the water treatment industry and in health and water-protection regulatory agencies be constantly vigilant to ensure that the hazards are understood and that questionable water treatment or delivery system construction or practices are avoided.

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Appendices

Table A Equipment useful for investigations[a]

Item	Examples
Investigation guidelines and investigative forms	IAFP manual, "Procedures to Investigate Waterborne Illness, 3rd ed"; 50 copies of Form C; one dozen copies each of Forms E and F; two copies of form D and all parts of Form G, Epi-Info software (CDC, Atlanta).
Sterile sample containers	Water sample bottles (bottles for chlorinated water should contain enough sodium thiosulfate to provide a concentration of 100 mg of this compound per L of sample), plastic bags (Whirl-Pak® type), 250 mL, 1-L and 1-gal sized jars and jugs.
Sterile and wrapped sampling implements	Moore swabs (compact pads of gauze made from strips 120 cm [4 ft] by 15 cm [6 in.] tied in the center with a long, stout twine or wire—for sewer drain, stream or pipeline samples), fiberglass-epoxy bacterial filter cartridge, 0.3 µm; tongs, scoop or similar utensils for collecting ice.
Specimen-collecting equipment (for human specimens from cases and controls)	Sterile containers (with lids) for stool specimens, bottles containing a bacterial preservative and transport medium, mailer tubes or styrofoam box, sterile swabs, rectal swab units, tubes of bacterial transport medium, stool preservative medium for parasites, phlebotomy supplies for blood specimens.
Kits for testing chemical disinfectants and pH	DPD (*N*,*N*-diethyl-*p*-phenylenediamine) chlorine comparator with color disc for chlorine (0.1 ppm) and chlorine test papers; field-type pH meter or pH comparator with color disc or pH test papers; applicable pH indicator solutions and DPD reagent solution; dissolved oxygen testing unit.
Dye tracing study equipment	Fluorescein (yellow-green fluorescent) dye in powder form (10 packages containing 300 g each), in tablet form (100 tablets), or in liquid form (prepared by mixing 300 g in 1 L of water); fluorometer; filters (primary and secondary) for use with fluorometer; sample holder for continuous sampling or individual sampling; fluorometer recorder.
Disinfectant and neutralizer	0.5% w/v solution of calcium hypochlorite or 5.25% household liquid bleach; 50% w/v sodium thiosulfate.
Virus filtration equipment for viruses and parasites[b]	Large plastic container for storing water sample prior to concentration; portable electric or gasoline powered water pump with quick disconnect brass or stainless steel plumbing adapters or hose couplings; two filter holders for 10-in. water filter cartridges fitted for adapters or couplings; portable water meter fitted for adapters or couplings; four lengths of fiber-reinforced garden hose fitted with adapters or couplings; one length of a strong-walled supply hose fitted with adapters or couplings; 10-in. prefilter (3 µm nominal porosity wound polypropylene yarn filter with hollow perforated stainless steel core) cartridge filter; 10-in. virus absorbing filter pleated 0.2 µm porosity nylon membrane type (positively charged) for waters of pH values up to 8.5, or pleated 0.45 µm porosity glass fiber membrane type (positively charged) for waters of pH value of 7.5 or lower (e.g., Virosorb, 1-MDS, AMF/Cuno Meriden, pleated, 0.45 µm, glass filter); 1600 mL sterile, pH 7, 3% beef extract solution in 1 gal wide-mouth screw capped autoclavable polypropylene container for each sample to be collected; stands to support filter holders during filtration; for parasites 10-in. polypropylene yarn-wound cartridge filter, 1.0 µm porosity (e.g., Micro Wynd II™, AMF/Cuno; Meriden, CT. 1.0 µm normal porosity).

(continued)

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Table A (continued)

Item	Examples
Supporting equipment	Laptop or tablet, with software; thermocouples of varying lengths with either recording potentiometer, data logger, or digital indicator; devices to take samples below surface and sediment samples; chemical smoke kit and/or micromanometer; Occupational Safety and Health Administration (OSHA) or equivalent approved respirator; sterile plastic gloves; plastic container liners for ice; waterproof marking pens; waterproof test tube rack; pencils, note pad; roll of adhesive or masking tape; labels; waterproof cardboard tags with eyelets and wire ties; flashlight; matches; test tube rack to fit tubes used; insulated chest or styrofoam container; packing material; camera with flash; spare batteries for all equipment; 95% ethyl alcohol; propane torch; refrigerant in plastic bags, liquid in cans, rubber or heavy plastic bags that can be filled with water and frozen; heavy-duty bags for ice, "canned ice," or cold-packs (blue ice).

[a]Assemble a kit to be kept in the agency responsible for investigating waterborne illness. It should include at least ten water sample bottles; ten 1-L, or gal jars or jugs; ten specimen collection containers or devices; and one each of the following supporting equipment and sterilizing equipment. Date of sterilization should be marked. Periodic resterilization or replacement of sterile supplies, media, or transport media is required to maintain the kit in a ready-to-use condition
[b]Similar equipment for sampling for either viruses or parasites may be available from national water, environmental, or health agencies

Table B Illness acquired by ingestion of contaminated water: a condensed classification by symptoms, incubation periods, and types of agents

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
UPPER GASTROINTESTINAL SIGNS AND SYMPTOMS [NAUSEA, VOMITING] PREDOMINATE Incubation period usually less than 1 h *Chemicals*						
Arsenic poisoning, acute (see also below CHRONIC ILLNESSES)	Arsenic (inorganic)	A few hours	Nausea, vomiting, headaches, weakness, delirium, hypotension, shock, anemia, and leukopenia	Natural deposits in the earth or from agricultural and industrial practices	Urine, blood	Indiscriminate disposal of arsenic compounds; back siphonage; contaminated groundwater
Cadmium poisoning, acute (see also below CHRONIC ILLNESSES)	Cadmium	15–30 min	Nausea, vomiting, abdominal cramps, diarrhea, shock	Natural deposits such as ores containing other elements, enters water through mining and industry	Blood, urine, hair, or nails	Corrosion of galvanized pipes; erosion of natural deposits (sea salt aerosols); volcanic eruptions; discharge from metal refineries; runoff from waste batteries and paints
Copper poisoning, acute (see also below CHRONIC ILLNESSES)	Copper	A few minutes to a few hours	Acute, gastrointestinal distress	Copper pipes and fittings	Vomitus, gastric washing, urine, blood	Corrosion of the plumbing materials; carbonated beverages; acidic water

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Fluoride poisoning (see also below CHRONIC ILLNESSES)	Sodium fluoride	A few minutes to 2 h	Acute, salty or soapy taste, numbness of mouth, vomiting, diarrhea, dilated pupils, spasms, pallor, shock, collapse	Excess addition of sodium fluoride for dental health; natural deposits	Vomitus, gastric washing	Malfunctioning fluoride equipment at water treatment plant; erosion of natural deposits
Incubation(latency) period between 13 and 72 h *Viruses*						
Norovirus infection (Norwalk virus, Norwalk-like virus, Small Round Structured Viruses [SRSV])	Norovirus, Caliciviruses, serotypes, strains and isolates include Norwalk virus; Hawaii virus; Snow Mountain virus; Mexico virus; Desert Shield virus; Southampton virus; Lordsdale virus; Wilkinson virus	Typically 24–48 h	Nausea, vomiting, diarrhea, abdominal pain, myalgia, headache, malaise, low-grade fever, duration up to 60 h	Human feces	Stools, vomitus	Inadequate sewage disposal; using contaminated water
LOWER GASTROINTESTINAL TRACT SIGNS AND SYMPTOMS [ABDOMINAL CRAMPS, DIARRHEA] PREDOMINATE Incubation (latency) period usually between 18 and 72 h *Bacteria*						
Aeromonas diarrhea	*Aeromonas hydrophila*	1–2 days	Watery diarrhea, abdominal pain, nausea, chills, headache	Aquatic environment, both freshwater and marine	Stools	Drinking contaminated, untreated surface water; contamination of foods by sea or surface water

(continued)

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Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Campylobacteriosis	*Campylobacter jejuni, C. coli*	2–7 days, usually 3–5 days	Abdominal cramps, diarrhea (blood and mucus frequently in stools), malaise, headache, myalgia, fever, anorexia, nausea, vomiting. Sequela: Guillain-Barré syndrome	Mammal, poultry, waterfowl and human feces	Stools, rectal swabs, blood	Using contaminated water supplies (e.g., streams, untreated surface supplies); inadequate disposal of animal feces (also, foodborne)
Cholera	*Vibrio cholerae* serogroup O1 classical and El Tor biotypes; serogroup O139	1–5 days, usually 2–3 days	Profuse, watery diarrhea, (rice-water stools), vomiting, abdominal pain, rapid dehydration, thirst, collapse, reduced skin turgor, wrinkled fingers, sunken eyes, acidosis	Human feces; foods washed or prepared with contaminated water	Stools, rectal swabs	Obtaining fish and shellfish from sewage-contaminated waters in endemic areas; using contaminated water to wash or freshen foods; improper sewage disposal; (also, foodborne)
Cholera-like vibrio gastroenteritis	Non-O1/O139 *V. cholerae* and related spp. (e.g., *V. mimicus*, *V. fluvialis*, *V. hollisae*)	1–5 days	Watery diarrhea (varies from loose stools to cholera-like diarrhea)	Human feces, domestic sewage, sea water	Stools, rectal swabs	Inadequate sewage disposal; using contaminated water supply (also, foodborne)

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Enterohemorrhagic or Shiga-toxin producing *Escherichia coli* diarrhea	*E. coli* O157:H7, other serotypes non-O157 STEC O26, O45, O103, O104, O111, O113, O121, O128, O145	1–10 days, typically 2–5 days	Watery diarrhea, followed by bloody diarrhea; severe abdominal pain, blood in urine. Sequelae: hemorrhagic colitis, hemolytic uremic syndrome	Cattle, human feces, sewage, cattle manure	Stools, rectal swabs	Inadequate sewage disposal, using contaminated water supply; cattle waste reaching water (also, foodborne)
Enteroinvasive *Escherichia coli* diarrhea	Enteroinvasive *E. coli* strains	½–3 days	Severe abdominal cramps, fever, watery diarrhea (blood and mucus usually present), tenesmus, malaise	Human feces and sewage	Stools, rectal swab	Inadequate sewage disposal; using contaminated water supply (also, foodborne and person-to-person spread)
Enteropathogenic *Escherichia coli* (EPEC), infantile diarrhea	Enteropathogenic *E.coli* strains	Minimum 0.5 h – 34.0 h with a mean 12.9 h (range 4.5–24.0)	Profuse watery diarrhea, sometimes prolonged leading to dehydration, sometimes bloody diarrhea	Human feces and sewage	Stools, rectal swab	

(continued)

Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Enterotoxigenic *Escherichia coli* diarrhea	Enterotoxigenic *E. coli* strains	½–3 days	Profuse watery diarrhea (blood and mucus absent), abdominal pain, vomiting, prostration, dehydration, low-grade fever, vomiting in a small percentage of cases	Human feces and domestic sewage	Stools, rectal swabs	Inadequate sewage disposal; using contaminated water supply (also, foodborne and person-to-person spread)
Plesiomonas Enteritis	*Plesiomonas shigelloides*	1–2 days	Diarrhea (blood and mucus in stools), abdominal pain, nausea, chills, fever, headache, vomiting	Aquatic environment, both freshwater and marine	Stools, rectal swabs	Drinking contaminated; untreated surface water; contamination of foods by sea or surface water

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Salmonellosis	*Salmonella* (>2000 serovars) from feces of infected animals, environment	6–72 h, typically 18–36 h can be longer than 72 h	Abdominal pain, diarrhea, chills, fever, nausea, vomiting, malaise	Animal and human feces, domestic sewage, farm runoff, meat and poultry processing plant wastes	Stools, rectal swabs	Inadequate sewage disposal; access of animals into well pits and streams, using contaminated water supply (also, foodborne and person-to-person)
Shigellosis	*Shigella dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*	½–7 days, typically 1–3 days	Abdominal pain, diarrhea (stools may contain blood, pus, and mucus), tenesmus, fever, vomiting	Human feces, rectal swabs	Stools, rectal swabs	Inadequate sewage disposal; contaminated well water; cross connections; back siphonage; interrupted disinfection; swimming in polluted waters; freshening produce with contaminated water (also, foodborne and person-to-person)

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Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Yersiniosis	*Yersinia enterocolitica* *Y. pseudotuberculosis* (unknown as a cause of illness from water)	1–7 days	Abdominal pain (may simulate acute appendicitis); low-grade fever, headache, malaise, anorexia, chills, diarrhea, nausea, vomiting	Urine and feces of infected animals (pigs, rodents)	Stools, rectal swabs	Using contaminated surface or spring water; access of animals to surface water (also, foodborne)
Viruses						
Astrovirus gastroenteritis	Astroviruses	1–2 days	Diarrhea, sometimes accomplished by one or more enteric signs or symptoms	Human feces	Stools, acute and convalescent blood	Inadequate sewage disposal; using contaminated water supply (also, foodborne and person-to-person spread)
Enterovirus (see entry under "Generalized infection signs and symptoms")						
Norovirus (see entry under "Upper gastrointestinal signs and symptoms")						
Rotavirus infection	Rotavirus	1–3 days, usually 2 days	Severe watery diarrhea, often with vomiting, fever, abdominal pain, loss of appetite, dehydration	Human feces, domestic sewage	Stools, rectal swabs	Inadequate sewage disposal, using contaminated water supply (also, foodborne and person-to-person spread)

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Incubation periods from a few days to a few weeks *Parasites*						
Amebiasis	*Entamoeba histolytica*	Few days, to several months, typically 2–4 weeks	Mild to severe gastroenteritis, abdominal pain, constipation or diarrhea (stools contain blood and mucus), fever, chills, skin ulcers	Human feces, domestic sewage	Stools, rectal swabs, blood, imaging	Inadequate sewage disposal; using contaminated water supply; cross connections; back siphonage; water and sewer lines in same pits; inadequate disinfection; inadequate filtration of water (also, foodborne and person-to-person spread)
Balantidiasis	*Balantidium coli*		Non-bloody diarrhea, cramping, halitosis, abdominal pain, bloody and/or mucus stools	Human feces, domestic sewage, pig feces, pig manure	Stools	Inadequate sewage disposal; allowing pigs to access areas where crops for human consumption are grown; contact with water frequented by pigs
Cryptosporidiosis	*Cryptosporidium* spp.	1–12 days, typically 8 days	Prolonged watery diarrhea, abdominal pain, anorexia, vomiting, low-grade fever	Human, cattle, deer, sheep, cat, rodent, and other animal feces, domestic sewage, animal waste	Stools, intestinal biopsy	Inadequate sewage or animal waste disposal; contaminated water; inadequate filtration of water

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Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Cyclosporiasis	*Cyclospora cayetanensis*	1–11 days, typically 7 days	Prolonged watery diarrhea, weight loss, fatigue, nausea, anorexia, abdominal cramps	Human feces, domestic sewage	Stools	Sewage contaminated irrigation water or spraying water suspected; washing fruits with contaminated water; untreated water
Giardiasis	*Giardia intestinalis* (also known as *Giardia lamblia, Giardia duodenalis*)	5–25 days, typically 7–10 days	Diarrhea (pale, greasy, malodorous stools), abdominal pain, bloating, nausea, weakness, vomiting, dehydration, fatigue, weight loss, fever	Human feces, domestic sewage, animal (e.g., beaver) feces	Stools	Inadequate sewage disposal, using untreated surface water supplies (e.g., mountain streams and lakes); inadequate or interrupted disinfection; cross connections; improper filtration; using untreated surface water supplies as ingredient for processing (also, foodborne and person-to-person)

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SIGNS OF SUFFOCATION (CYANOSIS) OCCUR Variable incubation periods depending on concentration ingested *Chemicals*						
Methemoglobinemia (Blue baby syndrome)	Nitrites and nitrates	Variable depending on nitrate concentration in water and amount ingested	Bluish coloration of skin, brownish color of blood (usually occurs in infants less than 4 months of age)	Fertilizers, domestic sewage, animal feces	Blood	Shallow, unprotected, or cased wells; excessive use of fertilizers in water shed; large numbers of animals around well
NEUROLOGICAL SYMPTOMS AND SIGNS (VISUAL DISTURBANCES, TINGLING, AND/OR PARALYSIS OCCUR) Incubation (latency) period usually less than 1 h *Chemicals*						
Organophosphate poisoning	Organic phosphorous insecticides	Few minutes to a few hours	Nausea, vomiting, abdominal cramps, diarrhea, headache, nervousness, blurred vision, chest pain, cyanosis, confusion, twitching, convulsions	Pesticides	Blood	Back siphonage of insecticide compounds from hose application; seepage after soil-foundation spraying (also foodborne under similar situations of contamination)

(continued)

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Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Incubation (latency) period usually between 1 and 6 h *Chemicals*						
Chlorinated hydrocarbon poisoning	Chlorinated hydrocarbon insecticides	30 min to 6 h	Nausea, vomiting, paresthesia, dizziness, muscular weakness, anorexia, weight loss, confusion	Pesticides	Blood, urine, stools, gastric washings	Back siphonage of insecticide compounds from hose application; seepage after soil-foundation spraying (also foodborne under similar situations of contamination)
SKIN INVOLVEMENT Incubation period greater than 1 week *Parasites*						
Dracunculiasis	*Dracunculus medinensis*	8–14 months, usually 12 months	Blister, burning and itching at site of exit, especially the foot (vesicle and milky fluid), fever, nausea, vomiting, diarrhea, dyspnea, eosinophilia	Larvae discharge from worms protruding from skin of infected person	Visualization of adult worm under skin	*Cyclops* (crustacean) in water supply; unprotected water supply; frequently step wells and ponds

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Incubation period usually less than 1 week *Bacteria*						
Tularemia	*Francisella tularensis*	1–10 days, usually 3 days	Sore throat, mouth ulcers, tonsillitis, and swelling of lymph glands in the neck (from ingestion of contaminated water)	Blood and tissue of infected wild mammals	Blood	Access of animals to water supplies; using contaminated stream or untreated surface water; dead animals in surface water
Incubation period usually greater than 1 week *Bacteria*						
Typhoid and paratyphoid fevers	*Salmonella* Typhi for typhoid; *S.* Paratyphi A, B, and C for paratyphoid fever	7–28 days, usually 14 days	Continued fever, malaise, headache, cough, nausea, vomiting, anorexia, abdominal pain, chills, rose spots, constipation or bloody diarrhea. Sequela: reactive arthritis	Human feces and urine, domestic sewage	Stools, rectal swabs, blood in incubatory and early acute phase, urine in acute phase	Inadequate sewage disposal; back siphonage; cross contamination; using contaminated water supplies; chlorination failures (also, foodborne and person-to-person)

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Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Viruses						
Enterovirus infection	Poliovirus Coxsackieviruses Echoviruses	3–14 days, usually 5–10 days	Variable, including fever, respiratory tract symptoms, meningitis, herpangina, pleurodynia, conjunctivitis, myocardiopathy, diarrhea, paralysis, encephalitis, ataxia	Human feces and domestic sewage, respiratory discharges	Stools, rectal swabs, respiratory secretions, blood	Fecal contamination of water
Hepatitis A	Hepatitis A virus	15–50 days, usually 25–30 days	Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice, dark urine, light-colored stools	Human feces and urine, domestic sewage	Stools, urine, blood	Inadequate sewage disposal; using contaminated surface or groundwater supplies; cross connections, back siphonage, inadequate disinfection; harvesting shellfish from sewage contaminated water (also, foodborne and person-to-person)

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Hepatitis E	Hepatitis E virus	15–65 days, usually 35–40 days	Similar to above (high mortality for pregnant women in third trimester due to fulminant hepatitis)	Human feces and domestic sewage	Stools, blood	Inadequate sewage disposal; using contaminated surface water or groundwater supplies; cross connections, back siphonage, inadequate disinfection; harvesting shellfish from sewage contaminated water (also, foodborne and person-to-person)
Parasites						
Toxoplasmosis	*Toxoplasma gondii*	4–28 days, mean 9 days	Fever, headache, myalgia, rash	Cat (family) feces	Blood	Surface water not filtered

(continued)

Table B (continued)

Illness	Agent	Incubation or latency period[a]	Signs and symptoms[a]	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
ACUTE AND/OR CHRONIC ILLNESS Variable incubation periods *Algae*						
Cyanobacterial toxin poisoning (blue-green algae producing microcystins)	Some strains of the following cyanobacteria: Hepatotoxins—*Anabaena* spp., *Microcystis* spp. *Oscillatoria* spp., *Nodularia* spp., *Nostoc* spp., *Cylindrospermopsis* spp. and *Umezakia* spp., Neurotoxins—*Aphanizomenon* spp., *Oscillatoria* spp., Toxic alkaloids—*Cylindrospermopsis raciborskii*	Few hours, but can be weeks months, or years	Can be in stages: nausea, vomiting, fever, headache; followed by a pulmonary stage, then hepatotoxicosis and multiple organ failure. Other symptoms include muscle and joint pain, blisters, mouth ulcers, and allergic reactions, and seizures	Cyanobacterial blooms in water	Blood	Ingestion of water containing cyanobacterial toxins; water plant to change water source (if possible); adjust water intake depth; activated carbon to remove toxins
CHRONIC ILLNESS *Chemicals*						
Arsenic poisoning (see also above UPPER GASTROINTESTINAL SIGNS AND SYMPTOMS)	Arsenic (inorganic)		Skin changes, links to cancer of the bladder, lungs, skin, kidneys, nasal passages, liver and prostate (only some of signs listed here)	Natural deposits in the earth or from agricultural and industrial practices	Urine, blood	Contaminated groundwater

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Cadmium poisoning (see also above UPPER GASTROINTESTINAL SIGNS AND SYMPTOMS)	Cadmium	Several months	Kidney damage, lung damage, fragile bones	Natural deposits such as ores containing other elements, enters water through mining and industry	Blood, urine, hair, or nails	Corrosion of galvanized pipes; erosion of natural deposits (sea salt aerosols); volcanic eruptions; discharge from metal refineries; runoff from waste batteries and paints
Copper poisoning (see also above UPPER GASTROINTESTINAL SIGNS AND SYMPTOMS)	Copper		Liver or kidney damage	Natural deposits such as ores containing other elements	Vomitus, gastric washing, urine, blood	Corrosion of the plumbing materials
Fluoride poisoning (see also above UPPER GASTROINTESTINAL SIGNS AND SYMPTOMS)	Sodium fluoride	Months to years	Bone disease (including pain and tenderness of the bones); children may get mottled teeth	Excess addition of sodium fluoride for dental health	Vomitus, gastric washing	Malfunctioning fluoride equipment at water treatment plant; erosion of natural deposits
Lead poisoning	Lead	Weeks to months	Infants and children: delays in physical or mental development; children could show slight deficits in attention span and learning abilities; Adults: kidney problems, high blood pressure	Household plumbing materials in water service lines	Blood	Plumbing materials made of lead or with lead solder; potable water from a corrosive source without treatment reacting with lead pipes

[a]This list is not exhaustive. Not all of the listed illnesses will occur in all countries. Cultural, economic, and climatic conditions preclude or foster conditions that influence their occurrence

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Table C Illnesses acquired by contact with water: a condensed classification by, symptoms, incubation period, and types of agents

Illness	Etiologic agent	Incubation or latency period	Signs and Symptoms	Source of contaminated water	Specimens to collect	Factors contributing to waterborne outbreaks
SKIN INFECTIONS/CONDITIONS (RASHES, BLISTERS, AND/OR PUSTULES) OCCUR Incubation period of a few hours to a few days Bacteria						
	Aeromonas hydrophila	Few hours to a few days	Cellulits, septicemia	Fresh and brackish water	Exudate from skin lesions	Swimming in fresh water, breaks in skin
Pseudomonas aeruginosa infection (hot tub rash, *Pseudomonas* dermatitis, *Pseudomonas* folliculitis) (swimmer's ear, otitis externa)	*Pseudomonas aeruginosa*	Few hours to a few days	Generalized rash, pustules, most prevalent in areas covered by bathing suit, and in ears, earache	Water, skin	Exudate from skin lesion or ear	Inadequate cleaning of pools, water vessels, and devices; immersion in improperly cleaned and disinfected whirlpool baths or hot tubs
Staphylococcus infection (hot tub rash) (swimmer's ear, otitis externa)	*Staphylococcus* spp.	Within a few days of swimming	Rash, and in ears, earache	Water, skin	Exudate from skin lesion or ear	Inadequate cleaning of pools, water vessels, and devices; immersion in improperly cleaned and disinfected whirlpool baths or hot tubs
Tularemia	*Francisella tularensis*	1–10 days, usually 3 days	Ulcers on skin, chills, high fever	Blood and tissue of infected wild mammals, or arthropods	Blood, sputum, lymph node biopsy, muscle tissue	Access of animals to water courses, dead animals in surface water, contact with contaminated water
Vibriosis	*Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio mimicus*	Few hours to few days	Wound and ear infections	Sea water	Exudates from skin lesions	Swimming in sea water, breaks in skin, warm weather

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Vibrio vulnificus infections	*Vibrio vulnificus*	Average 16 h	Wound infections, septicemia; fever, chills, malaise, prostration; metastatic cutaneous lesions (preexisting liver disease is often associated with illness)	Sea water	Exudates from skin lesions, blood	Swimming in sea water, breaks in skin, chronic liver illness with declining immune defense mechanisms
Parasites						
Amebic abscesses	*Acanthamoeba* spp. *Balamuthia* spp.	Unknown	Painful subcutaneous abscesses	Water	Swabs or aspirates of lesions	Exposure to warm muddy waters or industrial water wash solutions, patients are often immunocompromised
Schistosoma dermatitis (swimmer's itch)	*Schistosoma* cercaria, many spp.	Few minutes to hours	Dermatitis, prickly sensation and intense itching, redness, blisters	Feces and urine of infected animals and birds	None	Snails in water, swimming and wading in infested waters
Algae						
Cyanobacterial toxin poisoning	Cyanobacteria	Several days or more	Rash, hives, skin blisters (especially on the lips and under swimsuits), mouth ulcers, eye/ear irritation	Algal blooms	None	Skin exposure during bathing and swimming to water where algae has bloomed

(continued)

Table C (continued)

Illness	Etiologic agent	Incubation or latency period	Signs and Symptoms	Source of contaminated water	Specimens to collect	Factors contributing to waterborne outbreaks
Seaweed dermatitis (swimmer's itch)	*Lyngbya majuscula*	Few minutes to hours	Redness, blisters and lesions containing pus on skin	Sea water natural habitat	None	Warm weather, swimming in sea water with blue-green algae bloom
EYE INFECTIONS (CONJUNCTIVITIS, PURULENT DISCHARGES, REDNESS, SWELLING OCCUR) Incubation period usually less than a week Bacteria						
Conjunctivitis	*Chlamydia trachomatis*	5–12 days	Conjunctivitis, purulent discharge	Genitourinary exudates	Skin or conjunctival swabs	Unchlorinated swimming pool water
Pseudomonas aeruginosa infection	*Pseudomonas aeruginosa*	Few hours to a few days	Conjunctivitis	Water, skin	Corneal scraping	Rubbing eyes while immersed in hot tubs, contaminated lens solution
Viruses						
Enteroviral hemorrhagic conjunctivitis	Adenoviruses, Picornaviruses	4–12 days ½–3 days	Redness, swelling, pain in eye; hemorrhages on conjunctiva	Discharges from infected eyes	Exudates from eye, blood	Unchlorinated swimming pool water
Parasites						
Amebic keratitis	*Acanthamoeba* spp.	Unknown—probably weeks to months	Keratitis, severe eye pain, eye redness, blurred vision, sensitivity to light	Shallow muddy ponds	Corneal scrapings	Wearing contact lenses while swimming in fresh water, using non-sterile saline (homemade) for contact lenses
Chemicals						
Swimming pool conjunctivitis	Chemical added to cause maladjusted pH	Few minutes	Redness of eyes, swelling around eyes	Water and chemicals used for treatment of pools		Improper pH adjustment of swimming pool water

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GENERALIZED INFECTION SIGNS AND SYMPTOMS (FEVER, CHILLS AND/OR MALAISE OCCUR)						
Incubation period less than 1 week Parasites						
Primary amebic meningoencephalitis	*Naegleria fowleri*, *Balamuthia mandrillaris*	1–7 days	Severe frontal headache, nausea, high fever, meningitis, death	Warm fresh bodies of water, hot springs	Spinal fluid, biopsy	Recent swimming in fresh water during hot water, sniffing fresh water up nose
Incubation period greater than 1 week Parasites						
Granulomatous amebic encephalitis (GAE)	*Acanthamoeba* spp. *Balamuthia* spp.	Weeks to months	Parasites invade through skin or lungs and then CNS. Headaches, seizures, stiff neck. Lesions may form in internal organs	Water		Affects chronically ill or debilitated
Bacteria						
Chromobacterium violaceum infection	*Chromobacterium violaceum*	3–14 days	Fever, malaise, myalgia, abdominal pain, pustular rash, sepsis with hepatic abscess	Soil, water	Blood	Breaks in skin, puncture wound

(continued)

Table C (continued)

Illness	Etiologic agent	Incubation or latency period	Signs and Symptoms	Source of contaminated water	Specimens to collect	Factors contributing to waterborne outbreaks
Granulomatous tuberculous lesions	*Mycobacterium marinum*	3–6 weeks	Small papular lesion of spongy consistency with crusty scale surrounding it. Thick secretions under crust, leaving bluish red, soft scar at elbow, knees, and ankles, foot, hand, bridge of nose	Soil, water	Exudate from skin lesions	Rough walls of swimming pools, inadequate chlorination of pool water, injury, scrape, puncture during swimming; abrasions when cleaning aquariums, immersion in natural bathing waters (fresh and salt)
Leptospirosis (Weil's disease)	*Leptospira* spp.	4–19 days, usually 10 days	Fever, chills, malaise, muscular aches, headache, vomiting, stiff neck, occasionally a rash	Animal (e.g., dog, rodent, cattle, deer, swine, fox, squirrel, skunk, raccoon, opossum, muskrat urine)	Urine, blood	Wound and abrasion and mucus membrane entrance. Wading or swimming in farm ponds or water to which animals have access

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Parasites						
Schistosomiasis (Bilharziasis)	*Schistosoma haemotobium*	4–6 weeks	Blood in urine, fever, hepatic pain, epigastric distress, diarrhea, bladder obstruction	Urine of infected human	Urine	Inadequate sewage control, snails in water, water pollution, swimming or bathing in human sewage contaminated water
	S. japonicum *S. mansoni* *S. intercalatum* *S. mekongi*	4–6 weeks	Fever, chills, night sweats, enlarged tender liver, pain in back, groin, legs, diarrhea, high eosinophilic count, epigastric distress	Feces of infected person; feces of pigs, cattle, buffalo, dogs, rodents, cats, horses	Stool, urine	Inadequate sewage control, access of animals to water courses, snails in water, water pollution, swimming, wading, or bathing in sewage contaminated water

Table D Illnesses acquired by inhalation of microorganisms aerosolized from water[a]. A classification by symptoms, incubation period and type of agent

Illness and classification	Agent	Incubation or latency period	Signs and symptoms	Source of contaminated water	Specimens to collect	Factors contributing to outbreaks
Legionellosis, Legionnaires' Disease	*Legionella pneumophila*, other *Legionella* spp.	2–10 days	Muscle pain, loss of appetite, headache, high fever, dry cough, chills, confusion, disorientation, nausea, diarrhea, and vomiting, chest pain difficulty breathing	Water, including groundwater, fresh and marine surface waters, potable (treated) water, cooling towers, evaporative condensers and whirlpools	Respiratory secretions, lung biopsy, urine, abnormal X-ray, blood serology	Breathing aerosol produced from water containing systems; lack of maintenance of water systems; dead ends in water systems; lack of disinfection in water systems; lack of cleaning biofilm from water system surfaces
Pontiac Fever	*Legionella* spp.	24–48 h	Many of the above symptoms, but less severe, no pneumonia or death	Water, including groundwater, fresh and marine surface waters, potable (treated) water, cooling towers, evaporative condensers and whirlpools	Urine, blood serology	Breathing aerosol produced from water containing systems such as jacuzzis, decorative fountains, cooling towers; cleaning in a confined space with high pressure water; taking a shower

[a]Refers to infections caused by airborne bacteria that are amplified in water

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Table E Guidelines for Specimen Collection[a]

Instructions for collecting stool specimens[b]

Instructions	Bacterial	Parasitic[c]	Viral[d]	Chemical
When to collect	During period of active diarrhea (preferably as soon as possible after onset of illness)	Anytime after onset of illness (preferably as soon as possible)	Within 48–72 h after onset of illness	Soon after onset of illness (preferably within 48 h of exposure to contaminant)
How much to collect	Two rectal swabs or swabs of fresh stool from ten ill persons; samples from ten controls also can be submitted. Whole stool is preferred if nonbacterial stool testing considered	A fresh stool sample from ten ill persons; samples from ten controls can also be submitted. To enhanced detection, three stool specimens per patient can be collected >48 h apart	As much stool sample as possible from ten ill persons (a minimum of 10 mL of stool sample from each); samples also can be obtained from ten controls.	A fresh urine sample (50 mL) from ten ill persons; samples from ten controls also can be submitted. Collect vomitus, if vomiting occurs within 12 h of exposure. Collect 5–10 mL whole blood if a toxin/poison is suspected that is not excreted in urine
Method for collection	For rectal swabs, moisten two swabs in an appropriate transport medium (e.g., Cary-Blair, Stuart, Amies; buffered glycerol-saline is suitable for *E. coli*, *Salmonella*, *Shigella*, and *Y. enterocolitica* (but not for *Campylobacter* and *Vibrio*). Insert swab 2.5–4 cm (1–1.5 in) into rectum and gently rotate. Place both swabs into the same tube deep enough that medium covers the cotton tips. Break off top portion of sticks and discard. Alternatively, swab whole stools and put them into Cary-Blair medium	Collect bulk stool specimens, unmixed with urine, in a clean container. Place a portion of each stool sample into 10 % formalin and polyvinyl alcohol preservative (PVA) at a ratio of one part stool to three parts preservative. Mix well. Save portion of the unpreserved stool placed into leak proof container for antigen or PCR testing	Place fresh stool specimens (liquid preferable), unmixed with urine, into clean, dry containers, e.g., urine specimen cups	Collect urine, blood, or vomitus in prescreened containers[e]. If prescreened containers are not available, submit field blanks with samples[f]. Most analyses from blood requires separation of serum from red cells. Cyanide, lead and mercury analyses require whole blood collected in prescreened EDTA tubes. Volatile organic compounds require whole blood collected in a specially prepared gray-top tube

(continued)

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Table E (continued)

Instructions for collecting stool specimens[b]				
Instructions	Bacterial	Parasitic[c]	Viral[d]	Chemical
Storage of specimens after collection	Refrigerate swabs in transport media at 4 °C. When possible test within 48 h after collection; otherwise, freeze samples at −70 °C. Refrigerate whole stool, process it within 2 h after collection. Store portion of each stool specimen frozen at less than −15 °C for antigen or PCR testing	Store specimen in fixative at room temperature, or refrigerate unpreserved specimen at 4 °C. A portion of unpreserved stool specimen may be frozen at less than-15 °C for antigen or PCR testing	Immediately refrigerate at 4 °C. Store portion of each stool specimen frozen at less than −15 °C for antigen or PCR testing	Immediately refrigerate at 4 °C and if possible freeze urine, serum, and vomitus specimens at less than −15°C. Refrigerate whole blood for volatile organic compounds and metals at 4 °C
Transportation	For refrigeration: Follow instructions for viral samples. For frozen samples: Place bagged and sealed samples on dry ice. Mail in insulated box by overnight mail	For refrigeration: Follow instructions for viral samples. For room-temperature samples: Mail in waterproof container	Keep refrigerated. Place bagged and sealed specimens on ice or with frozen refrigerant packs in an insulated box. Send by overnight mail. Send frozen specimens on dry ice for antigen or PCR testing	Immediately refrigerate at 4 °C and if possible freeze urine, serum, and vomitus specimens at less than −15 °C. Refrigerate whole blood for volatile organic compounds and metals at 4 °C. Place double bagged and sealed urine, serum, and vomitus specimens on dry ice. Mail in an insulated box by overnight mail. Ship whole blood in an insulated container with prefrozen ice packs. Avoid placing specimens directly on ice packs

[a]From CDC webpage

[b]Label each specimen in a waterproof manner and put the samples in sealed, waterproof containers (i.e., plastic bags). Batch the collection and send in overnight mail to arrive at the testing laboratory on a weekday during business hours unless other arrangements have been made in advance with the testing laboratory. Contact the testing laboratory before shipping, and give the testing laboratory as much advance notice as possible so that testing can begin as soon as samples arrive. When etiology is unclear and syndrome is nonspecific, all four types of specimens may be appropriate to collect

[c]For more detailed instructions on how to collect specimens for specific parasites, please go to http://www.CDC.gov and search the website of key words

[d]For more detailed instructions on how to collect specimens for viral testing, please go to http://www.CDC.gov and search the website of key words

[e]The containers have been tested for the presence of the chemical of interest prior to use

[f]Unused specimen collection containers that have been brought in to the field and subjected to the same field conditions as the used containers. These containers are then tested for trace amounts of the chemical of interest

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Table F General instructions for collecting water samples for microbiological analysis

	Type of agent to be tested for		
	Viruses	Bacteria	Parasites
When to collect	As soon as hypothesis of waterborne outbreak formulated	As soon as hypothesis of waterborne outbreak formulated	As soon as hypothesis of waterborne outbreak formulated
How much to collect	400 L (surface water up to 360 L; groundwater up to 1500–1800 L)	1 L per pathogen that is to be sought and an additional 200 mL for testing for indicator organisms. Collect at least three samples from each well and 8–10 samples from distribution system	400 L
Method of collection	Pump through electropositive cartridge filters (approximately 10 L per minute rate do not surpass manufacturers rated flow rate for filter type)	Collect in sample bottles or bags (see test for procedures)	Pump through yarn-wound cartridge filters
Storage of sample after collection	Immediately refrigerate and hold at 4°C. Process within 72 h. Do not freeze	Immediately refrigerate and hold at 4°C. Testing should be done within 24 h after collection	Refrigerate at 4°C
Transportation	Keep at 4°C—use frozen refrigerant packs in an insulated box	Keep at 4°C—use frozen refrigerant packs in an insulated box. For frozen samples, put samples on dry ice in insulated box. Either bring to laboratory day of collection or send by courier	Keep at 4°C—use frozen refrigerant packs in an insulated box

Table G Guidelines for confirmation of waterborne outbreaks[a]

Bacterial/chemical/parasitic/viral			
Etiologic agent	Incubation period	Clinical syndrome	Confirmation
Bacterial			
1. *Campylobacter jejuni/coli*	2–10 days; usually 2–5 days	Diarrhea (often bloody), abdominal pain, fever	Isolation of organism from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated water
2. *Escherichia coli* (a) Enterohemorrhagic (*E. coli* O157:H7 and others)	1–10 days; usually 3–4 days	Diarrhea (often bloody), abdominal cramps (often severe), little or no fever	Isolation of *E. coli* O157:H7 or other Shiga-like toxin (verocytotoxin) producing *E. coli* from clinical specimens from two or more ill persons OR Isolation of *E. coli* O157:H7 or other Shiga-like toxin (verocytotoxin) from epidemiologically implicated water
(b) Enteroinvasive (EIEC)	Variable	Diarrhea (might be bloody), fever, abdominal cramps	Isolation of same enteroinvasive serotype from stool of two or more ill persons
(c) Enterotoxigenic (ETEC)	6–48 h	Diarrhea, abdominal cramps, nausea, vomiting and fever less common	Isolation of organism of same serotype demonstrated to produce heat stable (ST) and/or heat-labile (LT) enterotoxin from stool of two or more ill persons
(d) Enteropathogenic (EPEC)	Variable	Diarrhea, fever, abdominal cramps	Isolation of organism of same enteropathogenic serotype from stool of two or more ill persons
3. Nontyphoidal *Salmonella*	6 h–10 days; usually 6–48 h	Diarrhea, often with fever and abdominal cramps	Isolation of organism of same serotype from clinical specimens from two or more ill persons OR Isolation of organisms from epidemiologically implicated water
4. Salmonella Typhi	3–60 days; usually 7–14 days	Fever, anorexia, malaise, headache and myalgia; sometimes diarrhea or constipation	Isolation of organism from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated water

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5. *Shigella* spp.	12 h–6 days; usually 2–4 days	Diarrhea (often bloody), often accompanied by fever and abdominal cramps	Isolation of organism of same serotype from clinical specimens from two or more ill persons OR Isolation of organism from epidemiologically implicated water
6. *Vibrio cholerae* (a) O1 or O139	1–5 days	Watery diarrhea, often accompanied by vomiting	Isolation of toxigenic organism from stool or vomitus of two or more persons OR Significant rise in vibriocidal, bacterial-agglutinating, or antitoxin antibodies in acute- and early convalescent-phase sera among persons not recently immunized OR Isolation of toxigenic organism from epidemiologically implicated water
(b) Non-O1 and non-O139	1–5 days	Watery diarrhea	Isolation of organism of same serotype from stool of two or more ill persons
7. *Yersinia enterocolitica*	1–10 days; usually 4–6 days	Gastrointestinal symptoms, abdominal pain (often severe) mimicking appendicitis), diarrhea	Isolation of organism from clinical specimens from two or more persons OR Isolation of pathogenic strain of organism from epidemiologically implicated water
Chemical			
1. Heavy metals • Antimony • Arsenic • Cadmium • Copper • Fluoride	5 min–8 h; usually <1 h	Vomiting, often metallic taste	Demonstration of high concentration of metal in epidemiologically linked water

(continued)

Table G (continued)

Bacterial/chemical/parasitic/viral			
Etiologic agent	Incubation period	Clinical syndrome	Confirmation
Parasitic			
1. *Cryptosporidium* spp.	2–28 days; median: 7 days	Diarrhea, nausea, vomiting, fever	Demonstration of oocysts in stool or in small-bowel biopsy of two or more ill persons OR Demonstration of organism from epidemiologically implicated water
2. *Cyclospora cayetanensis*	1–14 days; median: 7 days	Diarrhea, nausea, anorexia, weight loss, cramps, gas, fatigue, low-grade fever; may be relapsing or protracted	Demonstration of the parasite by microscopy or molecular methods in stool or in intestinal aspirate or biopsy specimens from two or more ill persons OR Demonstration of the parasite from epidemiologically implicated water
3. *Giardia intestinalis*	3–25 days; median: 7 days	Diarrhea, gas, cramps, nausea, fatigue	Demonstration of the parasite in stool or small-bowel biopsy specimen of two or more ill persons
Viral			
1. Astrovirus	12–48 h	Diarrhea, vomiting, nausea, abdominal cramps, low-grade fever	Detection of viral RNA in at least two bulk stool or vomitus specimens by real-time or conventional reverse transcriptase-polymerase chain reaction (RT-PCR) OR Visualization of viruses with characteristic morphology by electron microscopy in at least two or more bulk stool or vomitus specimens OR Two or more stools positive by commercial enzyme immunoassay (EIA)

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2. Hepatitis A	15–50 days; median: 28 days	Jaundice, dark urine, fatigue, anorexia, nausea	Detection of immunoglobulin M antibody to hepatitis A virus (IgM anti-HAV) in serum from two or more persons who consumed epidemiologically implicated water
3. Norovirus (NoV)	12–48 h; median: 33 h	Diarrhea, vomiting, nausea, abdominal cramps, low-grade fever	Detection of viral RNA in at least two bulk stool or vomitus specimens by real-time or conventional reverse transcriptase-polymerase chain reaction (RT-PCR) OR Visualization of viruses (NoV) with characteristic morphology by electron microscopy in at least two or more bulk stool or vomitus specimens OR Two or more stools positive by commercial enzyme immunoassay (EIA)

[a]Most etiologic agent descriptions based on information from the Centers for Disease Control and Prevention, Atlanta, GA, USA

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Table H Guidelines for confirmation of water responsible for illness

Confirmation status	Criteria
Confirmed vehicle	Isolation of agent from ill persons and from water and laboratory criteria for confirming etiologic agent as stated in Table G. Combination of on-site investigation, statistical evidence and laboratory analysis. (see entries below)
Presumptive vehicle	On-site investigation demonstrating source and mode of contamination of water and survival of etiologic agent in water. Also, desirable to have laboratory isolations from water of etiologic agent that causes syndrome similar to that observed during the investigation and other supportive epidemiologic data. If so, this might provide sufficient evidence for confirmation. OR p-value for water <0.05 when other epidemiologic data supports water hypothesis. Also, desirable to have either laboratory isolations from water or on-site investigation that demonstrates source and mode of contamination and survival of treatment that supports the hypothesis. If so, this might provide sufficient evidence for confirmation. OR Odds ratio or relative risk for water greater than 2 and the lower limit of the 95% confidence level greater than 1 when other epidemiologic data supports the water vehicle hypothesis. Also, desirable to have either laboratory isolations from water or on-site investigation that demonstrates source and mode of contamination and survival of treatment that supports the hypothesis. If so, this might provide sufficient evidence for confirmation.

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Table I $CT_{99.9}$(3-log) values for inactivation of *Giardia* cysts at different concentrations of disinfectants, temperatures and pH values[a]

		Disinfectant						
Concentration (mg/L)		Free chlorine				ClO_2	Ozone	Chloramine[b]
<0.5°C	pH	6	7	8	9	6–9	6–9	6–9
≤0.4		137	195	277	390			
0.6		141	200	286	407			
1		148	210	304	437			
2		165	236	346	500			
3		181	261	382	552	63	2.9	3800
5°C	pH	6	7	8	9	6–9	6–9	6–9
≤0.4		97	139	198	279			
0.6		100	143	204	291			
1		105	149	216	312			
2		116	165	243	353			
3		126	182	268	389	26	1.9	2200
10°C	pH	6	7	8	9	6–9	6–9	6-9
≤0.4		73	104	149	209			
0.6		75	107	153	218			
1		79	112	162	234			
2		87	124	182	265			
3		95	137	201	292	23	1.43	1850
15°C	pH	6	7	8	9	6–9	6–9	6-9
≤0.4		49	70	99	140			
0.6		50	72	102	146			
1		53	75	108	156			
2		58	83	122	177			
3		63	91	134	195	19	0.95	1500
20°C	pH	6	7	8	9	6–9	6–9	6–9
≤0.4		36	52	74	105			
0.6		38	54	77	109			
1		39	56	81	117			
2		44	62	91	132			
3		47	68	101	146	15	0.72	1100
25°C	pH	6	7	8	9	6–9	6–9	6-9
≤0.4		24	35	50	70			
0.6		25	36	51	73			
1		26	37	53	75			
2		29	41	61	89			
3		32	46	67	97	11	0.48	750

[a]Source: Environmental Protection Agency Technical Guidance Manual LT1ESWTR Disinfection Profiling and Benchmarking. March 2003
[b]Chloramines refer to all forms of chloramine. The *CT* values may be assumed to achieve greater than 99.99% inactivation of viruses only if chlorine is added and mixed in the water before addition of ammonia. If this condition is not met, the system must demonstrate by on-site studies or other information that it is achieving at least this much inactivation of viruses

Table J *CT* values for 99.99% inactivation of viruses at pH 6–9 at different temperatures with different disinfectants[a]

Disinfectant	Temperature (°C)						
	Log inactivation	0.5	5	10	15	20	25
Free chlorine	2	6	4	3	2	1	1
	3	9	6	4	3	2	1
	4	12	8	6	4	3	2
Ozone	2	0.9	0.6	0.5	0.3	0.25	0.15
	3	1.4	0.9	0.8	0.5	0.4	0.25
	4	1.8	1.2	1.0	0.6	0.5	0.3
Chlorine dioxide	2	8.4	5.6	4.2	2.8	2.1	1.4
	3	25.6	17.1	12.8	8.6	6.4	4.3
	4	50.1	33.4	25.1	16.7	12.5	8.4
Chloramines	2	1243	857	643	428	321	214
	3	2063	1423	1067	712	534	356
	4	2883	1988	1491	994	746	497

[a]Source: Environmental Protection Agency Guidance Manual for the Compliance with Filtration and Disinfection Requirements Public Water Systems Using Surface Water Sources. March 1991

Table K Estimated log removal of *Giardia* and viruses by various methods of filtration[a]

Method of filtration	Estimated log removal	
	Giardia (3-log inactivation is goal)	Viruses (4-log inactivation is goal)
Conventional (provided turbidity <0.5 NTU)	2.5	2.0
Direct	2.0	1.0
Slow sand	2.0	2.0
Diatomaceous earth	2.0	1.0

[a]Environmental Protection Agency (Federal Register, June 29, 1989, 40 CFR, Parts 141 and 142) http://water.epa.gov/lawsregs/rulesregs/sdwa/swtr/upload/SWTR.pdf. Accessed May 22, 2015

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FOODBORNE, WATERBORNE, ENTERIC ILLNESS COMPLAINT REPORT
Form A

Complaint no.*

Complaint received from	Address	Phone Home Work
Person to contact for more information	Address	Phone Home Work e-mail

Complaint
Type of complaint:* ☐ Illness ☐ Contaminated/spoiled/adulterated food ☐ Poor quality drinking water
☐ Poor quality recreational water ☐ Unsanitary establishment ☐ Complaint related to media publicity
☐ Disaster ☐ Other (specify)

Illness: ☐ Yes,[1,2*] ☐ No Number ill* ______ Number exposed ______ Time first symptom: Date* ______
Hour ______
Predominant symptoms:* ☐ Vomiting ☐ Diarrhea ☐ Fever ☐ Neurological ☐ Skin ☐ Other (specify)

Physician consulted: ☐ Yes ☐ No If yes, Name	Address	Phone

Hospitalized: ☐ Yes ☐ No Emergency Room visit: ☐ Yes ☐ No
If yes,
Hospital name ______________________________ Address ______________________________
______________________________ Phone ______________
Physician's name ______________________________ Phone ______________
Laboratory examination of specimen: Type specimen Organism/Toxin detected*

Suspect food/water* ______________________________ Source of food/water † ______________________
Brand identification † Code/Lot no. †

Suspect meal, event or place:* ______________________ Date ___________ Time ________
Address Phone

NAME	STATUS	ADDRESS	PHONE
1.	☐ ill ☐ well		
2.	☐ ill ☐ well		
3.	☐ ill ☐ well		
4.	☐ ill ☐ well		

Domestic water source: ☐ Community ☐ Non-community ☐ Bottled water
☐ Stream/lake ☐ Vended ☐ Well ☐ Untreated ☐ Other (specify)

Places and locations where foods eaten past 72 hours, other than home *[3]	Place and locations where water ingested past 2 weeks, other than home *[3]	Place and locations where recreation water contacted past 2 weeks *[3]

History of exposures within past six weeks:* ☐ Domestic travel (Place) ________________
☐ International travel (Place) ____________________ ☐ Child care ☐ Contact with ill person outside household or ill person visited household (indicate name) ☐ Contact with ill person within household (indicate name) ☐ Ill animal ______________

Received by	Date of complaint/alert	Time	Disposition

Investigator's name	Comments

[1] If yes, public health professional staff member should obtain information about patient which should be put on Form C.
[2] Ask person to collect vomitus and/or stool in a clean jar, wrap, identify, and refrigerate; hold until health official makes further arrangements.
[3] Ask person to refrigerate all available food eaten during the 72 hours before onset of illness; save or retrieve original containers or packages; sample should be properly identified; hold until health official makes further arrangements. Save any water in refrigerator and trays of ice cubes in freezer; collect was sample from suspect supply in clean jar; put on lid and refrigerate.
[*] Enter onto complaint log (Form B).
[†] Enter onto complaint log (Form B) under comments. USE REVERSE SIDE OR ATTACHED SHEET IF MORE SPACE REQUIRED FOR ANY ENTRY

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FOODBORNE, WATERBORNE, ENTERIC ILLNESS & COMPLAINT LOG

Form B

COMPLAINT[1]			ILLNESSES			FOOD		WATER[2]			HISTORY OF EXPOSURE[3]	COMMENTS (Specify place, location)
No.	Date	Type	Onset date	No. ill	Predominant symptom/ sign	Alleged/ Suspected	Where eaten within 72 hrs*	Where ingested within 2 wks*	Where contacted within 2 wks*	Source*		

Legend: [1]Type complaint – I = illness; CF = contaminated/adulterated/spoiledfood; UE = unsanitary food establishment; DW = poor quality drinking water; RW = poor quality recreational water; MP = complaint related to media publicity; D = disaster.

[2]Water source – C = community; NC = non-community; O = other; W = well; B = bottled; S/L = stream/lake; V = vended; U = untreated; O = other

[3]History of exposure (specify by name) – DT = domestic travel (out of town but within country); IT = international travel; CC = child care; CI = contact with ill person outside household or visitor to household; C = contact with ill person within household; AN = exposure to ill animal.

*Enter each place or source on separate line under the complaint number.

CASE HISTORY: CLINICAL DATA
Form C1

Source or place of outbreak, if known	Complaint number	Case identification no.

Name	Address	Phone: Home Work

Age	Sex	Occupation	Place of work	Ethnic group, special dietary habits, immunocompromised or other pertinent personal or health data

Signs and Symptoms† *(Check appropriate signs and symptoms and circle those that occurred first)*

INTOXICATIONS (Acute and chronic)

☐ Nausea
☐ Vomiting
☐ Anemia
☐ Bloating
☐ Burning sensation (mouth)
☐ Cyanosis
☐ Dehydration
☐ Excessive salivation
☐ Flushing
☐ Foot/wrist drop
☐ Insomnia
☐ Metallic taste
☐ Pallor
☐ Pigmentation
☐ Prostration
☐ Scaling of skin
☐ Soapy/Salty taste
☐ Thirst
☐ Weight loss
☐ White bands on fingernails
☐ Others (specify)
____
____
____

ENTERIC INFECTIONS

*☐ Abdominal cramps
*☐ Diarrhea
☐ bloody #
☐ greasy
☐ mucoid
☐ watery
No./day ________
☐ Chills
☐ Constipation
*☐ Fever _____ °C/°F
☐ Tenesmus
____

GENERALIZED INFECTIONS

☐ Cough
☐ Edema
☐ Headache
☐ Jaundice
☐ Lack of appetite
☐ Malaise
☐ Muscular aching
☐ Perspiration
☐ Stiff neck joints
☐ Swollen lymph nodes
☐ Weakness
☐ Decrease urine output
☐ Pain in back/kidney

Other INFECTIONS

☐ Ear
☐ Eye
☐ Itching
☐ Mouth
☐ Rash
☐ Skin lesion
☐ Pneumonia
Describe:
____
____
____
____

NEUROLOGICAL ILLNESSES

☐ Blurred vision
☐ Coma
☐ Delirium
Difficulty in: ☐ speaking ☐ swallowing ☐ breathing
☐ Dizziness
☐ Double vision ☐ Irritability
☐ Disorientation/loss of memory
☐ Hot/cold reversal syndrome
☐ Numbness ☐ Paralysis
Pupils ☐ dilated, ☐ fixed, or ☐ constricted
☐ Tingling

Other symptoms	Time of onset Date Hour	Incubation period	Duration of illness	Residual symptoms	Fatal Yes ☐ No ☐

Known allergies	Medications taken for illness	Amount	Dates	Medications/inoculations prior to illness

Physician consulted	Address	Phone	Hospital attended	Address	Phone

Contacts with known cases before illness (names)	Address	Phone

Cases in household occurring subsequently (names)	Dates of onset	Child care exposure (place)

Type of specimens obtained	Date collected	Specimen number	Laboratory results / Laboratory method / Laboratory where analysis performed	Case
1. 2. 3.			Laboratory results Laboratory method Laboratory where analysis performed	☐ Confirmed ☐ Presumptive ☐ Suspect

†Signs and symptoms are listed in columns to suggest classification of the disease; their occurrence is not necessarily limited to the category in which they appear on this form.
*Ask if these symptoms occurred, even if they were not mentioned in the interview.
#Ask whether there was decreased urine output.

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CASE HISTORY: FOOD/WATER HISTORY AND COMMON SOURCES
Form C2

☐ Ill ☐ Well

Date of illness/outbreak[1] Date ___	Day before illness outbreak Date ___	Two days before illness Date ___
Breakfast[2] Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]	**Breakfast[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]	**Breakfast[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]
Lunch[2] Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]	**Lunch[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]	**Lunch[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]
Dinner[2] Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]	**Dinner[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]	**Dinner[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ ___ ___ Companions[4]
Non-meal snacks/water ingested[2] Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]	**Non-meal snacks/water ingested[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]	**Non-meal snacks/water ingested[2]** Place ___ Hour ___ Item[3] ___ ___ ___ ___ Companions[4]

History of ingesting suspect food or water or contact with water from suspect source			Source	Address
Item	Time of eating, drinking or contact			
	Date	Hour		

Common events or gatherings	Date	Persons attending[4]	Ill	Well	Addresses	Phone

Nonroutine travel past month (international or domestic/ locations)	Water supply[5]	Sewage disposal	Pet/Animals (kind and number of each)

Water contacted during recreation or work in last 2 weeks	Unusual water supplies ingested in last 2 weeks

Investigator	Title	Agency	Date

[1] If ill before all meals eaten, complete column for three days before illness and so indicate to obtain 72-hour history.
[2] If water suspected, number of glasses of water, number of cold beverages made with water, number of beverages with ice ingested per day.
[3] Include all foods, ice, water, and other beverages.
[4] Record names of persons eating same meal and whether or not Ill.
[5] Specify C for community, SP for semipublic, U for untreated, B for bottled water, NC = Non-community, S/L = Stream/Lake, W = Well, V = Vended and O = Other.

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CASE HISTORIES SUMMARY: CLINICAL DATA
Form D1

Place of outbreak	Dates of outbreak	Complaint number

ID no.	Name of exposed persons whether or not ill	Address	Phone				Time of Ingesting food or water or contacting water		Onset of initial Symptom		Incubation period (differences between time of ingesting/-contact and onset)	Signs and symptoms									Date		
				Sex	Age	Ill	Day	Hour	Day	Hour		Nausea	Vomiting	Diarrhea	Abdominal cramps	Fever				Duration (no. days)	Physician seen	Hospitalized	Death

Investigator	Title	Median IP	Remarks

(fold mark)

CASE HISTORIES SUMMARY: WATER/LABORATORY DATA
Form D2

NOTE: Line up with appropriate identification number on Form D1

Water ingested at suspect meal or event									Amount of water ingested			Laboratory tests			Specific comments or additional information about any ill, not ill persons. (Record all information where space does not permit in other sections, such as additional symptoms, physician, and hospital names.)	Case			ID #
									Glasses water per day	Cold beverages made with water per day	Beverages made with ice per day	Specimen	Date collected	Result		Suspect	Presumptive	Confirmed	
Suspect water									Confirmed etiology			Remarks							

CLINICAL SPECIMEN COLLECTION REPORT Form E			Complaint no.	Specimen no.
Place of outbreak	Address		Case I.D. no.	Type of specimen
Patient name	Address			Phone
Reason for collecting specimen □ Victim of outbreak □ Person at risk but not ill □ Handler of suspect food or water □ Suspected carrier □ Animal □ Other (specify)				
Physician	Address			Phone
Symptoms: □ Nausea □ Vomiting □ Diarrhea □ Fever □ Other (specify)				

Time of ingesting/ contacting suspect food, meal, or water Day Hour	Time of onset Day Hour	Incubation period	Duration of illness	Medications Type	Amount	Dates

Method of collecting specimen	Method of preservation	Method of shipment
Other Information		

Investigator collecting specimen	Title	Agency	Date Hour collected/submitted
Test requested	Presence/Absence	Count/Titer/ Concentration	Definitive type

Comments and interpretations

Laboratory analyst	Lab name & location	Date/Hour received	Date started	Date completed	Etiologic agent as determined by analyst

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<table>
<tr><td colspan="2">Water/Ice Sample Collection Report
Form F</td><td>Complaint No.</td><td colspan="2">Sample No.</td></tr>
<tr><td>Identification of water supply</td><td>Location</td><td>Sampling Point</td><td colspan="2">Date/Hour Collected</td></tr>
<tr><td>Person in Charge</td><td>Phone/e-mail</td><td colspan="3">Description of Sample Including Amount Sample or Filtered</td></tr>
<tr><td colspan="2">Method of Sterilizing Containers[1] and/or Collection Utensils[2]</td><td colspan="3">Method of Transportation of Sample</td></tr>
<tr><td colspan="2">Shipped
☐ Refrigerated ☐ Frozen ☐ Ambient temperature</td><td>Identification marks</td><td colspan="2">Date/Hour Shipped</td></tr>
<tr><td>Estimated Chlorine Contact Time Before Sampling</td><td>Chlorine
Free _____
Total ______</td><td>Temperature of water</td><td colspan="2">Other Field Test Results</td></tr>
<tr><td colspan="5">Symptoms of victims ☐ Nausea ☐ Vomiting ☐ Abdominal Cramps ☐ Fever ☐ Diarrhea ☐ Conjunctivitis ☐ Other (specify)</td></tr>
<tr><td>Time of Ingesting/Contacting Suspect Water
Date Hour</td><td>Time of onset
Date Hour</td><td>Incubation period</td><td colspan="2">Duration of illness</td></tr>
<tr><td>Investigator</td><td>Title</td><td>Agency</td><td colspan="2">Date/Hour</td></tr>
<tr><td>Test Requested</td><td>Presence/Absence</td><td>Count/Concentration</td><td colspan="2">Serotype</td></tr>
<tr><td>☐ Campylobacter</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Cryptosporidium</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ E. coli (specify type)</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Giardia</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Legionella</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Salmonella</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Shigella</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ V. cholerae</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ V. parahaemolyticus</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Yersinia enterocolitica</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Others (Bacteria, viruses, parasites, toxic chemicals specify)</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Heterotrophic Plate Count</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Coliphage</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Total coliform</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Enterococci</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ E. coli (indicator)</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Total culturable viruses</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>☐ Other (specify)</td><td></td><td></td><td colspan="2"></td></tr>
<tr><td>Physical properties of Water: (Turbidity)</td><td>pH</td><td colspan="3">Chemical Properties of Water</td></tr>
<tr><td colspan="5">Comments and Interpretation</td></tr>
<tr><td>Laboratory Analyst</td><td>Agency</td><td>Date Received</td><td>Started</td><td>Completed</td></tr>
<tr><td colspan="5">Etiologic Agent</td></tr>
</table>

[1]Attach a list of number, sample, and tests desired for other samples collected at the same establishment during the same investigation

[2]Specify only if unusual (such as field) method of sterilizing/sanitizing collection container or utensil or if an unusual method of collecting sample

A50795008

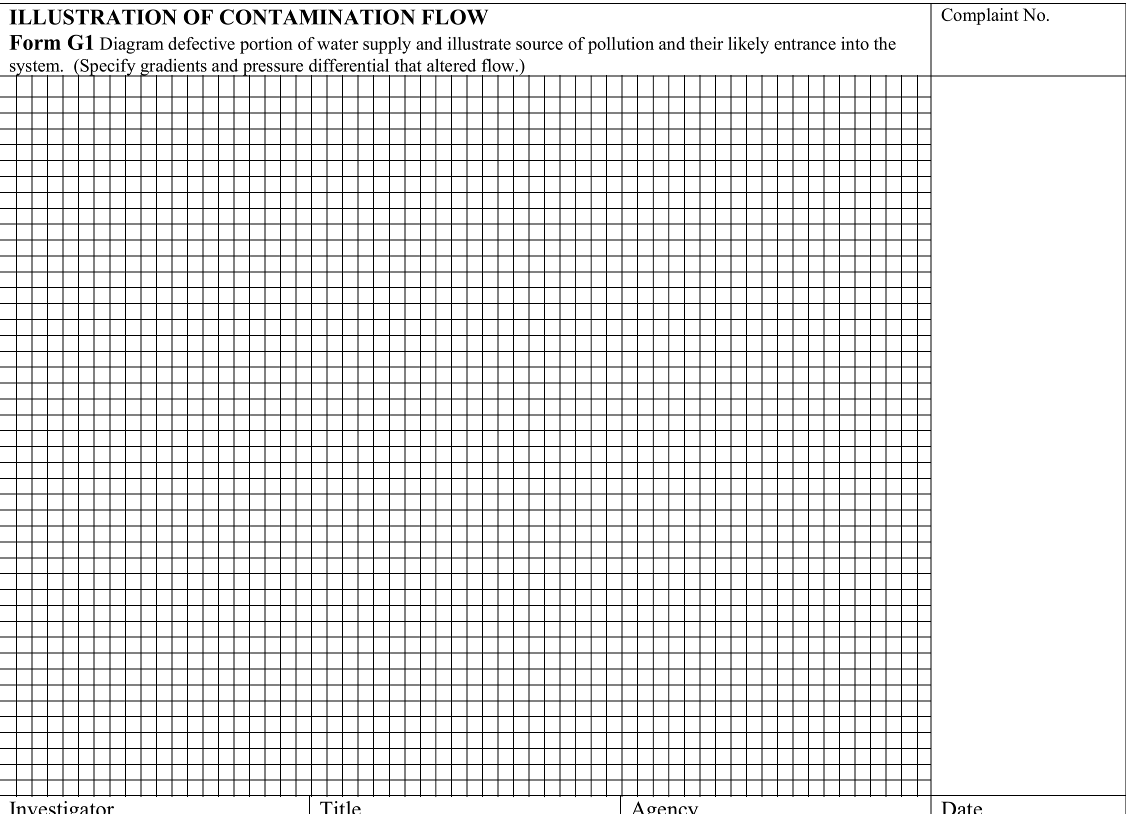

ILLUSTRATION OF CONTAMINATION FLOW

Form G1 Diagram defective portion of water supply and illustrate source of pollution and their likely entrance into the system. (Specify gradients and pressure differential that altered flow.)

Complaint No.

Investigator	Title	Agency	Date

Scale 1 block =

RECORD REVIEW OF ON-SITE INVESTIGATION AND TEST RESULTS PRIOR TO AND DURING OUTBREAK (Data from on-site record review and Forms F and G – **Form G2**)						Complaint No		
Heterotrophic Plate Count (plant/distribution)			**E. coli Counts/(raw/plant/distribution)**			**Other Chemical Tests** (e.g. ozone)		
Location	Date	Results	Location	Date	Results	Specify	Date	Result
Total Coliform Count (raw water)			**Other Microbiological Tests (specify)**			**Physical/Organoleptic Tests (pH, turbidity, UV light)**		
Location	Date	Results	Type	Date	Results	Specify	Date	Result
Total Coliform Count (finished)			**Chlorine Residuals (plant)**			**Other Examinations**		
Location	Date	Results	Location	Date	Results ☐ Free ☐ Total	Specify	Date	Result
Total Coliform Count (distribution)			**Chlorine Residuals (distribution)**					
Location	Date	Results	Location	Date	Results ☐ Free ☐ Total			
Interpretations								
Reviewer			Title			Date		

<table>
<tr><td colspan="4">SOURCE AND MODE OF CONTAMINATION OF SURFACE SOURCES[1]
Form G3</td><td colspan="2">Complaint No</td></tr>
<tr><td>Name of surface supply</td><td colspan="2">Location</td><td>Person-in-charge</td><td colspan="2">Phone/e-mail</td></tr>
<tr><td>LAND USE OF WATERSHED

☐ Cultivated ☐ Feedlot
☐ Forested ☐ Industrial
☐ Irrigated ☐ Mining
☐ Oil fields ☐ Pasture
☐ Recreation ☐ Thickly settled
☐ Other (describe) ____________</td><td>RECENT DATES

☐ Flooding ______________

☐ Drought ______________</td><td colspan="2">TYPE SEWAGE FOR POPULATED AREAS

☐ Primary
☐ Secondary
☐ Oxidation Pond
☐ Untreated/raw
☐ Septic Tanks
☐ Other (describe)
________________</td><td>DISCHARGES INTO SURFACE WATER

☐ Yes ☐ No
☐ Yes ☐ No
☐ Yes ☐ No
☐ Yes ☐ No
☐ Yes ☐ No
☐ Yes ☐ No</td><td>TYPES OF ANIMALS IN WATERSHED

☐ Livestock
☐ Poultry
☐ Aquatic mammals
☐ Waterfowl
☐ Snails
☐ Other (list)
___________________</td></tr>
<tr><td colspan="6">Sewage outfalls or seepage water (give location and distance from water intake or point of use)</td></tr>
<tr><td colspan="6">Source of pollution (give location and distance from water intake or point of use)
☐ Feedlot ☐ Slaughterhouse ☐ Pasture runoff into surface water</td></tr>
<tr><td colspan="6">Results of dye test from outlets or seepage to intake or point of use or other means of evaluation of movement of contaminants</td></tr>
<tr><td colspan="6">Results of any physical/chemical/microbial test of source water (See Form G2)</td></tr>
<tr><td colspan="6">Factors contributing to surface water pollution/contamination and outbreak*: ☐ Ingestion of untreated water ☐ Pollution of watershed ☐ Dead animal in water
☐ Animals have direct access to water ☐ Use of contaminated water as an alternative source ☐ Overflow of sewage or outfall near water intake ☐ Drought
☐ Flooding ☐ Other (specify)</td></tr>
<tr><td>Investigator</td><td colspan="2">Title</td><td>Agency</td><td colspan="2">Date</td></tr>
</table>

[1] Note all that apply. Explain source/mode and describe entry in more detail on back or separate attached sheet
*Record on Form L

SOURCE AND MODE OF CONTAMINATION FOR GROUND WATERS[1]
Form G4

Complaint No

Location	Person-in-charge/Owner	Phone/e-mail

TYPE OF GROUND SUPPLY	TYPE OF WELL	TYPE OF SOIL AND AQUIFER	DEPTH
☐ Well ☐ Spring ☐ Other (specify) State source of information: ____________	☐ Drilled ☐ Bored ☐ Driven ☐ Dug ☐ Step ☐ Other ____________	☐ Sand ☐ Clay ☐ Loam ☐ Peat ☐ Gravel ☐ Rocky ☐ Limestone ☐ Other (specify) ____________	Static water ________ Well ____________ ____________

Excreta disposal; in vicinity of well which may have contaminated ground water:

Type: ☐ Community primary ☐ Community secondary ☐ Leaking sewer line ☐ Septic tank ☐ Cesspool/ seepage pit ☐ Absorption field ☐ Privy ☐ Toxic waste disposal
Distance ________ ________ ________ ________ ________ ________ ________ ________

Type: ☐ Stream ☐ Surface water ☐ Animals ☐ Feedlot ☐ Manure piles ☐ Compost ☐ Dump/landfill ☐ Toxic waste storage
Distance ________ ________ ________ ________ ________ ________ ________ ________

Observed faults in construction/maintenance/operation/ of wells/springs/other ground water source:
☐ Casing Depth ________ ☐ Ground casing Depth ________ ☐ Casing not intact ☐ Open well/spring ☐ Animal holes around casing ☐ Flooding ☐ Platform/apron not intact ☐ Casing top below grade ☐ Pitless adapter faulty ☐ Other (specify)

Type of pump: ☐ Submersible ☐ Jet ☐ Turbine ☐ Reciprocating ☐ Hand ☐ Gravity ☐ Other (specify)	Source of priming water	Disinfection ☐ None ☐ Failure	Chlorine test ☐ Free ☐ Total
Contamination during pumping: ☐ Unsafe water for priming ☐ Leaks in system under vacuum ☐ Well pit flooded ☐ Pump not sealed to platform/top bushing not closed ☐ Other (specify)	Type repairs made Disinfection following repairs: ☐ Yes ☐ No	Date	

Results of dye test from outlets or seepage to intake or point of use; or other means of evaluation of movement of contaminants

Results of any physical /chemical/microbial test of ground water (give test done, dates, present/absent/count/concentration, as applicable; See Form G2

Factors contributing to ground water contamination and outbreak*
☐ Overflow or seepage of sewage into well/spring ☐ Surface runoff into well/spring ☐ Contamination through limestone or fissured rock
☐ Flooding/heavy rains ☐ Chemical/pesticide contamination ☐ Seepage from abandoned well ☐ Contamination through suction line
☐ Improper well/spring construction ☐ Unsafe water used for priming ☐ Other (specify)

Investigator	Title	Agency	Date

[1] NOTE ALL THAT APPLY. Explain source/mode of contamination and describe entry in more detail on back on separate sheet.

* Record on Form K

A50795008

DISINFECTION FAILURES THAT ALLOWED SURVIVAL OF PATHOGENS OR TOXIC SUBSTANCES[1]
Form G5a

Complaint No

Name of facility	Location	Person-in-charge	Phone/e-mail

Type of disinfection: ☐ None ☐ Simple chlorination ☐ Super chlorination ☐ Breakpoint chlorination ☐ Ultraviolet ☐ Hypochlorite
☐ Chloramine ☐ Chlorine dioxide ☐ Ozone ☐ Other (specify)

Deficiencies in: ☐ Disinfection equipment ☐ Disinfection operation ☐ Disinfectant contact time | Comments | Disinfectant demand
Interruption in: ☐ Disinfection equipment ☐ Disinfection operation
Dates __________ __________ __________

Disinfection tests at plant (give minimum values) Location: | Comments
During investigation day before 2 days before last week (date) last month (date)
Free ______ ______ ______ ______ ______

Disinfectant rate applied = disinfectant used per day / flow rate

Disinfectant demand (usage) = disinfectant dosage applied – disinfectant concentration measured downstream

☐ Sudden changes to disinfectant demand, If yes, date(s):

	Disinfectant concentration C (mg/L)	Disinfectant contact time T (minutes)	CT_{calc} C x T	pH	Water temperature (°C)	CT 99.9 (from Table I)	$CT_{calc}/CT_{99.9}$
Sequence 1st							
2nd							
3rd							
4th							
5th							
Sum							

Factors contributing to survival of pathogen or failure of inactivation of toxin during treatment and outbreak*
☐ Inadequate prefiltration treatment ☐ Inadequate filtration ☐ Inadequate chemical feeding ☐ No disinfection ☐ Inadequate disinfection
☐ Interruption of disinfection ☐ Other (specify)

Investigator	Title	Agency	Date

[1] Explain treatment failure and describe entry in more detail on back or on separate attached sheet
* Record on Form K

SOURCE OF CONTAMINATION AND TREATMENT FAILURES THAT ALLOWED SURVIVAL OF PATHOGENS OR TOXIC SUBSTANCES[1]
Form G5b

Complaint No

Name of facility	Location	Person-in-charge	Phone/e-mail

Raw water intake
☐ Excessive pollution in relation to treatment potential ☐ Bypass connection by which raw or partially treated water gets into distribution system ☐ Nearby uncontrolled pollution ☐ Other (specify)

Fluoridation feed deficiencies ☐

Sedimentation deficiencies: ☐ No sedimentation before filtration ☐ Turbidity not removed ☐ Tank not cleaned
☐ High proportion of microorganisms remain ☐ Retention time ☐ Other deficiencies (specify)

Sedimentation rate: Depth of water / Transit time from inlet to outlet = ___________________

Record review:	Coagulant dose	pH	Turbidity	Other tests (specify)
Date/value:	/	/	/	

Records show routine monitoring of measurements: ☐ Yes ☐ No

Filtration performance criteria: ☐ Media loss ☐ Media deterioration ☐ Mud ball formation ☐ Channeling ☐ Surface cracking
☐ Under drain failure ☐ Cross connections ☐ Chemical deficiencies (specify)

Type filtration: ☐ Conventional (rapid) ☐ Direct (rapid) ☐ Pressure ☐ Slow ☐ Bag cartridge ☐ Diatomaceous earth
☐ Other (specify)

Frequency of backwashing:

Deficiencies of filtration:

Recycling back-wash water: ☐ Yes ☐No

Source of backwash water:

Average filtered water turbidity:	Filter 1	Filter 2	Filter 3	Filter 4	Filter 5	Filter 6	Other filters (list on back)
	______	______	______	______	______	______	

Combined filter effluent Clearwell effluent Plant effluent

Nature of recent illnesses of staff (name of illness or recent symptoms) Name of employee

Other observations or measurement of treatment plant operations

Factors contributing to survival or failure of inactivation of toxin during treatment of outbreak*
☐ Inadequate prefiltration treatment ☐ Inadequate filtration ☐ Inadequate chemical feeding ☐ No disinfection ☐ Inadequate disinfection
☐ Interruption of disinfection ☐ Other (specify)

Investigator	Title	Agency	Date

[1] Explain treatment failure and describe entry in more detail on back or separate attached sheet
* Record on Form K

A50795008

SOURCE AND MODES OF CONTAMINATION DURING DISTRIBUTION AND AT POINT-OF-USE[1]
Form G6

Complaint No

Location	Person-in-charge	Phone/e-mail

Type cross connections: ☐ Sewer lines ☐ Waste lines ☐ Fire water supply ☐ Boilers ☐ Carbonated water lines ☐ Cooling water
☐ Hydraulic operations ☐ Other (specify)

Cross connection deficiencies
☐ Deficiency of double check valve arrangement ☐ Defective check valve ☐ Defective other backflow prevention devices ☐ Temporary attachment not detached ☐ Other (specify)

Comments

Backsiphonage detected: ☐ Inlets without air gap ☐ Inlet too close to fixture side/well ☐ Submersed inlet ☐ Hose attachment in vessel
☐ Defective vacuum breakers ☐ Connections to sprinkler system used to spray pesticide or toxic substances ☐ Negative pressure
☐ Other (specify)

Negative pressure occurred due to:
☐ Water shut off due to repairs
Date(s) ________________
Typical repair(s) __________
Disinfection afterwards ☐ Yes ☐ No
☐ Nearby fires
Date(s) _____________
☐ Negative pressure on upper floors
Date(s) _____________
☐ Repumping
Date(s) ________________
☐ Intermittent service
Date(s) _____________
☐ Other (specify)
Date(s) _____________

Sites sampled
1_________ 6 ________
2_________ 7 ________
3_________ 8 ________
4_________ 9 ________
5_________ 10 ________

Recent illness of persons in building Type of illness/major symptoms	Date(s)	Name of person(s)	Address	Phone/e-mail

Chlorine residuals in distribution system Free Total	Location	Line pressure testing results	Results of other tests (specify test)

Previous month

No. sites where disinfected residual was measured (a)	No. sites where no disinfectant residual measured., but HPC measured (b)	No. sites where disinfectant residual not detected and HPC not measured (c)	No. sites where disinfectant residual not detected, HPC criteria (e.g. > 500/mL exceeded) (d).	No. sites disinfectant residual not measured, HPC > criteria (e.g. > 500/mL) (e)

v = (c + d + e) / (a + b) x 100 = ___________%
(_____ + _____ + _____) / (_____ + _____) x 100 = ___________%

Type of storage/transportation facility contaminated: ☐ Community storage tank ☐ Cistern ☐ Transportation tank ☐ Household storage container
☐ Other (specify)

Factors contributing to distribution line contamination and outbreak*
☐ Cross connections and defective backflow prevention devices ☐ Submerged inlet and backsiphonage ☐ Contamination of storage tank, cistern, storage containers ☐ Improper or no disinfection of mains, plumbing or storage facility, transportation container after repairs or new connection
☐ Line pressure loss
☐ Other (specify)

Comments

Investigator	Title	Agency	Date

[1] Explain source/mode of contamination and describe entry in more detail on back or on separate attached sheet
* Record on Form K

A50795008

CONTAMINATION SOURCE AND SURVIVAL OF PATHOGENS OR TOXICSUBSTANCES FOR RECREATIONAL WATERS[1]
Form G7

Complaint No.

Name of pool/lake/spa/spray pad/water course	Location	Person-in-charge	Phone/e-mail

Likely route of infection
☐ Contact ☐ Inhalation ☐ Accidental ingestion

Type of exposure
☐ Swimming pool ☐ River/stream ☐ Lake/pond ☐ Whirlpool ☐ Hot tub
☐ Spray pad ☐ Other (specify)

Type filtration: ☐ Rapid ☐ Pressure ☐ Diatomaceous earth ☐ Other (specify)	Frequency of backwashing	Deficiencies of filtration	Recycling backwash water ☐ Yes ☐ No	Source of backwash water	Turbidity

Type of disinfection
☐ None ☐ Injected hypochlorite ☐ Batch hypochlorite ☐ Other (specify)

Deficiencies in: ☐ Disinfection equipment ☐ Disinfection operation
Interruption in: ☐ Disinfection equipment ☐ Disinfection operation
Date(s) ____________ ____________

Comments about deficiencies or interruption:

Disinfectant residuals

	During investigation	Day before onset first case	2 days before	3 days before	Last week	Last month (date)
Free:	______	______	______	______	______	______
Total:	______	______	______	______	______	______

Calculated usage

Sudden changes in disinfection usage
Date(s)

Data for CT calculations: (Compare with data on disinfectants and microbes of concern in Table I and J)

Disinfectant residual (C) Exposure time (T) CT_{calc} (C x T) Water temperature °C pH of water $CT_{99.9}$ $CT_{calc}/CT_{99.9}$

Factors contributing to WATER CONTAMINATION AND/OR SURVIVAL (check all that apply)*

☐ Sewage outflows	☐ No filtration	☐ Improper pH adjustment	☐ Diving in water
☐ Flooding/heavy rain	☐ Inadequate filtration	☐ Snails present	☐ Wading/swimming/skiing
☐ Underground seepage of sewage	☐ No disinfection	☐ Puncture injuries or wounds	☐ Other (specify)
☐ Swimming in parasite-infested water	☐ Improper disinfectant	☐ Rough pool well construction	☐ Animals have access to watershed
☐ Uncapped wellhead			

Comments

Investigator	Title	Agency	Date

[1] Explain source of contamination and treatment failure and describe entry in more detail on back or on separate attached sheet
* Record on Form K

A50795008

CONTAMINATION SOURCE AND SITES OF AMPLIFICATION AND AEROSOLIZATION OF PATHOGENS[1]
Form G8

Complaint No.

Name of pool/lake/spa/spray pad/water course	Location	Person-in-charge	Phone/e-mail

Device(s) potentially producing aerosols: ☐ Cooling towers ☐ Evaporative condensers ☐ Humidifiers ☐ Water heaters and holding tanks ☐ Shower heads ☐ Decorative fountains ☐ Dusty environment ☐ Ultrasonic mist machines ☐ Irrigation systems ☐ Other (specify)__________

Condition of device: ☐ Dirt/dust observed ☐ Sediment ☐ Slime ☐ Other (Specify) (Explain)

Amplification potential (explain)

Disinfectant used	Concentration	Frequency	Date of last application

Results of air flow or sampling studies (explain)

Factors contributing to contamination, growth and/or survival (check all that apply)*
☐ Aerosols generated ☐ Close contact or air currents carried aerosols ☐ Susceptible persons (e.g. >50 years old, smokers, heavy drinkers, immunosuppressed) ☐ Warm water conducive to growth ☐Poorly operated/maintained water system ☐ Other (specify)

Water samples collected (number and location)	Comments

Investigator	Title	Agency	Date

[1] Explain source of contamination and treatment failure and describe entry in more detail on back or on separate attached sheet

* Record on Form K

FOOD/BEVERAGE/COMMUNITY ATTACK RATES
Form H1, Vehicle Attack Rate Table[1]

Place of Outbreak

Complaint No.

Food/Beverages	Number of Persons Who Ate or Drank				Number of Persons Who Did **Not** Eat or Drink				Difference in Percent	Relative Risk	Statistical Significance
	Ill	Well	Total	Attack Rate	Ill	Well	Total	Attack Rate			

[1]For calculation of data when all persons who may have been exposed to the suspect vehicle (but not all persons will have ingested every beverage/food) have been identified and interviewed (retrospective cohort)

COMMUNITY ATTACK RATE

Exposure	Name of Community	Number Ill	Population of Community	Attack Rate	Relative Risk	Statistical Significance
Exposed (at risk)						
Related, unexposed						
Total						

Remarks and interpretation (compare with laboratory results)

Prepared by:	Title	Date

A50795008

CASE-CONTROL VEHICLE EXPOSURE/DOSAGE
Form H2, EXPOSURE RATES

Place of Outbreak

Complaint No.

Beverages	Ill				Well				Difference in Percent	Odds Ratio	Statistical Significance
	a Ate/drank	c Did Not drink	a+c Total	Percent	b Ate/drank	d Did Not drink	b+d Total	Percent			

Remarks and Interpretations (compare with laboratory reports)

QUANTITY OF WATER INGESTED

Number of glasses ingested per day[1]	Ill	Well	Total	Attack Rate	Relative Risk	Statistical Significance
5 or more						
3-4						
1-2						
0						
Total						

[1] For calculation when a sample of ill and not illpersons may have been exposed to the suspect vehicleand have been identified and interviewed

Number of Beverages Containing Water[2]	Ill	Well	Total	Attack Rate	Relative Risk	Significance Statistical
5 or more						
3-4						
1-2						
0						
Total						

[2]Include all beverages made of water excluding hot beverages (these are to be included in the attack rate).

Prepared by:	Title	Date

LABORATORY RESULTS SUMMARY Form I	Complaint No.	Outbreak	Date

Cases (Data from Forms C, D, and E)

I.D. Number	Specimen	Organism/Test result	Marker (e.g. serotype, phage type, PFGE)

Water Environmental samples (Data from Form F)

Sample No.	Sample Description	Organism/Chemical recovered	Count	Marker

Interpretation and remarks

Etiologic agent	Vehicle	Source of contamination

A50795008

Form J1: Chi-sq. analysis can be easily completed using on-line calculators or statistics programs such as Epi-Info. However, to confirm the result or to do the whole thing yourself, here are the steps:

Calculation example: odds ratio and chi-square (χ^2) statistic

	Ill	Well	Totals
Exposed	18 (a)	12 (b)	30
N/exposed	3 (c)	16 (d)	19
Totals	21	28	49

Step 1: Create a 2x2 table as shown with observed data (O values) and marginal totals

Step 2: Calculate odds ratio:

(AxD)/(BxC) = (18x16)/(12x3)= 8.0
95% CL: (1.64 < OR < 44.23)[1]

	Ill	Well	Totals
Exposed	18 (12.857) (a)	12 (17.143) (b)	30
N/exposed	3 (18.429) (c)	16 (10.857) (d)	19
Totals	21	28	49

Step 3: Enter expected (E) numbers for each cell:

E = (Row total)x(column total) / (grand total).

e.g. For cell (a): (30x21)/49 = 12.857
Complete for all cells.

Note: Any E numbers less than 5? If yes, then stop and go to Fisher's Exact Test [Form J2]

Step 4: Calculate chi-sq. (χ^2) as the sum of $\frac{(O-E)^2}{E}$ for all 4 cells

e.g. for cell (a): $\frac{(18-12.857)^2}{12.857} = 2.057$

Chi-sq. (all four cells) $= 2.057 + 1.543 + 3.248 + 2.436 = 9.284$

Step 5: Compare your *calculated* chi-sq. value with the *critical* value to determine significance (Table 17):

Table 17 Critical values of the chi-sq. distribution

1. Locate the row showing your table size; 2. Begin at column $P<0.05$ … Your calculated chi-square value must meet or exceed the critical value to be considered statistically significant at that level. If you fail to meet or exceed the minimal value for $P<0.05$, the result is $P>0.05$, and the relationship is declared "*not significant*".

Table row × column	for $P<0.05$ chi-square must exceed …	for $P<0.025$ chi-square must exceed …	for $P<0.01$ chi-square must exceed …	for $P<0.005$ chi-square must exceed …	for $P<0.001$ chi-square must exceed …
for 2×2 tables (1 df)	3.841	5.024	6.635	7.879	10.828
for 2×3 tables (2 df)	5.991	7.378	9.210	10.597	13.816
for 2×4 tables (3 df)	7.815	9.348	11.345	12.838	16.266
3×3 or 5×2 tables (4 df)	9.488	11.143	13.277	14.860	18.467

[1] Odds ratio shown here with confidence limits. This is normally produced by software programs such as Ep-Info. If limits *include* 1.0 then the relationship *cannot be significant,* regardless of the Chi-sq. analysis.

Step 6: Summarize: *Exposure was related to illness.* ***Ill persons*** *were eight times more likely to have been exposed to this factor than non-ill persons. This relationship is statistically significant. The probability of these data occurring by chance alone is less than 0.5%. Reject the null hypothesis of "no association."*

[Odds ratio: 8.0, 95% CL: $1.64 < OR < 44.23$, chi-sq.: 9.28, 1 df, $P < 0.005$]

A50795008

Form J2: Fisher's exact test can be easily completed using on-line calculators or statistics programs such as Epi-Info. However, to confirm the result or to do the whole thing yourself, here are the steps.

Calculation example: odds ratio and fisher's exact test

	Ill	Well	Totals
Exposed	8 (a)	5 (b)	13
N/exposed	2 (c)	16 (d)	18
Totals	10	21	31

[Odds ratio: 12.8, 95% CL 1.60 < OR < 131.10]

Step 1: If all E numbers in 2x2 table ≥ 5, use Chi-sq. [see Form J1]
Here, cell 'a' has smallest E value at 4.2

Step 2: Calculate the Probability directly...

$$P_1 = \frac{10! \times 21! \times 13! \times 18!}{8! \times 5! \times 2! \times 16! \times 31!} = 0.0044$$

	Ill	Well	Totals
Exposed	~~8~~ **9** (a)	~~5~~ **4** (b)	13
N/exposed	~~2~~ **1** (c)	~~16~~ **17** (d)	18
Totals	10	21	31

Step 3: If we don't yet have a zero in the cells, add +1 to larger of (a)x(d) or(b)x(c) andsubtract-1 from smaller pair. Keep marginal totals fixed. Recalculate:

$$P_2 = \frac{10! \times 21! \times 13! \times 18!}{9! \times 4! \times 1! \times 17! \times 31!} = 0.0003$$

	Ill	Well	Totals
Exposed	~~9~~ **10** (a)	~~4~~ **3** (b)	13
N/exposed	~~1~~ **0** (c)	~~17~~ **18** (d)	18
Totals	10	21	31

Step 4: We still don't yet have a zero in the cells, so again add 1 to (a)x(d) and subtract 1 from (b)x(c). Now a zero appears. Recalculate one last time:
Cancel where possible. (Note 1! =1 and 0! =1)

$$P_3 = \frac{\cancel{10!} \times 21! \times 13! \cancel{\times 18!}}{\cancel{10!} \times 3! \times 0! \cancel{\times 18!} \times 31!} = 0.000006$$

Step 5: The final probability (P) is the sum of all probabilities (in this case $P_1+P_2+P_3$) or approximately 0.0047.

Step 6: Summarize: *Exposure was related to illness. Ill people were almost 13 times more likely to have been exposed to this factor compared to non-ill people. The relationship is statistically significant. The probability of these data occurring by chance alone is less than 0.5% (<0.005). Reject null hypothesis of "no association".*
[Odds ratio: 12.8, 95% CL: 1.6<OR<131.1, P=0.0047].

Notes:

1. When deciding which cells to increase by +1, always multiply (a) × (d) and compare with (b) × (c). *Increase* each cell of the pair with the higher product and *decrease* each cell of the pair with the smaller product, while keeping all marginal totals unchanged.
2. The final P is an "exact" P (probability) and may be reported as such (P = 0.0047). In this example, it is also <0.005 of course, and can be reported in this way if preferred.
3. The Fisher's test is used when the Chi-Square test is invalid due to any "E" values <5 in a 2 × 2 table. In all other circumstances, Chi-Sq. is an excellent approximation for the FE test.
4. If original data include a zero in one of the cells, you will calculate only one P value. (The O.R. will be reported as "undefinable" but the direction of the effect will be very clear).

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5. This P is calculated for a one-tailed FE test. It is adequate for this application. Two-tailed FE test will require further calculation.
6. Should a relationship NOT meet the critical value for significance (that is, $P > 0.05$), it is described *as* "*not statistically significant*". Note that a relationship may be observed, but this result is telling you that it could have occurred by chance alone more than 5% of time if you were to repeat the analysis. That may still require further investigation, but from a statistical standpoint, it cannot be claimed as a *statistically significant relationship*.

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WATERBORNE ILLNESS SUMMARY REPORT
Form K, page 1 of 2

Complaint No. | Disease

TYPE OF EXPOSURE	LOCATION OF OUTBREAK	DATE OF OUTBREAK (Date first case became ill)	NUMBER OF:	ACTUAL	ESTIMATED
			Persons exposed	_______	_______
			Persons ill	_______	_______
☐ Ingestion	State/Province ____________	Mo. Day Yr.	Hospitalized	_______	_______
☐ Contact	City or town ____________		Fatalities	_______	_______
☐ Inhalation	County ____________				

HISTORY OF EXPOSED PERSONS — NUMBER OF HISTORIES OBTAINED ________

Enter the percent of persons with the following symptoms

Diarrhea (>3 stools/day) ________
Visible blood in stools ________
Vomiting ________
Nausea ________

Diarrhea (other): No. ____/definition ________
Cramps ______ Conjunctivitis ______ Other (specify)
Fever ______ Otitis externa ______ ________
Rash ______ Cough ______ ________

INCUBATION PERIOD (hours)
Shortest ________
Longest ________
Median ________

DURATION OF ILLNESS (days)
Shortest ________
Longest ________
Median ________

ATTACH SUMMARY OF CASE HISTORIES (Form D2) ☐

ETIOLOGY OF OUTBREAK (ATTACH FORM I WITH LISTING OF SPECIMENTS EXAMINED AND EPIDEMIC CURVE)

Agent (if not known enter, "unk")	Diagnosis Confirmed	Diagnosis Presumptive	Diagnosis Suspected	COMMENTS:
Pathogen ____________	______	______	______	____________
Chemical ____________	______	______	______	____________
Other ____________	______	______	______	____________

CHARACTERISTICS OF WATER SUPPLY (if illness acquired by ingesting water). ATTACH ALL APPLICABLE PARTS OF FORM G

TYPE OF WATER SUPPLY
☐ Community or municipal
☐ Subdivision
☐ Trailer Park
Name ____________
☐ Noncommunity (with own water supply)
☐ Camp/cabin/recreation area
☐ School
☐ Restaurant
☐ Hotel/motel
☐ Church
☐ Other (specify) ________
☐ Individual household supply
☐ Bottled water (brand) ________
☐ Other (specify) ________
☐ ozone

WATER SOURCE
☐ Well
☐ River/stream
☐ Lake/pond/reservoir
☐ Spring
☐ Other (specify) ________

WATER TREATMENT PROVIDED
(check all that apply)
☐ No treatment
☐ Disinfection
☐ Chlorine
☐ Chlorine and ammonia (chloramines)
☐ Ozone
☐ Corrosion inhibitor
☐ Unknown
☐ Other (specify) ________

WATER TREATMENT PROVIDED (continued)
☐ Coagulation and/or Flocculation
☐ Settling (sedimentation)
☐ Filtration at purification plant
(do not include home filters)
☐ Rapid sand
☐ Slow sand
☐ Diatomaceous earth
☐ Other (specify) ________
☐ Unknown
☐ Other (specify)
☐ Unknown

COMMENTS: ________

WATERBORNE ILLNESS SUMMARY REPORT (continued)
Form K, part 2 of 2

Complaint No.

Disease

VEHICLE

CONFIRMATION BY (check all that apply)
☐ Laboratory
☐ Attack rate table and high confidence
☐ Other (specify) ____________________

COMMENTS: ____________________
____
____
____
____

ATTACH ATTACK RATE TABLE (form H1): ATTACH LISTING OF WATER SAMPLES EXAMINED AND FINDINGS (Form I)

RECREATION EXPOSURE (If illness acquired by contacting water or ingesting recreation water during activities)

Route of entry

☐ Intentional ingestion	☐ Contact	☐ Swimming pool	☐ River/stream	☐ Lake/pond
☐ Accidental ingestion	☐ Inhalation	☐ Hot tub	☐ Whirlpool	☐ Other (specify) _________________

ATTACH ALL APPLICABLE PARTS OF FORM F AND DESCRIBE SETTING

FACTORS CONTRIBUTING TO WATER CONTAMINATION AND/OR SURVIVAL (check all that apply)

AT SOURCE

☐ Overflow of sewage	☐ Use of a backup source of water by a water utility	☐ Other (specify) ________________
☐ Flooding heavy/rains	☐ Improper construction or location of well or spring	☐ Unknown
☐ Underground seepage of sewage	☐ Contamination through creviced limestone or fissured rock	

AT TREATMENT PLANT

☐ No disinfection	☐ No filtration	☐ Other (specify) ________________
☐ Temporary interruption of disinfection	☐ Inadequate filtration	☐ Unknown
☐ Chronically inadequate disinfection	☐ Deficiencies in other treatment processes	

IN DISTRIBUTION

☐ Cross contamination	☐ Contamination of mains during construction or repair	☐ Other (specify) _________________
☐ Back siphonage	☐ Contamination of storage facility	☐ Unknown

OTHER REASONS FOR CONTAMINATION OR SURVIVAL OF ETIOLOGICAL AGENT IN WATER
(include recreational exposure)

ATTACH NARRATIVE REPORT (include unusual aspects of outbreak or investigation not covered above and recommendations given for prevention)
REMARKS:

NAME OF REPORTING AGENCY	PERSON COMPLETING FORM (Please print)	DATE INVESTIGATION INITIATED	
ADDRESS	TITLE	PHONE/E-MAIL/FAX	DATE OF REPORT

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Multiple Types of *Legionella pneumophila* Serogroup 6 in a Hospital Heated-Water System Associated with Sporadic Infections

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Five sporadic cases of nosocomial Legionnaires' disease were documented from 1989 to 1997 in a hospital in northern Italy. Two of them, which occurred in a 75-year-old man suffering from ischemic cardiopathy and in an 8-year-old girl suffering from acute leukemia, had fatal outcomes. *Legionella pneumophila* serogroup 6 was isolated from both patients and from hot-water samples taken at different sites in the hospital. These facts led us to consider the possibility that a single clone of *L. pneumophila* serogroup 6 had persisted in the hospital environment for 8 years and had caused sporadic infections. Comparison of clinical and environmental strains by monoclonal subtyping, macrorestriction analysis (MRA), and arbitrarily primed PCR (AP-PCR) showed that the strains were clustered into three different epidemiological types, of which only two types caused infection. An excellent correspondence between the MRA and AP-PCR results was observed, with both techniques having high discriminatory powers. However, it was not possible to differentiate the isolates by means of ribotyping and analysis of *rrn* operon polymorphism. Environmental strains that antigenically and chromosomally matched the infecting organism were present at the time of infection in hot-water samples taken from the ward where the patients had stayed. Interpretation of the temporal sequence of events on the basis of the typing results for clinical and environmental isolates enabled the identification of the ward where the patients became infected and the modes of transmission of *Legionella* infection. The long-term persistence in the hot-water system of different clones of *L. pneumophila* serogroup 6 indicates that repeated heat-based control measures were ineffective in eradicating the organism.

Legionella pneumophila is a well-known cause of bacterial pneumonia (22), accounting for up to 30% of cases of nosocomially acquired pneumonia, which most frequently occur in immunologically deficient subjects (10, 12, 24). Fifteen serogroups of *L. pneumophila* have been described (1, 3). *L. pneumophila* serogroup 1 is the most frequent among human isolates, and 12 or 15 antigenic subtypes have been recognized with different sets of monoclonal antibodies (MAbs) (references 4 and 11, respectively). *L. pneumophila* serogroup 6, the second most common serogroup according to the frequency of isolation from clinical samples (17), shows a lower antigenic variability, and up to five antigenic subtypes have been detected in different studies (11, 15, 18).

Epidemiological investigations of legionellosis are complicated by the ubiquity of legionellae in nature. Discriminatory molecular subtyping methods should be applied to clinical and presumptively linked environmental strains in order to detect the source of the infection. MAb subtyping is insufficiently discriminatory when a given serogroup comprises only a few antigenically distinct subtypes, as for *L. pneumophila* serogroup 6. Furthermore, phenotypic differences have been reported in genotypically similar organisms (9, 23, 25). However, at least in one of these instances (9), it was later shown that macrorestriction analysis (MRA) could differentiate strains showing identical restriction fragment length polymorphism profiles (14). A simultaneous infection with multiple genomic types of a single *L. pneumophila* serogroup has recently been described (16), leading to discussions as to the number of colonies which should be typed after primary isolation and to the preferable typing method(s). Thus, a combination of antigenic and genomic typing systems has been recommended for the definition of patterns of colonization, and the clone(s) involved in the transmission of the infection (6, 9, 14, 16, 19, 21, 23, 27).

Here we report on an investigation of *L. pneumophila* serogroup 6 isolates from a hospital in which five sporadic cases of Legionnaires' disease occurred from 1989 to 1997. In order to determine whether a given clone of *L. pneumophila* serogroup 6 had found an ecological niche that enabled it to survive over a long period of time in the nosocomial environment and/or whether derivatives of the same organism had infected susceptible people over several years, the human isolates of *L. pneumophila* serogroup 6 were compared with their environmental counterparts by MAb subtyping, MRA, and arbitrarily primed PCR (AP-PCR).

MATERIALS AND METHODS

Water distribution system and specimen collection and processing. The hospital consists of a single building with 24 wards and a total of approximately 1,200 beds. The building is 18 years old. It receives water from a single municipal supply. The hot water system consists of four portions that serve three main sections (designated sections A, B, and C) and a minor part (section D) of the

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TABLE 1. Clinical and environmental *L. pneumophila* serogroup 6 isolates from the hospital under survey, by time of isolation and origin

Strain no.	Date (mo/yr)	Source of isolate	Location (block/ward)	Heating tank pressure[a]	*Legionella* count (cfu/liter)
1	April/1989	Patient 1	C/cardiology, medicine		
2	April/1989	Sink tap water	C/cardiology	MP	3×10^3
3	April/1989	Sink tap water	C/medicine	MP	ND[b]
4	April/1989	Bath tub water	C/cardiology	MP	1×10^3
5	July/1995	Shower water	C/pediatric hematology	HP	ND
6	August/1995	Shower water	C/pediatric hematology	HP	8×10^2
7	July/1995	Patient 2	C/pediatric hematology, room 5		
8	July/1995	Patient 2	C/pediatric hematology		
9	July/1995	Patient 2	C/pediatric hematology		
10	January/1996	Sink tap water	C/pediatric hematology, room 5	HP	1×10^2
11	January/1996	Sink tap water	C/pediatric hematology, room 1	HP	5×10^2
12	January/1996	Sink tap water	C/pneumology	MP	2×10^2
13	January/1996	Sink tap water	B/hematology	HP	1×10^3
14	January/1996	Heating tank	B	MP	$>1 \times 10^4$
15	January/1996	Heating tank	B	LP	7×10^2
16	January/1996	Heating tank	A	HP	4×10^3
17	January/1996	Heating tank	A	MP	1×10^4
18	January/1996	Sink tap water	A/radiology	LP	3×10^3
19	January/1996	Sink tap water	D/ICU	LP	2×10^2

[a] HP, high pressure; MP, medium pressure; LP, low pressure.
[b] ND, not determined.

hospital. The hot-water temperature is 55 to 56°C. For sections A, B, and C, each section has four 5,000-liter vertical heating tanks. From three of them, vertical pipes deliver hot water at different pressures, depending on the floor served: from the second underground floor to the 1st floor, low pressure, from the 2nd to the 6th floors, medium pressure, and from the 7th to the 12th floors, high pressure. Recirculation of water within each section is accomplished by pumps. The fourth heating tank acts as a reservoir to meet extra demands. Section D of the hospital is served by two 3,000-liter heating tanks with a thermostat-set point to mix warm and cold water and a recirculation pump.

Five-liter hot-water specimens from individual sections of the hospital were collected on the same day in sterile containers from distal outlets after allowing the water to flow for 10 min, and they were collected from the heating tanks through a bottom valve after allowing the water to flow for 2 min. The sampling started on the upper floors and continued to the lower levels.

Total bacterial counts were evaluated as numbers of CFU by the membrane (pore size, 0.45 μm; Millipore, Milan, Italy) filtration method of 10-fold serial dilutions of the samples after transfer of the membranes to the surfaces of two separate tryptone soy agar (Oxoid, Garbagnate Milanese, Italy) plates and incubation at 37°C for 24 h. The concentration of *Legionella* spp. was determined as the numbers of CFU per liter on buffered charcoal yeast extract (BCYE) agar plates (Oxoid). The water was concentrated by membrane (pore size, 0.2 μm; Millipore) filtration, and then diluted and undiluted specimens were plated and incubated at 37°C in humidified air for at least 10 days. Suspect *Legionella* colonies, which failed to grow in the absence of cysteine, were further checked by direct immunofluorescence with an *L. pneumophila* species-specific MAb (Diagnostics Pasteur, Marnes-La-Coquette, France) and then with *L. pneumophila* serogroup 1 to 6 monovalent fluorescein-labeled antisera (SCIMEDX; Dasit, Cornaredo, Italy, and BIOS; Daltec, San Vittore Olone, Italy).

Strains. Three groups of *L. pneumophila* serogroup 6 strains were examined. Their designations and origins are listed in Table 1. The first group consisted of four strains (one from patient 1 and three from two putatively associated hospital environmental sources) isolated in 1989 (26), and the second group consisted of five strains (three from patient 2 and two from the epidemiologically linked source of infection) isolated in 1995. The third group consisted of 15 strains isolated in January 1996 from different sites of the same hospital but not associated in time with human infection. In fact, no isolates were available from three patients with serologically confirmed cases of infection which occurred in 1994, 1995, and 1997. *L. pneumophila* serogroup 6 reference strains Chicago 2 (ATCC 33215) and Dresden (15) were used as internal controls in molecular typing experiments. Suspensions of strains from cultures derived from a single colony were kept at −80°C in skim milk until they were used.

Subtyping with MAbs. Strains typed as *L. pneumophila* serogroup 6 by direct immunofluorescence with the monovalent antiserum were further subtyped by indirect immunofluorescence with MAbs 9/2, 4/5, 18/2, and 54/2. These MAbs recognize different epitopes on the lipopolysaccharide of this organism (11, 15). MAb 9/2 specifically recognizes all *L. pneumophila* serogroup 6 strains. MAb 4/5 also reacts specifically only with serogroup 6 strains, but not with all serogroup 6 strains. MAbs 18/2 and 54/2 recognize antigenic variants of serogroup 6, but they also react with some strains belonging to other serogroups of *L. pneumophila* (15).

DNA fingerprinting. MRA, AP-PCR, and ribotyping were performed. For MRA, genomic DNA was prepared by the method described by Lück and coworkers (13). Briefly, the bacteria were grown for 72 h on BCYE agar plates and were then washed twice and suspended in SE buffer (75 mM NaCl, 25 mM EDTA [pH 7.4]). The bacterial suspensions ($A_{600} \cong 1.5$) were mixed with equal volumes of molten 2.0% low-melting-point agarose (Bio-Rad, Milan, Italy) in SE buffer, and the mixture was poured into acrylic casting wells. After the agarose gelled, the blocks were immersed in a digestion solution of 1% sodium lauroylsarcosine, 0.5 M EDTA, and 2 mg of proteinase K (Boehringer Mannheim, Milan, Italy) per ml (pH 9.5) and incubated at 50°C overnight. Agarose blocks were washed four times in TE buffer (10 mM Tris, 1.0 mM EDTA [pH 8.0]) and were stored in the same buffer at 4°C. DNAs were cleaved with *Not*I, *Sfi*I, and *Asc*I (New England Biolabs, Schwahlbach, Germany) following the manufacturer's instructions. The blocks were then loaded on 1% agarose (FMC, BIOSPA, Milan, Italy) in 0.25× Tris-borate-EDTA buffer (pH 8.3). Pulsed-field gel electrophoresis (PFGE) was carried out with Rotaphor Type V equipment (Biometra, Gottingen, Germany) at 12°C for 36 h with a voltage decrease from 200 to 180 V and with a constant angle of 135°. Pulse times were 100 to 2, 50 to 2, and 60 to 2 s for DNAs cleaved with *Not*I, *Sfi*I, and *Asc*I, respectively. Bacteriophage lambda concatemers and *Saccharomyces cerevisiae* WAY 5-4A (Biometra) were used as DNA size markers. Genomic fragments were stained with ethidium bromide and were photographed under UV illumination.

AP-PCRs were carried out with a set of four oligonucleotides, designated AP5 (5′-TCCCGCTGCG-3′), AP12 (5′-CGGCCCCTGC-3′), CD1 (5′-GGATCCTG AC-3′), and 1247 (5′-AAGAGCCCGT-3′). Amplification reactions were performed in a 50-μl volume containing 10 mM Tris-HCl (pH 8.3), 4.0 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at 200 μM, each primer at 2.5 μM, 2 ng of genomic DNA, and 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim). PCRs were performed in a Perkin-Elmer model 9600 thermal cycler with the fastest available transition times between each temperature. After incubation at 90°C for 60 s and at 95°C for 90 s, the reaction mixtures were cycled 45 times through the following temperature profile: 95°C for 30 s, 37°C for 1 min, and 74°C for 1.5 min. The samples were then incubated at 74°C for 3.5 min and were then held at 4°C. Samples of 10 μl of each amplification mixture were loaded onto a 2.0% (wt/vol) agarose gel with TBE (Tris-borate-EDTA) buffer containing 0.5 mg (wt/vol) of ethidium bromide per ml, and the gel was electrophoresed at 3 V/cm for approximately 5 h. Each strain was tested in three independent experiments performed under identical conditions. Gel

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photographs were scanned with a Hewlett-Packard Scanjet IIcx scanner. The PFGE and AP-PCR patterns were analyzed by GelCompar, version 4.0, computer software (Applied Maths, Kortrijk, Belgium). Similarity between pairs of strains was calculated as the Dice coefficient, which corresponds to the ratio of twice the number of common fragments to the total number of fragments in the two patterns. Clustering and the linkage level between pairs or groups of strains were calculated by the unweighted pair group method with arithmetic averages and are represented as a dendrogram.

For the analysis of *rrn* operon polymorphism, the chromosomal DNAs of the strains were digested with *Hin*dIII and *Pst*I, and the fragments were separated by electrophoresis through a 0.8% agarose gel in TBE buffer at 40 V for approximately 16 h. Restriction fragments were Southern blotted onto a nylon membrane (Hybond-N; Amersham) and were cross-linked by exposure to UV light. Prehybridization, hybridization with the digoxigenin (DIG)-labelled 7.5-kb *Bam*HI fragment of pKK3535 (5), posthybridization washing, and immunologic detection were performed according to the manufacturer's instructions (Boehringer Mannheim). DIG-labelled hybrids were detected with an anti-DIG alkaline phosphatase antibody conjugate and the chemiluminescent substrate Lumigen PPD (Boehringer) according to the manufacturer's specifications. For the detection of the chemiluminescent signal, the membranes were exposed to Kodak XAR film.

Analysis of the 16S-23S rRNA gene spacer regions was performed with primer 2 (5′-TTGTACACACCGCCCGTC-3′), which annealed to the 16S rRNA gene from base pairs 1390 to 1407, and primer 7 (5′-GGTACTTAGATGTTTCAGTTC-3′), which annealed to the 23S rRNA gene from base pairs 188 to 208, according to Gürtler and Stanisich (8). Amplification reactions were carried out by using 10 ng of genomic DNA in a 100-μl reaction mixture containing 1× PCR buffer (Promega), 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 50 μM, each primer at 1 μM, and 0.25 U of *Taq* DNA polymerase (Promega). After incubation at 94°C for 4 min, 30 cycles were performed in a Perkin-Elmer model 9600 thermal cycler, with each cycle comprising 45 s at 94°C, 1 min at 55°C, and 45 s at 72°C. The samples were then incubated at 72°C for 5 min and were then held at 4°C. The amplification products were electrophoresed and visualized as outlined above.

RESULTS

Clinical and environmental investigations. On 9 March, 1989 a 75-year-old man (patient 1) with a history of nephrosclerosis, hypertension, and ischemic cardiopathy was admitted to the cardiology ward of a hospital in northern Italy. He presented with unstable angina and ulcerative rectocolitis. On 15 March he was transferred to the medicine ward, and then on 2 April he was transferred to the coronary care unit in the cardiology ward due to acute myocardial infarction. Ten days later, after improvement in his clinical condition, he was transferred back to the medicine ward. On 19 April the patient developed acute dyspnea for pulmonary edema, and the chest X ray disclosed a bronchopneumonic picture in the right upper lobe. Ten days later the patient was transferred to the intensive care unit (ICU), where he died 5 h after admission. Antibiotic therapy was not given. *L. pneumophila* serogroup 6 was isolated from lung tissue obtained at autopsy and from water samples of the cardiology and medicine wards. A semiquantitative evaluation by CFU counts of the samples from the cardiology ward showed 3×10^3 legionellae/liter in the sink tap water and 1×10^3 legionellae/liter in the bathtub water (Table 1).

On 5 June 1995 an 8-year-old girl (patient 2) suffering from acute lymphocytic leukemia was admitted to the pediatric hematology ward of the same hospital to initiate the conditioning regimen prior to bone marrow transplantation. On 14 June she received the transplant, and on the following day she developed a fever (>38°C). Despite antimicrobial therapy, on the 14th posttransplantation day respiratory symptoms appeared and a chest X ray disclosed lower left pulmonary infiltrates. Forced respiration was started, antibiotic therapy was implemented, and the patient was transferred to the ICU. On the 29th posttransplantation day, culture results became available and indicated positivity for *L. pneumophila*. Despite addition of rifampin to the antibiotic regimen, the patient died from respiratory failure 3 days later. *L. pneumophila* serogroup 6 was isolated from bronchoalveolar lavage specimens obtained on 7 and 10 July and on the day of death. A microorganism of the same species and serogroup (8×10^2 CFU/liter) as the microorganism isolated from patient 2 was cultured from hot water from the shower of the pediatric hematology ward where the patient had stayed (Table 1).

Three cases of nosocomially acquired Legionnaires' disease were clinically diagnosed in 1994, 1995, and 1997 and were confirmed by seroconversion of the patients, but cultures of respiratory specimens did not yield *Legionella* isolates. Since a polyvalent *L. pneumophila* serogroup 1 to 6 antigen was used for the indirect immunofluorescence test, it was not possible to determine the serogroup that caused the infection.

An environmental investigation was further performed in 1996. *L. pneumophila* serogroup 6 was isolated from 15 (62.5%) of 24 sites examined. The legionella concentration at the different sites examined did not correlate with the total bacterial counts in the samples (data not shown). No other *L. pneumophila* serogroups or *Legionella* species were isolated.

After the first documented case of nosocomial Legionnaires' disease in 1989, active surveillance was implemented in the hospital. In May 1989 and August 1995 control measures were undertaken by superheating the heating tanks, and water was flushed for 15 min at the distal outlets of the system at a temperature >65°C.

Subtyping with MAbs. All the strains examined reacted with MAb 9/2, which is specific for *L. pneumophila* serogroup 6. The other three MAbs were selected because they recognize subgroup-determining epitopes of serogroup 6 strains (11, 15). None of the strains reacted with MAb 18/2. Two strains isolated in 1989 and all those isolated in 1995 were positive with the subgroup-specific MAbs 4/5 and 54/2, as was the Chicago 2 type strain. Two of the strains isolated in 1989 and 11 of the 15 strains isolated in January 1996 were negative with these MAbs (Table 2 and data not shown).

Genomic analysis. The results obtained by PFGE analysis of *Sfi*I-digested genomic DNAs of 19 representative strains are shown in Fig. 1 and are summarized in Table 2. Among the three enzymes tested, *Sfi*I gave the most complex macrorestriction pattern, allowing the identification of three main clusters, designated clusters A, B, and C. Cluster C included strains characterized by a similarity score of >80%, as deduced by computer-assisted analysis of electropherograms. Within this group, some differences were observed among isolates (at the level of up to four bands), and these differences determined a further subdivision into five subtypes (subtypes C^{I} to C^{V}; Table 2), each of which was composed of strains with >85% similarity. Also, *Not*I and *Asc*I digestions made it possible to identify three pulsotypes (pulsotypes D, E, and uncut for *Not*I and pulsotypes F, G, and H for *Asc*I; Table 2). Each pulsotype included strains with identical macrorestriction patterns (data not shown). In addition, the data reported in Table 2 indicate a complete correspondence between groups of strains clustered on the basis of macrorestriction profiles following digestion with the three enzymes.

The AP-PCR results obtained by the use of four different oligonucleotide primers are shown in Fig. 2 and are summarized in Table 2. Primers CD1 and 1247 defined three different groups each (groups g, h, and i and groups j, k, and l, respectively), and the groups matched those previously identified by PFGE. Primer AP12 was less discriminatory, since it differentiated the strains into two groups (groups e and f), while AP5 was more discriminatory, allowing the definition of four different groups (groups a, b, c, and d).

There was an excellent correspondence of the results obtained by AP-PCR with primer AP5 and MRA with *Sfi*I. As shown in Table 2, cluster a matched subtype A, cluster b matched subtype B, cluster c matched subtypes C^{I} to C^{III}, and

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TABLE 2. MAb, PFGE, and AP-PCR subtyping of clinical and environmental isolates of *L. pneumophila* serogroup 6

Strain no.[a]	Reactivity against MAb:				PFGE type after digestion with[b]:			AP-PCR type with primer:				Combined type code[c]
	9/2	4/5[d]	18/2[d]	54/2[d]	*Sfi*I	*Not*I	*Asc*I	AP5	AP12	CD1	1247	
1	+	+	−	+	A	NC	F	a	e	g	j	I
2	+	−	−	−	C^{III}	D	G	c	e	i	l	III^{I}
3	+	−	−	−	C^{I}	D	G	c	e	i	l	III^{I}
4	+	+	−	+	A	NC	F	a	e	g	j	l
5	+	+	−	+	B	E	H	b	f	h	k	II
6	+	+	−	+	B	E	H	b	f	h	k	II
7	+	+	−	+	B	E	H	b	f	h	k	II
8	+	+	−	+	B	E	H	b	f	h	k	II
9	+	+	−	+	B	E	H	b	f	h	k	II
10	+	−	−	−	C^{I}	D	G	c	e	i	l	III^{I}
11	+	−	−	−	C^{V}	D	G	d	e	i	l	III^{II}
12	+	−	−	−	C^{I}	D	G	c	e	i	l	III^{I}
13	+	−	−	−	C^{V}	D	G	d	e	i	l	III^{II}
14	+	−	−	−	C^{II}	D	G	c	e	i	l	III^{I}
15	+	−	−	−	C^{IV}	D	G	d	e	i	l	III^{II}
16	+	+	−	+	A	NC	F	a	e	g	j	I
17	+	+	−	+	A	NC	F	a	e	g	j	I
18	+	−	−	−	C^{IV}	D	G	d	e	i	l	III^{II}
19	+	−	−	−	C^{II}	D	G	c	e	i	l	III^{I}
Chicago 2	+	+	−	+								
Dresden	+	−	−	−								

[a] Strains were numbered as in Table 1.
[b] Indistinguishable patterns are indicated with the same letter code. Minor differences (<20%) within a single PFGE type are indicated by superscript roman numerals; NC, genomic DNA is not cut by the enzyme.
[c] Obtained by combining MAb, PFGE, and AP-PCR types; strains with >80% and 90% similarities at the macrorestriction and AP-PCR levels, respectively, were considered to belong to a single type. Superscript roman numerals indicate minor differences within a single type.
[d] MAbs that recognize monoclonal subgroups of serogroup 6.

cluster d matched subtypes C^{IV} and C^{V}. The degree of similarity between subtypes C^{I}, C^{II}, and C^{III} (corresponding to AP-PCR type c) was nearly 85%. This value is similar to that determined by comparing types C^{IV} and C^{V}, which correspond to AP-PCR type d, and is higher than the value obtained by comparison of all C subtypes (80%; see Fig. 1). Likewise, the degree of similarity between types c and d was 90%, which is the highest value among those determined by analysis of AP-PCR patterns (Fig. 2).

Southern hybridization of *Hin*dIII- and *Pst*I-digested genomic DNA with the *rrnB* gene probe did not differentiate the 19 *L. pneumophila* isolates listed in Table 2. In addition, PCR of the 16S-23S *rrn* intergenic region (8) did not reveal any amplicon length polymorphism (data not shown).

By combining the MAb, PFGE, and AP-PCR types presented in Table 2, the strains were subdivided into three different combined type codes, designated types I, II, and III. When the data in Table 1 were examined in light of the typing results (Table 2), it appeared that two unrelated clones of *L. pneumophila* serogroup 6 were responsible for the infection episodes in 1989 and 1995. The type I strain, which caused the infection in 1989, was found to persist in the water system of the hospital until 1996, when the last environmental sampling was performed. The type II isolate was found to contaminate the high-pressure heating tank of hospital section C only during the summer of 1995, when it was also responsible for the infection of patient 2. Interestingly, from 1989 to 1996 a third clone of *L. pneumophila* serogroup 6 (type III) was found in the water supply system of the hospital and was found to contaminate different heating tanks and sections but did not cause any documented infection episode.

DISCUSSION

From 1989 to 1997, nosocomially acquired Legionnaires' disease was documented in five patients in the hospital under study. *L. pneumophila* serogroup 6 was isolated from clinical specimens from two patients who died, while for the other three patients, all of whom recovered, only seroconversion against a *L. pneumophila* serogroup 1 to 6 polyvalent antigen was evidenced. Serological typing of presumptively associated environmental strains revealed that *L. pneumophila* serogroup 6 was responsible for extensive contamination of the hospital hot-water supply system. The legionella concentration at the different sites examined ranged from 10^2 to $>10^4$ CFU per liter, which is an amount considered to be able to cause one or more sporadic cases per year (7). These facts led us to wonder whether a single clone of *L. pneumophila* serogroup 6 had persisted in the hospital environment for over 7 years and had caused sporadic infections. Therefore, we compared the MAb types, MRA patterns, and AP-PCR types for all the nosocomial *L. pneumophila* serogroup 6 isolates. Although the discriminatory power of MAb typing is relatively poor for *L. pneumophila* serogroup 6, it is interesting that strains belonging to the two subtypes Chicago (5 strains) and Dresden (10 strains) contaminated the water system of the hospital over the period of time examined but that only MAb type Chicago had caused infection.

DNA-based typing techniques made it possible to differentiate the isolates into three distinct epidemiological types. The type I and II isolates belonged to MAb type Chicago. Type I was responsible for the infection in 1989 and persisted until 1996. Type II was associated with the infectious episode in 1995 and showed significant differences from type I (<50% similarity at the level of the *Sfi*I MRA and AP-PCR patterns).

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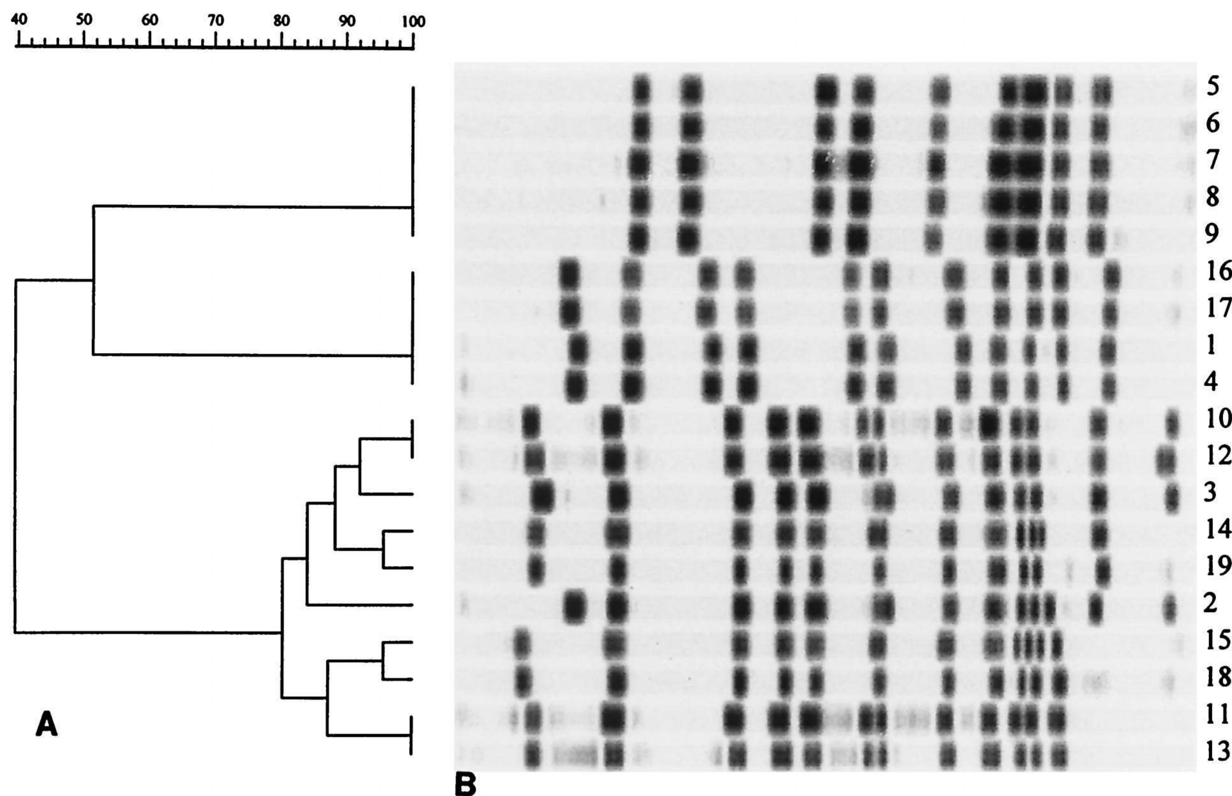

FIG. 1. PFGE analysis of *Sfi*I-cleaved genomic DNA of the *L. pneumophila* serogroup 6 strains listed in Table 1. (A) Dendrogram showing the genetic distance relationships of the 19 isolates designated as indicated in Table 1. (B) Macrorestriction patterns of the isolates.

All the strains included within type III belonged to MAb type Dresden and were indistinguishable when analyzed by PFGE of *Not*I- and *Asc*I-cleaved DNA or by AP-PCR with primers AP12, CD1, and 1247 but were closely related when analyzed by MRA with *Sfi*I or by AP-PCR with primer AP5 (Fig. 1 and 2).

The results reported here provide useful information. First, the long-term persistence in the water system of the hospital of multiple clones of *L. pneumophila* serogroup 6, one of which was responsible for a sporadic case of nosocomial legionellosis, is demonstrated. Type I was confined to the hot-water supply system of sections C and A during the years 1989 and 1996, respectively, being responsible for one sporadic human infection, which occurred in 1989, whereas type III was found in the heating tanks of all four hospital sections examined in the 1995 and 1996 period, as well as in the hot water supplied from the tank of section C during the 1989 sampling, but did not cause any documented case of nosocomial legionellosis. It can be speculated that eradication procedures performed after the first case was diagnosed may have altered the relative levels of individual types within the hospital hot-water system. Although the hot water of the hospital was maintained at 5 to 6°C above the thermal threshold for suppression of *Legionella* multiplication (23) and superheating was performed in May 1989 and August 1995, both control measures failed to eradicate the microorganism. However, while types I and III were able to persist in the hospital water system during the whole period examined, type II was probably eradicated since it was no longer isolated from the water taken during the extensive sampling of 1996. Whether types I and III are more resistant than type II to the thermal shock or whether they are endowed with a greater ecological fitness is still an open question.

Second, this study adds further information on the discriminatory power of DNA-based techniques for the typing of *L. pneumophila* serogroup 6. Analysis of the 16S *rrn* operon and of the 16S-23S spacer region did not reveal appreciable genomic polymorphism for the 19 strains examined, suggesting that these two techniques may be inadequate for DNA fingerprinting of *L. pneumophila* serogroup 6 strains. Digestion of genomic DNAs with either *Sfi*I or *Asc*I gave unique and complex PFGE patterns (nine or more fragments), enabling an accurate discrimination between pulsotypes (Simpson's index of diversity [D] = 0.37). The complexities of the electropherograms obtained upon *Not*I digestion were lower (the enzyme either did not cut the DNA or generated two to five fragments), but they were still adequate for differentiation of the isolates (D = 0.37). Interestingly, repeated attempts to obtain *Not*I digestion of DNA extracted from type I strains were unsuccessful. *Not*I recognizes and cuts the sequence 5′-GC ↓ G GCCGC-3′ (the arrow represents the cleavage site) and is sensitive to methylation of the CG residues at positions 4 and 5 of the restriction site. Whether an *Sss*I-like GpC methylase (20) is present in the type I isolates is still unknown, but this activity would certainly block cleavage at all *Not*I genome sites.

The amplification patterns obtained by AP-PCR with all four primers tested did not differ significantly in terms of complexity, because they produced 3 to 10 major amplicons for each type strain, but the level of discrimination achieved was dependent on the primer used. Thus, primer AP5 gave the best results, in that it generated four distinct patterns (patterns a to d; D = 0.21), while primer AP12 had the lowest discriminatory power and produced only two patterns (patterns e and f; D = 0.59), which, in turn, did not correlate with the observed MAb

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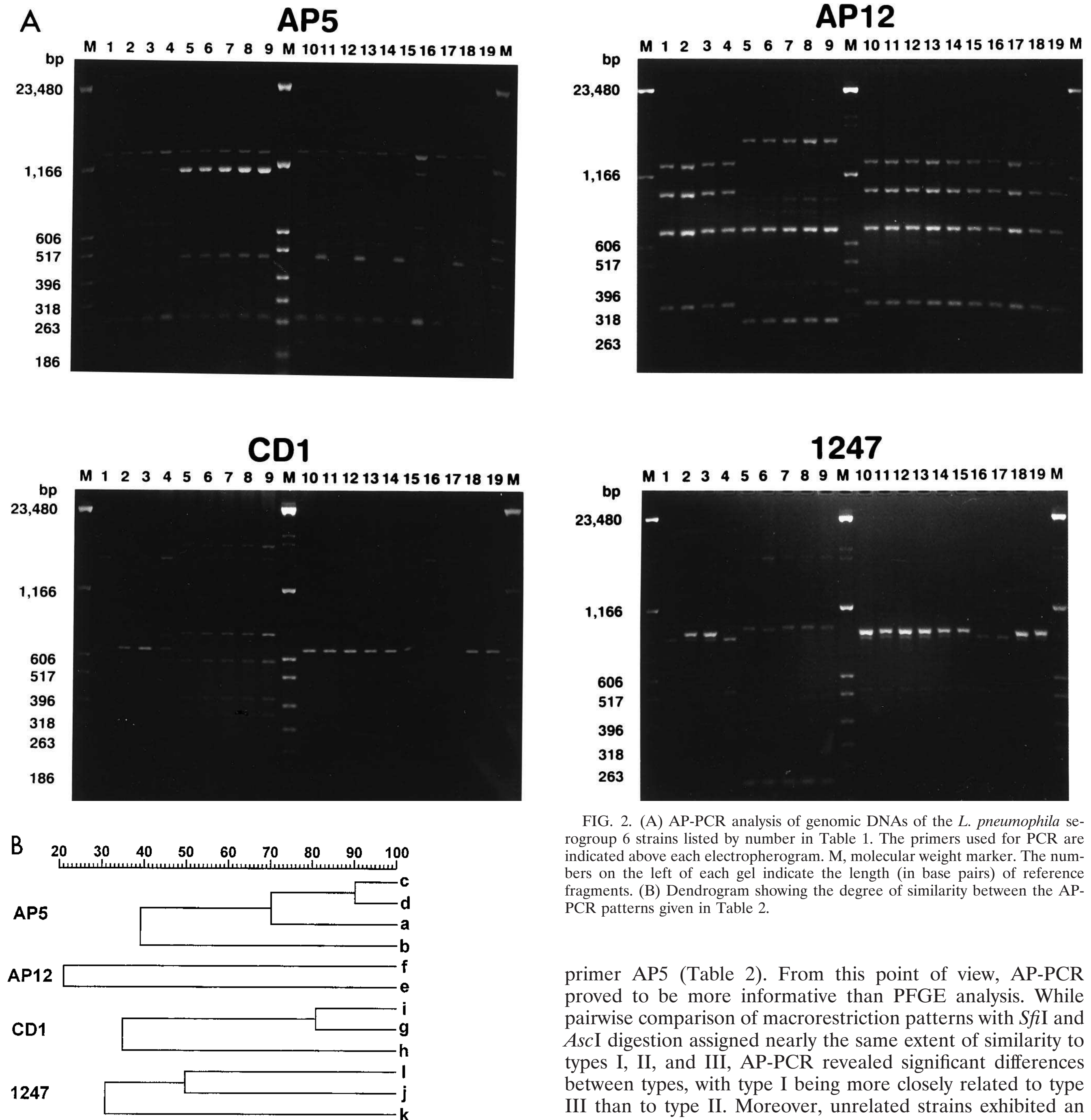

FIG. 2. (A) AP-PCR analysis of genomic DNAs of the *L. pneumophila* serogroup 6 strains listed by number in Table 1. The primers used for PCR are indicated above each electropherogram. M, molecular weight marker. The numbers on the left of each gel indicate the length (in base pairs) of reference fragments. (B) Dendrogram showing the degree of similarity between the AP-PCR patterns given in Table 2.

types. An interesting observation derived from this study is the excellent agreement between MRA with *Sfi*I and AP-PCR with primer AP5. Type III strains show some heterogeneity when analyzed by *Sfi*I digestion and can be considered a single type when an 80% similarity cutoff is imposed on the PFGE analysis, while they are resolved into two clusters at a similarity cutoff of 85%. In the latter case, strains 2, 3, 10, 12, 14, and 19 would be included in one subgroup, while strains 11, 13, 15, and 18 would be split into another subgroup. Of note, these two subgroups perfectly match with the c and d subtypes defined for type III isolates by AP-PCR fingerprinting with primer AP5 (Table 2). From this point of view, AP-PCR proved to be more informative than PFGE analysis. While pairwise comparison of macrorestriction patterns with *Sfi*I and *Asc*I digestion assigned nearly the same extent of similarity to types I, II, and III, AP-PCR revealed significant differences between types, with type I being more closely related to type III than to type II. Moreover, unrelated strains exhibited an overall high degree of polymorphism when tested by both MRA and AP-PCR, indicating comparable discriminatory powers for both techniques. AP-PCR is occasionally reported to suffer from poor reproducibility, but in our study strains tested on more than one occasion with the same primer consistently gave identical results. Thus, a major drawback derived from this observation is that AP-PCR analysis with appropriate primers can provide easily interpretable patterns (consisting of a maximum of 10 major amplicons) and can reach a discriminatory level comparable or even superior to that of MRA.

On the basis of the typing results, we may conclude that the infecting strains were transmitted from the hospital hot-water supply system. High densities of legionellae were found in the hot-water samples and in the heating tanks, which are known

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to be usual reservoirs of legionellae in hospital settings (28). In addition, legionellae were not isolated from the cold water or from the cooling towers of the hospital air-conditioning system during multiple samplings performed from 1989 to 1996. Strains identical to those isolated from the two patients were present in the central and peripheral hot-water supply system, and there is a close temporal relationship between the isolates from humans and the corresponding isolates from the hot water. Taking into account the temporal sequence of events, it can be assumed that patient 1 became infected in the medicine ward, where he resided during the week preceding the onset of symptoms. Although transmission from the water system (medium-pressure heating tank) of hospital section C to the patient can be hypothesized, we were unable to detect the type I infecting strain from the hot water taken from the medicine ward. However, we have shown that it was present in the hot water of the cardiology ward, where the patient had stayed before being transferred and which is served by the same heating tank as the medicine ward (Table 1). It is therefore possible that the type I strain was also present in the sample taken from the latter ward but that it escaped detection because only one randomly selected colony of *L. pneumophila* serogroup 6 was sent to the reference laboratory in Rome for typing. The mode of disease acquisition was presumably aspiration. The patient did not shower but was exposed to water by bed bathing, which is a known risk factor (2). For patient 2, who was infected while staying in the pediatric hematology ward, inhalation of aerosol generated from showering appears to be the most likely mode of transmission of the infection. In fact, the causative strain was present in the hot water taken from the shower, and the hospital staff confirmed that the patient took showers on the days preceding the onset of symptoms. Moreover, it was ruled out that she might have drunk tap water and that floor-washing procedures might have constituted a risk factor.

The finding that antigenically similar but genetically different serogroup 6 strains were isolated from the environment associated with the infection has two important consequences. First, serological and MAb typing may be insufficient for discrimination of individual isolates of *L. pneumophila* serogroup 6. Second, sampling bias can occur, and a large number of environmental isolates should be genotyped to ensure that all types of legionellae present in the sample are recovered and characterized. Despite its undisputed discriminatory power, PFGE typing is time-consuming, relatively expensive, and available only to specialized laboratories. In contrast, AP-PCR is cost-effective, time-saving, and easy to perform. The single-primer reaction can rapidly discriminate large panels of *L. pneumophila* isolates and can be used for the quick screening of isolates from different sources in local settings.

In conclusion, our results highlight the value of combined MAb typing and genomic analysis in comparing *L. pneumophila* serogroup 6 strains. In particular, the high discriminatory power and feasibility of AP-PCR make this technique suitable for routine comparison of *L. pneumophila* serogroup 6 isolates in epidemiological studies aimed at detection of the infection source and validation of the effectiveness of control measures.

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The Microbiology of Waters and Associated Materials (2017) - 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 undertaking microbiological examinations of drinking waters, environmental and recreational waters and sewage sludge. This document replaces the Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories.

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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, soils (including contaminated land) 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 (established 1972 by the Department of the Environment). At present, there are seven 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, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 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 these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA's web-page:- http://www.standingcommitteeofanalysts.co.uk/Contact.html

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Rob Carter
Secretary
June 2017

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 2002 (SI 2002/2677). 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 resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety "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 "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

Glossary

ANOVA	Analysis of variance
AQC	Analytical Quality Control
Broth	A liquid medium design for the selective or non-selective recovery of bacteria
BSI	British Standards Institute
CEN	European Committee for Standardization (Comité Européen de Normalisation)
cfu	Colony forming units
CI	Confidence interval
COSHH	Control of Substances Hazardous to Health
E. coli	*Escherichia coli*
EQA	External Quality Assessment
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
kPa	Kilopascals
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time of Flight, Mass Spectrometer
MPN	Most probable number
MPR	Most probable range
NCTC	National Collection of Type Cultures (UK)
PTS	Proficiency Testing Scheme
QA	Quality Assurance
QC	Quality Control
Reference Material	A material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials
RO	Reverse Osmosis
Sample matrix	A sample description relating to its derivation, being specifically defined and of distinct relevance to its analysis, for example water type: potable water
UKAS	The United Kingdom Accreditation Service
UM	Uncertainty of Measurement
UV	Ultraviolet
WDCM	World data centre for microorganisms

Abbreviations of media names (examples only for table 5.12)

MLSB / MLSA	Membrane lauryl sulphate broth / agar
MLGA	Membrane lactose glucuronide agar
MEA	Membrane Enterococcus agar
TSCA	Tryptose sulphite cycloserine agar
TCA	Tryptose cycloserine agar
YEA	Yeast extract agar
PSA	*Pseudomonas* selective agar
LPW	Lactose peptone water
TW	Tryptone water
TNA	Tryptone nutrient agar
KAAA	Kanamycin aesculin azide agar
BA	Blood agar
BAA	Bile aesculin agar
BPW	Buffered peptone water

XLDA	Xylose lysine deoxycholate agar
CCDA	Charcoal cefoperazone deoxycholate agar

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. These include the stressed or damaged states (due to environmental or disinfectant challenge) the micro-organisms may be in, and the presence of competing and non-target organisms (which may result in restricted growth or false-positive colonies). It is, therefore, important that the media used by a laboratory are prepared, and the procedures conducted, in such a way that the results truly reflect, for example, the quality of the water being tested and that the data generated are reliable.

This booklet has been revised with the recognition that laboratory practices and procedures used for the microbiological analysis of water and associated materials are largely independent of the sample character, matrix or water type. It is intended that the document should support application of methods published in the series 'Microbiology of', whether drinking water, recreational and environmental waters or sewage sludge. While many of the examples given in the booklet relate to drinking water, reflecting the importance of this matrix and the origin of the document, specific guidance is also provided where appropriate for other water types and associated materials including sewage sludge.

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. In the UK the regulator has issued guidance[(1)] on 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 may 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 a class of micro-organisms in a given sample volume, and to estimate their numbers. The detection of small numbers of organisms is particularly important for drinking water and environmental samples from unpolluted sources. An 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 external quality assurance proficiency testing scheme.

Any laboratory where the analysis of water and associated materials is undertaken should operate a quality system. The main function of such a system is to define the processes that have been put into place to ensure that results are reliable and which must be 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 reliable within the constraints of microbiological testing. 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 or modified method within a laboratory.

Laboratories wishing to be accredited under a national accreditation scheme need to fulfil the requirements of BS EN ISO/IEC 17025(2). This document provides a framework for establishing appropriate documentation and procedures. Further information on requirements for accreditation in the UK under the Drinking Water Testing Specification (DWTS) has been provided by UKAS(3) and specific information and guidance for microbiological laboratories, on how to fulfil the requirements of ISO 17025 published by Eurachem(4).

2 The quality manual

The foundation of a quality system which aims to meet the requirements of ISO 17025 on general requirements for the competence of testing and calibration laboratories(2) is a quality manual that defines the laboratory's quality management system and its policy towards quality in relation to its testing and, where appropriate, sampling. The manual should be broad in its approach, establishing the basis of a management system that is appropriate to the scope of the laboratory's activities. It should be simple in that it is easily read and understood by all members of staff, and it should be easy to maintain in the ever-changing circumstances of the laboratory. In broad outline, the manual should document the laboratory's policies, and summarise its systems, programmes, procedures and instructions to an appropriate extent. It should contain a quality statement, details of the laboratory in terms of location and staff structure, and should define senior level responsibilities such as those of technical and quality management. Every laboratory should have an organisational chart showing staff posts and associated role profiles, 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 their role and responsibilities.

The quality system will require a record of staff training which should be maintained and regularly updated to provide a record of staff competence. There should also be a defined plan for individual staff development and the provision of cover for work when staff members are absent. In addition to the requirements of ISO 17025(2), the quality manual may also incorporate health and safety policies, safe working procedures and environmental policies and how it will set, maintain and check quality standards.

The quality manual should define records that the laboratory will keep, and maintain, the nature and frequency of measurement calibrations of equipment critical to the testing scope, the format of analytical procedures and strategies for internal and external quality assurance.

The protocol for assuring the quality of test results should be fully documented within the quality system and should include participation in appropriate external proficiency schemes where such schemes exist. A robust internal quality assurance system is essential. These areas are covered in more detail within other sections of this booklet.

Suppliers and materials purchased for use during analysis must be appropriately evaluated to confirm their suitability and to ensure that the quality of the testing activities are not compromised. New batches of, for example, membrane filters whether from the same or a different supplier to those ‘in use’ should be tested to verify that performance is both acceptable and consistent.

A procedure relating to the handling of items under test needs to be included within the quality system and referenced from the quality manual. The use of appropriate sample containers and preservatives, details of sample handling, reception and suitable transportation conditions are all factors which need to be considered.

One of the critical components of the quality management system is an effective internal audit process. This process must be documented to provide guidance on the audit process and should require that audits are undertaken by appropriately trained staff. Internal auditors should be knowledgeable of, but not directly involved in, the activity, process or procedure being audited. The procedure should include the requirement to take remedial action, which must include investigations, identification of root causes, implementation of appropriate corrective actions and a check on the effectiveness of these implemented corrective actions.

The importance of effective interactions between the laboratory and its customers cannot be underestimated, as understanding the needs of the customer and their use of the final result can have a significant influence on method selection and guidance provided to explain results. Documented policies on defining customer requirements with regard to service and contract set up through to contract execution should therefore be contained within the quality manual.

The method of reporting results to customers should be clearly defined and enable results which require immediate remedial action to be communicated without delay to appropriate persons. Records relating to laboratory results should be kept for as long as is necessary to comply with requirements for archive and audit trails. These should include, for example; the date, place and time of sampling, the members of staff undertaking the sampling and analysis, the test result with appropriate units and a reference to the methodologies used along with full details of testing.

The quality management system can only be successful if all constituent parts are well documented, understood and supported by staff. The manual should provide policies covering all activities of the laboratory and requires periodic review.

The information that is produced by all areas of the quality management system, for example the results of the audit process, should feed into the laboratory planning system and should include goals, objectives and action plans for the coming year. This information is usually assessed by top management of the organisation and other staff as appropriate, during the management review meeting, which is usually held on an annual basis.

3 Laboratory staff

The nature of microbiological testing requires that the work should be performed by or under the supervision of an experienced person qualified in microbiology.

Laboratories should have a documented policy, and associated procedures, that detail staff responsibilities, training and on-going competency assessment. All laboratory staff

should have training records that detail relevant education, qualifications, training received, on-going competency and experience acquired.

New employees should be made aware of key laboratory hygiene practices that are very important in minimising the risks of infection when handling samples or cultures. These include requirements for wearing of laboratory coats, the need for hand washing and personal hygiene, disinfection of laboratory work surfaces and cleaning up of spillages and basic aseptic techniques. These practices should be observed and maintained at all times.

Analytical staff should be trained in the principles and rationale of the tests being conducted in addition to receiving training in each analytical method. Training in ancillary techniques and the operation of major items of equipment should also be described and recorded.

Wherever possible, staff should be encouraged to broaden their understanding and to make contact with people from similar organisations, including participation at appropriate meetings, seminars and conferences. It is important that staff should understand the principles of the tests being conducted, the reasons why they are carried out and the significance of results.

3.1 Staff training and records

Staff training records should show appropriate training for each documented method where training has been given, including training in the use of major items of equipment and basic microbiological techniques. Evidence that training has been both adequate and successful should be documented in training records.

New analysts under training should be supervised during any analysis, counting and recording of results performed. Any counting by a trainee should be carefully checked by a competent analyst.

Assessment of successful training may involve staff analysing external quality assurance samples where their data can be compared with data from other analysts or laboratories. Alternatively, for water analysis, spiked or raw water samples, containing low numbers of target organisms (for drinking water) or higher numbers requiring dilution (for environmental waters), may be used provided that replicate samples are analysed in parallel by a fully trained member of staff. In order to demonstrate satisfactory performance, an appropriate number of replicates containing the target organism should be analysed using the full analytical procedure so as to provide statistical confidence in the assessment. There should be no significant difference between the results obtained by the two analysts. Details of the comparisons of the test results should also be documented in the training record. Further guidance on the criteria for assessment of competency is given by the UK Drinking Water Inspectorate [(5)].

Following the successful delivery of training, laboratory management can authorise staff to perform particular test methods and this is generally documented within the individuals 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 On-going competency and development

Training records should be reviewed regularly to ensure completeness and to identify any training needs for an analyst. On-going competency of trained analysts may be assessed by performance in appropriate internal quality control testing including: method demonstration during internal audits, duplicate split samples, spike recovery testing, externally sourced reference preparations and, where available, an external proficiency testing scheme. Failure by an analyst to perform satisfactorily should lead to a thorough investigation including both the adequacy of, and response to, the training received.

Where appropriate, on-going professional development should be encouraged and include attendance at appropriate meetings, workshops, seminars, training courses and similar events. A record of such events should be maintained and kept up to date by the analyst to demonstrate their continuing professional development.

4 General laboratory environment

4.1 Laboratory Organisation

The nature of microbiological examinations places requirements, particularly in the context of health and safety, on the design and organisation of the laboratory space. These include the progression of samples and materials through the laboratory, controlling ventilation, facilitating good microbiological practice and hygiene and managing contaminated materials. In many cases consideration should be given to restricting access to authorised personnel and supervised visitors only.

The laboratory environment for the microbiological examination of water should comply with guidelines(6,7,8) 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. Floors and work surfaces should be easy to clean and cleaning should be undertaken frequently. Work surfaces should be disinfected often and immediately if contamination is known to have occurred. Laboratories where micro-organisms in category 3 containment level are intentionally sought and isolated (for example S*almonella* Typhi) need to comply with separate and additional requirements(7) including security.

Whilst laboratory-acquired infection is rare, staff should be adequately trained in good microbiological practice including aseptic technique and the prevention of infection, not only to themselves but also to their colleagues. Training should include the understanding of risks from micro-organisms associated with ingestion, inhalation and skin absorption. Further guidance is given elsewhere (6,8,9,10).

4.2 Environmental monitoring

The ubiquitous nature of most of the microbes of interest makes it essential to ensure that any organisms that are detected have originated from the sample being analysed and have not been introduced inadvertently during sampling or subsequent testing. It is also essential to protect laboratory users from any pathogenic microbes likely to be found in samples.

Laboratories should therefore consider appropriate microbiological monitoring of the environment, relating both to the sampling procedure and the analysis within the laboratory. The objective of this monitoring should be to ensure that the working environment meets suitable standards for hygiene. Such standards are defined to minimise the risk of cross contamination of samples and protect the health and safety of laboratory employees. More detailed information on environmental monitoring can be found elsewhere[11].

The environmental monitoring programme should be designed to provide feedback about the efficacy of cleaning regimes including the disinfection of work surfaces and equipment. It should be relevant to the sample matrices and organisms being sought as well as the conditions under which the analysis is being performed. There are a number of techniques used within an appropriate programme to monitor both the air and surfaces. These include:

- Air sampling devices
- ‘Settle’ plates (Air settlement plates)
- Contact plates
- Surface swabs

Dependent on the work being carried out by the laboratory and the monitoring strategy adopted these techniques are used in conjunction with non-selective and selective agar media to determine when and where contamination of samples and the working environment may have occurred.

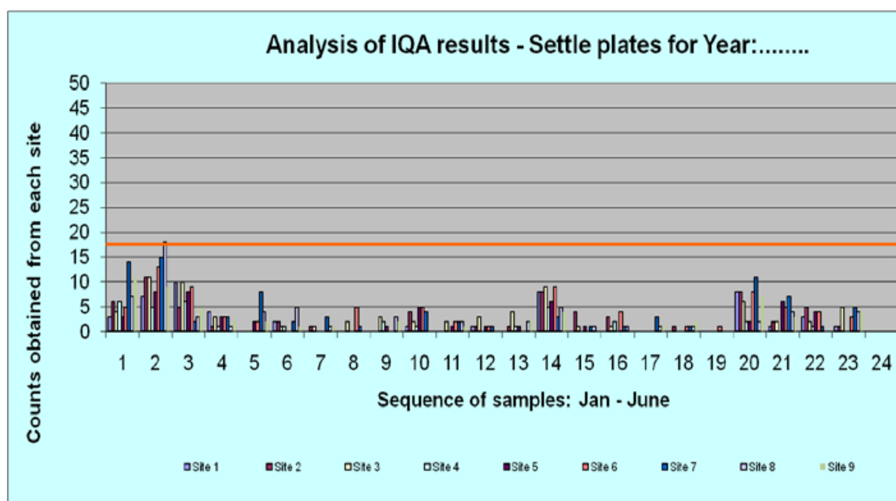
Settle and contact plates should be sterile and quality controlled before use. They should be checked visually for any sign of deterioration or contamination before use. The plates should be located so as to be relevant to the testing activities being performed but without interfering with them. The conditions of plate exposure and exposure time should ideally reflect those perceived to present the greatest risk of contamination, with testing activities in progress, and have regard to the potential deterioration or drying out of the plate during exposure. After exposure the plates should be incubated at temperatures and for times appropriate to the tests performed and the organisms of concern.

Environmental monitoring is not a replacement for the routine practice of aseptic technique or good hygiene and cleaning practices. Monitoring provides a means of verifying the effectiveness of these activities and an alert mechanism when changes have occurred and improvements are necessary. The emphasis should be on maintaining the environment, work surfaces and equipment to a suitable standard defined within the laboratory.

The monitoring programme should be sufficiently frequent to establish background counts, and be designed to demonstrate compliance with laboratory defined acceptable levels based on experience and appropriate to the scope and type of analysis performed. Trigger levels should be set for initiating further investigation and remedial action such as cleaning and disinfection where appropriate. The conclusions of the investigation may then be used to review and amend routine hygiene and cleaning practices and the environmental monitoring strategy.

Records should be kept of all the environmental monitoring undertaken and the results should be reviewed regularly. Laboratories should consider the use of guidance charts (see Figure 4.2) which may aid the interpretation of results and facilitate the identification of trends or patterns of contamination.

Figure 4.2 An example guidance chart:



4.3 Management and disposal of waste

Laboratories should have clear policies for the handling and segregation of waste and contaminated materials and equipment. Contaminated materials and waste cultures should be kept separate from preparation and testing areas. They should be discarded to suitable, labelled, receptacles which should not be overfilled. Consideration should be given to the categorisation and labelling of waste and the use of an appropriate recognised colour coding system.

ISO 14001[12] contains information on environmental and waste management which may be of use to laboratories in formulating their own policies and procedures. In the UK, guidance on the management of healthcare waste has been provided by the Department of Health[13,14] including application to laboratory facilities such as those testing environmental samples. In general, unless a laboratory is involved with testing clinical specimens or dealing with category 3 containment level organisms, it is usually sufficient to autoclave the material and dispose of the suitably bagged residue along with general laboratory waste.

Alternatively, it can be disposed of as offensive/hygiene waste, category code 18 01 04, 18 01 03 or equivalent in the appropriate colour coded waste bags. Depending on local policy it can also be described as ‘autoclaved laboratory waste’ and disposed of either by incineration or to non-hazardous landfill. It can also be sent for incineration without prior autoclaving.

5 Laboratory equipment

In accordance with good laboratory practice it is important that all equipment is verified as being fit for purpose and installed so as to facilitate operation. All equipment should be clearly identified and uniquely labelled so that comprehensive records can be kept of all relevant information and data allowing it to be retrieved quickly when necessary. Equipment should be kept clean and checked regularly for correct operation, as detailed in sections below. Any spillages should be cleaned up immediately. Equipment should be maintained according to manufacturer's instructions to ensure safety and reliability.

Items of equipment that are critical to measurements and analytical performance should be catalogued and include records of, for example, the date of purchase, the name of the supplier, the frequency of servicing and calibration, and, where appropriate, the location of instruction manuals. Examples of the type of equipment typically covered would be; incubators, water baths, autoclaves, refrigerators and microscopes. Service records of such equipment should be stored and include reports and details of any calibration carried out on the equipment. Details of equipment faults, modifications, repairs and upgrades should also be kept.

Equipment used for measurements or where specifications are important should be calibrated to ensure the appropriate degree of accuracy and reliability demanded for the analysis performed. Laboratories should have in place documented procedures for the calibration of all equipment involving, for example, recording weight, volume, temperature or time. Calibration equipment and standards used for monitoring of calibration for such equipment, for example thermometers, may include certified standards, and should include certification traceable to national standards. Certified standards need not be used routinely, but should be used to calibrate uncertified working standards to a regular programme. Certified standards and equipment used for this purpose (laboratory reference standards) should never be used for any other purpose. Once initially calibrated all certified standards, whether working or reference, should also be programmed for regular recalibration to national standards or replacement. In the case of reference standards this should always be carried out by a competent calibrating laboratory. Records of calibration and maintenance should be securely maintained.

5.1 Autoclaves

The principle of sterilisation to destroy micro-organisms by autoclaving is based on moist heat transfer. Autoclaving is used to sterilise media, bottles and other equipment used in microbiological analysis. 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 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 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 placed 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 should be equipped with at least one safety valve, a drain cock, temperature regulation device, timer, temperature probe and recorder. A safety/thermal

lock is usually activated at temperatures above 80°C. Autoclaves are pressure vessels and are subject to annual inspections for safety and insurance purposes.

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 and performance of laboratory autoclaves is given elsewhere[14,15].

For both autoclaves and media preparators (see 5.15 below) it is important that the correct time and temperature are achieved during each sterilisation cycle and that these are monitored and recorded. Details of the load, operator's identity and batch number, where appropriate, may also be recorded and retained. Each operating cycle and load configuration should have a performance validation undertaken initially and after significant repair or modification and all data recorded and stored. This may also be repeated at set intervals and can be achieved, for example using a multi-point thermocouple calibration procedure traceable to national standards.

Many media require a sterilisation cycle of 121°C for 15 minutes, although 115°C for 10 minutes and other cycles are also used. Sterilisation cycles for other materials may require a different holding time. The target temperature and time should have defined limits; typically the target temperature should be within +/- 3°C and the target time within +/-3 minutes for a 15 minute cycle. Autoclaves should not be overloaded and the loading pattern should not restrict the free passage of steam around the contents of the chamber.

The internal temperature of the autoclave/preparator should be established and verified during a sterilisation cycle using thermocouples. These should be calibrated to national standards and details of each cycle of the autoclave should be recorded, together with the contents of the unit. A temperature cycle or sterilisation time-temperature record provides an audit trail to show the time/temperature used. Whether these are satisfactory can only be shown by subsequent tests for sterility. Individual autoclave loads can, in addition, be marked with heat-indicating tape to demonstrate that they have been subject to a moist heat process. Other heat treatment indicators, for example Brownes tubes, and spore tests may also be used. Spore tests are typically purchased as preparations of *Geobacillus stearothermophilus* spores which are resistant to heat, in vials containing the spores suspended in an indicating growth medium. If the sterilisation cycle results in the kill of these spores, then complete sterilisation has been achieved. (On cycles at temperature/times less than 121°C for 15 minutes this may not result in the total destruction of spores in all cases).Other types of spore tests are also available. Results of these tests and other evidence of sterilisation efficacy may be recorded and retained.

Different types of loads such as contaminated materials 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. Some autoclaves have a holding temperature to keep agar molten if it is not possible to unload soon after the cycle has finished. It is not good practice though to hold prepared agar media for long as this can change the state of some ingredients and thereby the properties of the medium.

5.2 Balances and Gravimetric Devices

Balances are generally used to weigh out components of culture media and test portions of samples. They may also be used for gravimetric checks of pipettes, pipettors, dispensers, etc. Other gravimetric devices may include gravimetric diluters consisting of a balance and programmable dispenser that can prepare dilutions and moisture analysers used to determine the moisture content of a sample.

Weighing devices should possess a sensitivity that is appropriate for the substance being weighed. They should always be kept clean and serviced at pre-determined frequencies. They should be located in a suitable position on a level surface 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, an approach appropriate to the application should be taken. In many cases accuracy to at least ± 0.001 g is sufficient but accuracy to at least ± 0.0001 g may be necessary in specific instances. Verification of performance should be determined by using a range of calibrated weights, traceable to national standards, appropriate for the balance in use at least once a year. The permissible error will vary depending on the weight used and purpose for which the device is used. Further guidance on calibration of balances and weighing machines is given in UKAS publication 14(16). Calibration checks using working standard weights should be undertaken on a regular basis, for example daily or weekly, depending on use of the device. Continuity of calibration should also be demonstrated immediately following maintenance, relocation (including moving and replacing) or accidental movement of the balance. Balances and other gravimetric devices not within specified tolerances should not be used until re-calibrated.

5.3 Centrifuges

Centrifuges provide a means of separating substances of different density by centrifugal force. In microbiology laboratories they are frequently used for the separation of micro-organisms, including algae, from their surrounding fluid.

Bench top models are generally used in the microbiology laboratory and employ speeds in the range of 200-6000 rpm and different volume capacities. Micro-centrifuges are also available for handling bacterial cultures and accommodate Eppendorf tubes which are used in *Cryptosporidium* and *Giardia* analyses. If the speed, time and temperature of centrifuging are crucial to a method these should be independently verified at least annually or after significant repair or modification. Centrifugal force is determined by speed and rotor diameter, this is usually quoted in terms of rcf- relative centrifugal force.

It is important that centrifuge tubes and their contents are equally balanced and rubber cushions (where required) are placed in buckets before use. Prevention of aerosol generation and cross-contamination by correct operation of the equipment is essential. Centrifuges should be cleaned and disinfected regularly especially after any spillage or breakage and be well maintained and serviced and records kept.

5.4 Colony counting devices

These may be manual units or automated electronic devices.

5.4.1 Manual counting

Tally counters can be used for simple manual counting either separate from or in association with marker pens. Many manual counters use an illuminated contact operated grid surface with an audible indication and digital readout. A magnifying screen aids colony detection. At least annually, the calibration of the tally counter should be checked and the result recorded. As an example this can be achieved by using a 'standardised' plate if available or creating a reference plate with a known number of coloured dots (for example 25 to 75) simulating colonies on the back of a petri dish. This may be used to ensure that the reader is not over or under sensitive and that the digital readout is functioning correctly. The plate should be counted by at least two analysts, for example using different coloured marker pens, and there should be no difference for either analyst compared to the known initial count.

5.4.2 Automated electronic counting devices

Automated counters may be sophisticated image analysers which use a camera detection device connected to software that calculates the numbers of colonies present on a plate. The manufacturer's instructions for set up and use should be carefully followed. Sensitivity can usually be adjusted manually to ensure all target colonies are counted. A compromise usually has to be reached for counting very small colonies to avoid the unit 'counting' air bubbles or imperfections in the agar plate. Each type of agar plate should be set up and verified to ensure adequate discrimination of target colonies. All units must be kept as clean and free of dust as possible and avoid scratching surfaces that are essential to the counting process.

Although calibration plates, with a known number of countable particles present (for example 0, 1, 20, 100, 250), may be available it is usually better to compare an agreed manual count (for at least two analysts) to the count an automated instrument produces. Checks should be performed with these plates in addition to blank plates.

5.5 Dry Heat Sterilising Ovens

A sterilising oven uses a temperature of around 160-180°C to destroy bacteria and other micro-organisms by dry heat. Glass and metal ware are generally sterilised by this method as the temperature employed makes it unsuitable for many other materials. All metal and glassware should be clean before placing in the oven. The sterility of these items can be maintained on removal by putting them in suitable canisters or wrapping items individually or in batches in foil or craft paper. The oven should be equipped with a thermostat, temperature recorder and timing device. When the oven reaches temperature it is usually held for one hour. Details (date, time, temperature setting, sterilisation time, oven contents and batch number where appropriate) of each load should be documented and maintained. The temperature controlling system should be calibrated to national standards. Steriliser control tubes are available that change colour to give a visual indication that the correct temperature has been achieved. These can be placed throughout the load. After sterilisation, glassware should be allowed to cool in the oven before removal.

5.6 Filtration systems

Membrane filtration is a technique that is frequently employed in water microbiology for capturing bacteria in a liquid sample. Membrane filters having a pore size of 0.45μm are

often suitable and are the most frequently used but for some bacteria such as *Campylobacter* and *Legionella* a pore size of 0.2µm is required.

In addition to filtration manifolds a vacuum source is required and a receiver to collect filtered water. This can consist of a fairly simple set up, up to a large plumbed-in commercial system with automated emptying of the water reservoir. Systems need to be well maintained and kept clean according to manufacturer's instructions. It is recommended that back-up systems are available in case of break down. The vacuum source should not exceed 70 kPa [17] to avoid damaging the membranes and compromising their porosity and performance.

Filtration funnels should be free from cracks and have visible calibration marks at appropriate intervals for the range of volumes typically filtered. Calibration checks to verify volumes should be carried out on a random selection of funnels at regular intervals. Funnels are sterilised before each use by autoclaving or disinfected between uses by boiling, steaming or other means suitable for the application, for example ultraviolet irradiation. Alternatively, pre-sterilised, single use units may be used.

5.7 Flow Cytometry

Flow cytometry has numerous applications but in the context of water microbiology, it is a method by which suspensions of cells (for example bacteria or cryptosporidium oocysts) can be accurately enumerated and if required, separated out into known concentrations by particle characteristics (cell sorting). Cells can be fluorescently stained to identify distinguishing characteristics allowing analysis of communities of micro-organisms or categorised as to whether the cells are intact with implications for whether they are 'live' or dead cells.

Cells are guided to an 'interrogation point' within a flow cell where the stream of fluid is so narrow that the cells move in single file. The cells pass 'interrogation points' where laser light is applied and scattered in response to fluorochromes applied to the cells. The light response generated is converted into an electrical signal by a photodiode or photomultiplier tube. The electronic signals are proportional to the amount of light detected and displayed using analysis software within the flow cytometer. The cells are displayed as scatter pattern on a graph with cells with similar properties, for example size or fluorescence signal, appearing as clusters. A definitive cell concentration is also calculated.

5.8 Gas burners

Gas (Bunsen) burners have been used in microbiology laboratories from the earliest days to sterilise metal loops or straight wires and to flame necks of bottles and tubes as part of aseptic technique.

Gas burners produce a narrow naked flame using either mains or bottled gas. The type of flame produced can be achieved by varying the gas/air mixture by the means of a collar at the base of the burner.

As flaming loops can cause splatter disposable plastic loops may be used instead. (There are also advantages in terms of speed and efficiency as well as health and safety reasons for using plastic loops). In protective cabinets the use of burners should be avoided. Pipework and connections should be checked regularly. Gas detection devices are available to detect leaks.

5.9 Glassware

All items of glassware, such as pipettes, flasks, beakers and Petri dishes etc., used in the preparation of media or handling of samples should be of suitable quality and not cracked, chipped or broken. They should also be free from inhibitory substances, adequately cleaned, and when appropriate, sterilised before use.

Pipettes can be placed in canisters and other materials wrapped in special paper (such as craft paper) or foil, but generally access to free steam should be allowed to ensure sterilisation. Dry heat sterilisation in an oven can also be used.

Glassware should be stored in such a way as to protect against dust and breakage and, if sterilised, protected to maintain its cleanliness. In many instances, pre-sterilised plastic items provide an acceptable alternative.

The accuracy of volumetric equipment should be appropriate to the application and traceable to national standards. Class A glassware conforming to BS EN ISO 4788 is preferred where the accuracy is specified in the method(18). Calibrated glassware should never be heat sterilised as this invalidates the calibration. Any calibrated glassware that is subject to significant temperature change should have its calibration verified before it is used.

5.10 Glass washers

Many different types of electronically controlled glass washers are available for washing general laboratory glassware and bottles. Because washers subject glassware to physical and temperature stress, they are not suitable for cleaning calibrated glassware.

Some units can incorporate a purified water or acid/alkali rinse stage. Different cleaning agents can be used, the choice determined by the type of material being washed and the degree of soiling. All machines should be installed and serviced according to manufacturers' instructions.

The efficacy of cleaning is usually checked by visual inspection but if an acid/alkali rinse stage has been used a pH check may also be appropriate.

5.11 Hotplates and heating mantles

Hotplates and heating mantles are thermostatically controlled heating devices and may incorporate magnetic stirring units. They may be of ceramic, glass halogen or other design. They are used to prepare volumes of culture media and reagents.

Care should be exercised to ensure that only the appropriate quality of glassware is used on these units. They should have good heat resilience and be of robust construction with no chips or cracks (see section 5.9). Also ensure, even if stirring units are used, that localised charring does not occur at the base of a flask where solid media is not properly mixed with water.

Any spillages should be cleaned up as soon as the unit is cool. Units should be clearly signed to alert to the danger when still hot.

5.12 Immunomagnetic separators

Commercial units are available which are used to separate and concentrate target micro-organisms in liquid cultures by means of paramagnetic beads coated with an appropriate antibody.

Manual and automated separators are available. Manual units consist of a rotary mixer and particle concentrator with removable magnetic bar. Automatic systems perform the whole operation in an enclosed environment.

All equipment should be clean and free from inhibitory or interfering substances.

5.13 Incubators

Incubators are temperature controlled insulated cabinets and are available in many sizes with, or without, internal fan assisted circulation to provide a more even temperature distribution inside the cabinet. The inside of the incubator should be made of material that facilitates easy cleaning for example stainless steel. A glass or perspex inner door helps to minimise temperature loss whilst the main door is opened, for example for viewing the contents of the incubator. If the ambient temperature is close to or higher than that of the incubator, it is necessary that the unit has a cooling system in addition to a heating system to achieve the required temperature. This is usually required, for example for incubators maintained at 22 °C. Incubators sited in draughts, bright sunlight or other locations where environmental temperature fluctuations occur may not be able to maintain temperature adequately. A temperature-controlled environment may be needed to maintain tight temperature tolerances in incubators.

Specific maintenance and servicing arrangements are not usually required but units should be cleaned and disinfected inside and out regularly and particularly following any culture spillage. The approach to cleaning the inside of incubator chambers and fridge cabinets is similar. The inside may first be cleaned with warm tap water followed by liberally spraying all internal surfaces with a fresh solution of, for example, dilute sodium hypochlorite. It may be advantageous to alternate between two different disinfectants. If a suitable cleaning regime is followed it may only be necessary to use the disinfectant spray. The surfaces should be wiped dry immediately with absorbent paper towel. The disinfectant should leave no residue inside the chamber. For verification of cleanliness, if required, an appropriate programme of swab testing of internal surfaces may be undertaken, with swabs tested for a suitable range of bacteria and limits applied to the levels found for acceptance or triggering additional cleaning and disinfection.

Incubation chambers should not be over-loaded, the pattern of loading can markedly affect heat distribution, and thereby temperature, around the chamber and for example within stacks of plates and trays or in secondary containers such as jars or boxes.

The temperature of the incubator should be measured at regular intervals. The minimum number of readings that should be taken includes one at the beginning of the working day, before cultures have been removed, and one at the end of the working day or when samples are placed in the incubator, (these checks are particularly important with temperature cycling incubators), using a calibrated thermometer or temperature measuring device. An integral temperature display can only be used if its accuracy has been verified. Continuous temperature monitoring (with associated alarm systems) of the internal environment provides a complete appraisal of incubator performance, particularly for incubators with temperature cycling. On cycling incubators the rise in temperature from 30

°C to 37 °C or 44 °C should occur within 30 minutes and the time is counted as part of the higher incubation time. Monitoring throughout the incubation cycle allows a realistic assessment of temperature fluctuations within the incubator. Whether fan-assisted or not, it is important that an even temperature distribution is established within the incubator. This can be assessed by placing thermometers, or temperature recording devices, in different parts of the incubator over a period of time, for example over a 24h period, and recording the temperatures at regular intervals. This can also be achieved by using a multipoint instrument that is traceable to national standards. The temperature profile of the incubator should show no significant differences wherever the temperature measuring devices are placed.

A loading pattern should be established and any unusually hot or cold areas within the incubator identified. Such areas should be avoided as far as possible and designated as places where plates, etc. should not be incubated. Repeat profiling should be undertaken at regular pre-scheduled intervals and when the incubator is moved to another location or following repair.

The temperature distribution may also depend upon the manner in which the incubator is loaded. For example, stacking Petri dishes to greater than six dishes may affect the temperature distribution and result in the temperature profile of individual dishes being variable. Correct incubator temperature control is vital for the satisfactory performance of microbiological enumeration and detection. Maximum fluctuation around a given temperature for an incubator and thermometers and temperature measuring devices generally are described in section 5.26.

5.14 Media and reagent dispensers

There are a variety of devices that are employed to dispense culture media and reagents to tubes, bottles or plates. These range from calibrated pipettes, syringes and glassware to peristaltic pumps and programmable electronic devices with variable automated delivery.

All equipment must be clean and fit for purpose both in terms of volume delivered and suitability to the matrix being dispensed. For aseptic distribution of sterile culture media the parts of the equipment that will come into contact with the medium must be sterile. It is good practice to have separate tubing sets for selective media to minimise chances of tainting or carryover of inhibitory substances.

The dispensing equipment must be calibrated either before use or at regular intervals and in each case if a change in volume is made. The accuracy of the volume being dispensed needs to be determined in proportion to the volume being dispensed, in general it should not exceed +/- 5% (for volumes of 5 ml or greater).

5.15 Media Preparators

Media preparators operate on similar principals to autoclaves and are specially designed sterilising devices used to prepare larger volumes of media (>1 litre). Media preparators are stand-alone devices that allow controlled preparation, sterilisation, cooling and dispensing of culture media with minimal operator involvement. Advantages of such equipment include thorough mixing of the components during preparation, short heating and cooling stages which minimize denaturation of ingredients, improved safety for workers as handling of hot glassware is avoided and improved consistency of finished media. Like autoclaves they have a heating vessel, temperature and pressure gauge,

timer and safety valve. They are also fitted with a continuous stirring device. The entire process takes place within the sterilising unit once media ingredients and water have been added. Once started, the machine will heat the contents of the chamber to the target temperature whilst mixing. The medium is then held at this value for the specified duration of the sterilisation phase. After sterilisation is complete the instrument will enter the cooling phase and quickly bring the contents down to around 50 °C. The media preparator will then hold this temperature for the duration of the dispensing phase. At this stage additives or supplements may be aseptically added through the filling port. Addition at this stage ensures that heat-labile supplements are not deactivated and because the machine continues to mix, ensures homogeneity in the finished medium. A specially designed pouring and stacking unit may be used in conjunction with the sterilisation unit to aseptically dispense media to Petri dishes. The finished medium is usually dispensed by fitting a clean sterile dispensing tube to the integral peristaltic pump. It is good practice to have separate tubing sets for selective media to minimise chances of carryover of inhibitory substances. Spare sterile tubing sets should be kept bagged or wrapped in autoclave paper ready for use. Foil wrapping the connectors and dispensing nozzle may help to prevent contamination when fitting to the pump and stacker module. Many nozzle sets include a sliding sleeve to achieve this. To dispense the medium the tubing must first be primed and then calibrated to deliver the required volume per plate or bottle. Once dispensed, media may be allowed to remain on the stacker carousel until solidified; after which it should be promptly removed and stored as described in 6.8.

Records for each cycle and performance criteria must be maintained as described in 5.2 above.

Many media preparators have UV lamps which operate when dispensing to provide some protection from contamination. It is important that the equipment is kept very clean and that spills are cleaned up after each use. Tubing sets should be rinsed well with hot water to flush out any residual medium before bagging and re-autoclaving. The mixing chamber and stirrer should also be thoroughly rinsed and cleaned after every use.

Before placing into service, media preparators should be validated for typical runs by an accredited engineer. Preparators should be regularly serviced and have an annual calibration which is traceable to national standards and all data recorded and stored. Media preparators are pressure vessels and like autoclaves, are subject to annual safety inspections.

5.16 Microscopes (optical)

Microscopes are used for the detailed study of material too small to be seen with the eye. Such study may include the examination of sediments or colony morphology on agar plates, enabling of counting of very small colonies, performing counting and identification of algae and intestinal parasites or viewing Gram stained slides. There are many types of microscope including stereoscopic, inverted and immunofluorescent microscopes.

The modern microscope has a number of easily identified parts many of which require optimisation if the microscope is to work correctly. The light source is usually a tungsten filament bulb at the base of the instrument to provide a constant source of light. The intensity of light can be controlled by a rheostat. The base of the light source usually has an iris diaphragm to vary the amount of light that reaches the condenser. The condenser, situated beneath the stage, contains lenses which allow light to be focused onto the specimen. The condenser contains two screws to permit it to be centred, and it can be focused up and down. It also has a diaphragm. The stage is the part where the specimen

rests. It usually contains a clip to hold the slide in place and a rack and pinion system to permit the slide to be moved in the x and y axes. Both axes have a micrometer to permit the user to take a positional reading during scanning to enable the user to go back and find objects of interest.

The magnification is achieved by two lenses. The first of these is located in a rotating 'nosepiece' and is called the objective lens. This gathers light from the specimen. A number of objectives are usually screwed into the 'nosepiece'. These can range in magnification from none at all to x 2, x 4, x 10 x, x 20, x 40 and x 100. The higher magnification objectives may be of a water or oil immersion type. The magnification is usually inscribed on each lens. In the binocular microscope the light from the objective lens is split by prisms to two eyepiece lenses. These usually have a magnification of x 10 and one may contain an eyepiece graticule to facilitate counting, or to allow measurement of the size of objects. The total magnification of the microscope is calculated by multiplying the magnification of the objective and eyepiece lenses.

Most microscopes contain two focus knobs. The coarse focus is used to bring the objective lens into the focal plane of the specimen and the fine focus is used to make the image sharp. In binocular microscopes, the inter-pupillary distance can be set by moving the eyepieces towards or away from each other. This enables the user to see a single image from the two eyepieces. With the image focused with one eyepiece, it is usually possible to adjust the other eyepiece by focusing up and down to give a clear image with both eyes.

Many objects, for example cells, contain water. When they are suspended in water they are difficult to see by bright field illumination. The contrast between the object and the fluid it is suspended in can be increased by modifying the light as it passes through the microscope. Dark field condensers produce a hollow cone of light which, under normal circumstances, does not enter the condenser. When a refractable object, for example a bacterium, enters the light path, the specimen appears intensely illuminated against a black background. In phase contrast microscopy, annular rings in the objective and the condenser separate the light into different phases. The light that travels through the central part of the light path is then combined with the light that travels round the periphery of the specimen. The interference produced by these two paths produces images in which the dense structure appears darker than the background. Objectives with annular rings can also be used for bright field microscopy. Differential interference contrast (DIC) uses polarising filters and prisms to separate and recombine the light paths giving a 3-dimensional appearance to the specimen. One of these systems is essential if unstained specimens are to be examined.

An incident light fluorescence microscope uses a shorter wavelength of light (usually ultra-violet light) to illuminate the object. Some parts of the object change the wavelength of the light to a longer wavelength in the visible light spectrum. Alternatively, a sample can be stained with a specific stain which achieves the same objective. These stains which absorb light of one wavelength and emit it at a longer wavelength are called fluorochromes. The light source is usually a high pressure mercury vapour or xenon lamp, however light emitting diode (LED) lamps are now available, these do not contain mercury and are gaining in popularity due to their energy efficiency and extended lamp life. In epifluorescence, the light which is produced is focused by the objective onto the specimen. The wavelengths of visible light which are produced travel back through the objective to the eyepiece. Filters within the microscope are used to generate light of a specific wavelength. These are called exciter filters. A dichroic mirror is used to reflect this light onto the specimen. The dichroic mirror allows the longer wavelength light from the sample

to pass back up the microscope. Unwanted UV light is then removed by a barrier filter to prevent it reaching the users eyes. Fluorochromes can be used to stain micro-organisms. Alternatively, the fluorochromes can be conjugated with proteins, for example antibodies. In this way *Cryptosporidium* can be stained and rendered visible.

When microscopes providing 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 bulb can explode during replacement. Correct disposal routes for bulbs should also be used. Direct contact between bulbs and fingers should be avoided. This minimises contamination or etching of the glass which would shorten the life of the bulb. Great care should also be taken not to scratch or otherwise damage glass optics.

5.16.1 Centring the light source and Kohler illumination

To centre the light source, the condenser is placed as close to the stage as possible. A sample slide is placed on the stage and a low power objective, for example x 5 or x 10, is used to focus on a sample. The lamp iris is reduced until it is minimal then the condenser is focused to bring the edges of the iris into sharp relief. The condenser is then centred using the two screws positioned on either side of the condenser until the light appears to be in the middle of the field of view. The lamp iris is opened until the edges just touch the outer field of view and any finer adjustments necessary are made using the centring screws. The lamp iris is now opened until it is just outside the field of view. The extent to which the lamp iris is opened relates to the objective lens that will be used for examination of the specimen. The process should be repeated, for example when assessing a slide for Cryptosporidium oocysts using the x100 objective for DIC microscopy. This will minimise exposure of the specimen to intense light.

The condenser iris may be adjusted to increase or decrease the image contrast. Once this is set the microscope has Kohler illumination. Specimen contrast is controlled by adjusting the condenser iris and light intensity by adjusting the rheostat on the lamp housing.

5.16.2 Calibration

Objects viewed under a microscope can be measured to determine their size. Such measurements are done by using a graticule inserted into one of the eyepieces. This is a measuring scale placed in the eye-piece which is usually sub-divided into 100 units. The graticule can be calibrated using a stage micrometer. This allows the microscopist to determine the size of the eyepiece units by comparing them with a scale on the stage micrometer which is of known length. The microscope should be calibrated for each of the magnifications normally used for measuring. The stage micrometer usually contains a ruled length of 1 mm (1000 μm). The ruled length is divided into 100 units, numbered from 0 to 100, each measuring 10 μm. If the eyepiece graticule being used can be focused independently of the eyepiece, this should be undertaken prior to the calibration.

The stage micrometer is placed on the microscope stage, the transmitted light turned on and the microscope focused on the micrometer image. Using the times 10 objective first, the microscope stage and the eyepiece are adjusted so that the zero line on the eyepiece graticule is exactly superimposed on the zero line of the stage micrometer. Without changing the stage adjustment, a point is found as distant as possible from the two zero lines where a line on the eyepiece graticule is again superimposed exactly on a line on the stage micrometer. The number of divisions on the eyepiece graticule and the number of divisions on the stage micrometer between the two points of superimposition is

determined. If, for example, 100 divisions on the eyepiece graticule measure 100 divisions (1000 µm) on the slide graticule, then one division on the eyepiece graticule measures 10 µm. This is usually the case for the x 10 objective.

The procedure is followed for each objective. For example, with a x 20 objective, 1 eyepiece graticule calibrates to 5 µm and a with a x 100 objective, 1 eyepiece graticule calibrates to 1 µm. Calibration information should be recorded and kept with the microscope. The microscope should be calibrated at regular intervals, for example, annually. The microscope calibration should remain constant. If the calibration were to change, the reason for this should be investigated.

5.16.3 Care of the microscope

Microscopes perform efficiently only when serviced regularly, at a frequency depending on usage and when correctly aligned [(19)]. They should be protected from environmental contamination and used and set up according to manufacturer's instructions. Details of servicing, including adjustments, replacement components and modifications should be recorded and the records maintained and stored. When not in use, the microscope should be protected with a dust cover to prevent optical surfaces from dust and other contaminants that might affect their performance. In addition, the optics and stage should be cleaned with lens tissue after use.

5.17 Microwave ovens

Microwave ovens heat by using microwave energy and 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 must always have an adequate headspace to allow expansion of contents without overflowing.

Ovens fitted with a turntable can achieve better heat distribution. It is therefore important to establish, for each media type to be processed in a microwave, the power setting, time and number of bottles to be processed. These standard processing times and heat settings should also be verified to ensure that the performance of medium is not impaired.

Microwave ovens should always be kept 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. (Self-check devices are available but a recognised service engineer should be employed especially for high wattage devices).

5.18 Modified atmosphere incubation equipment

Traditionally gas jars that can be sealed and that use commercially available gas generating packs to produce an anaerobic or micro-aerobic environment have been used. Systems are now available using sealed bags or other similar commercial products. These are suitable for the incubation of small numbers of petri dishes or similar items. For larger

quantities anaerobic cabinets and incubators are available. Commercial air-tight containers may be suitable providing they are of the correct volume for the gas generating pack (typically 2.5 or 3.5 litres).

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. Older systems employed a catalyst which combined hydrogen with oxygen to produce water. Hydrogen was generated by adding water to a pouch containing sodium borohydride. Commercial (catalyst-free) gas generating paper sachets are now available. These use a selection of chemicals to remove oxygen and generate carbon dioxide. They are supplied sealed in packets and once the packet is opened, the reaction starts. Bottles and plates should be placed in the jar first followed by the anaerobic indicator (see below) before the packet is opened and the sachet is added. The reaction generates heat and condensation may appear on the inside of the jar. Great care should be taken to ensure the correct size of sachet for the appropriate volume of jar is used. Anaerobic jars should be cleaned after use and when contamination is suspected. Similar sachets are available for the generation of a micro-aerobic atmosphere for the isolation of *Campylobacter*.

Cultures should be stacked loosely in the jar. Suitably vented petri dishes should be used (see section 6.7). These should be dried before use to prevent moisture collecting and inhibiting circulation. The caps of screw-topped containers should be loose enough to allow gas equilibration with the jar atmosphere. 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 QC 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 strip changes colour and the bacterial cultures show that suitable internal atmospheric conditions have been achieved. Before use, new batches of generators should be performance tested with appropriate anaerobic or micro-aerobic organisms.

Larger anaerobic cabinets should be operated according to manufacturer’s instructions and serviced at regular intervals.

In general anaerobic incubation requires an atmosphere of <1% oxygen and 9-13% carbon dioxide. Micro-aerobic incubation requires an atmosphere of 5-7% oxygen and ~10% carbon dioxide.

5.19 pH meters

pH meters are designed to measure the hydrogen ion concentration at ambient temperature (i.e. 15 - 25 °C). They should be capable of measuring to +/- 0.1 pH units and have either manual or automatic temperature compensation. The measuring and reference electrodes are usually grouped together to form a combined electrode. When not in use, pH electrodes should be stored according to manufacturer’s instructions.

In the microbiology laboratory the pH meter is mainly used to check the pH of each batch of culture media and reagents after sterilisation or preparation. On occasions it is used to adjust the pH of media before autoclaving.

The pH meter calibration should be checked before each use. When in daily use, and supporting calibration stability data are available, it may be sufficient to undertake a full calibration weekly. This should be performed according to manufacturer’s instructions using 2 (or more) buffer solutions, compliant with ISO 17034, and covering the appropriate

pH range. Buffer solutions, if purchased pre-prepared, should be used within their expiry date. A third standard buffer, usually mid-range between the two calibration points, may be used to verify the performance of the meter and the validity of the calibration. The calibration should be checked daily using the same pH buffers. This check should be undertaken daily or before each use of the meter if used less frequently. A full recalibration should be undertaken if this check gives unsatisfactory results. Calibration details and the results of calibration checks should be recorded and retained. Unused buffer solution should be discarded and not returned to the stock bottle. The response of electrodes (for example slope and millivolt output) should be checked daily. The meter should also be subject to routine internal AQC using a different value buffer from a different manufacturer. If results of the AQC or other checks are outside acceptable values, the pH meter must not be used unless a full recalibration rectifies the situation.

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 can leach 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. The electrode must not be allowed to dry out and wet storage, in a buffer or storage solution, recommended by the manufacturers should be used.

5.20 Pipettes and Pipettors

Many laboratories use sterile glass or plastic disposable 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 or mechanical device and pipettes can be 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 new batch or manufacturer should be checked to confirm delivery of correct volumes. This can be achieved by weighing volumes of water and verifying the weights against set tolerances. Ten replicate weighings are usually performed, the standard deviation, percent coefficient of variation (%COV), also known as relative standard deviation (%RSD), and inaccuracy can then be determined.

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 or a barrel filter 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 not be laid down on a bench but stored in suitable holders/chargers when not in use. Some automatic pipettors are autoclavable but particular care is required with calibration checks. They must be kept clean, particularly if there is any hint of internal contamination for example when dispensing media. Ideally individual pipettors should be dedicated to a particular task and location.

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 or plate dryer, 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, before further use.

New pipettors should be calibrated before use, and at suitable intervals, according to manufacturer's instructions. This can be achieved by weighing volumes of water, taking into account variations in the temperature and therefore density of the water used. 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, or less than 1 % where accuracy may be more critical, for example in the preparation of a standard. The coefficient of variation and bias required will vary depending the use and the general advice given above may not be applicable in some circumstances. It is for the laboratory to set fitness for purpose criteria based on its requirements. Intermediate calibration checks should be undertaken on a regular basis, for example daily or weekly, depending on the use of the automatic pipetting device. Details, for example dates and staff undertaking calibrations, should be recorded and stored for each pipettor. Pipettors can also be sent away to approved suppliers for re-calibration.

5.21 Protective cabinets

Protective cabinets can be either defined as a microbiological safety cabinet (MSC) or a laminar flow hood. A MSC can be defined as a ventilated enclosure intended to offer protection to the user and the environment, for example from aerosols arising from the handling of potentially hazardous and hazardous micro-organisms, with air discharged to the atmosphere being filtered. There are three classes of MSC. Class I cabinets are open fronted and designed to protect the operator by continuously drawing air into the front of the cabinet away from the worker then exhausting through a high efficiency particulate air (HEPA) filter. Class II is also open fronted and is designed so that the work area is kept clean by a down-flow of HEPA filtered air across the work. This protects the worker and the product but can be affected by air movements outside the cabinet. Class III cabinets are totally enclosed to contain hazardous agents on which work is conducted through gloves attached to ports. Air enters through a HEPA filter and is exhausted in a similar way to a class I cabinet.

Laminar flow hoods provide a filtered air flow that protects the worker and removes dust and other particles depending upon the type of filter installed. They can be used as powder weighing cabinets and to provide an environment for handling sterile products. They can also be used to reduce smells, when handling sewage sludge samples for example and protect the worker against certain chemical vapours providing the correct type of filter is installed. This is in effect a class 1 cabinet exhausted through an activated carbon filter.

The space inside cabinets should be kept as clear of equipment as possible and gas burners must not be used inside cabinets. Use of sterile disposable loops, etc., provide a suitable alternative to remove the need for a gas burner. Operators must be fully trained in the purpose for and operation of each cabinet and know the type of work that can be undertaken within it. All cabinets should be serviced, inspected and maintained according to the manufacturer's instructions and records should be retained. Formal inspections are required on an annual basis by authorised persons where air flows and general efficiency of the cabinet are measured. Spent filters should be replaced as required. Cabinets should be kept clean and disinfected prior to inspection.

5.22 Refrigerators and freezers

Refrigerators include chillers and cold storage rooms where the temperature is maintained at 5 ± 3 °C. They are used for the storage of media, reagents, cultures, materials & samples. Un-inoculated media, sterile materials and reagents should be stored in separate refrigerators or compartments to cultures, and should not be stored in such a manner that the temperature of the compartment is adversely affected. Ideally, samples should not be stored in the same refrigerator as media. Where this is not feasible they should be kept separate in dedicated areas so as to minimise the risk of contamination. Spark free units should be used for the storage of volatile or flammable reagents.

Each refrigerator should contain a calibrated thermometer or temperature measuring device which is used to record the temperature on a regular basis. Continuous monitoring devices are preferable provided they are checked regularly.

Even temperature distribution within the refrigeration space is important and for large capacity refrigerators should be established. This can be assessed by placing thermometers, or temperature recording devices, in different parts of the refrigerator over a period of time, for example over a 24-hour period, and recording the temperature at regular intervals. This can also be achieved by using a multipoint instrument that is traceable to national standards. The temperature profile of the refrigerator should show no significant differences wherever the temperature measuring devices are placed.

A freezer is a chamber which allows frozen storage to take place. Freezers usually operate at a temperature of around - 20 °C ± 5 °C, but deep-freeze cabinets that operate at a temperature of - 70 °C ± 10 °C and below are available. Freezers are used to store microbiological cultures, some reagents and chemicals as well as samples and sample preparations for analysis. The freezer should be loaded and unloaded so that a low temperature is maintained. As with refrigerators, the temperature of freezers should be checked regularly.

Modern refrigerators and freezers are usually available as frost-free items, but older equipment may require regular defrosting. Refrigerators and freezers should be defrosted when needed and kept clean. Routine cleaning should be undertaken, for example 3 monthly, with clean warm water and using a clean non-abrasive cloth. Detergents and disinfectants should only be used rarely, for example when a spillage has occurred or the cabinet is known to be contaminated. Spillages should be cleaned up immediately on discovery. Where detergents or disinfectants are used the surfaces should be thoroughly rinsed afterwards and allowed to air dry before reintroducing materials and cultures that might be affected by them. Periodically they should be inspected for leaks and damage.

5.23 Sample preparation devices (Blender, Homogeniser and Pulsifier®)

Equipment of this type is used to prepare initial suspensions of a variety of solid and semi-solid substances that can then be analysed by standard microbiological techniques. The choice of equipment depends upon the matrix of the material being analysed. Unless forming part of an established procedure the recovery performance characteristics of the device to be used should always be ascertained for each new matrix.

A blender has a blade in the base that rotates rapidly and samples are placed in a sterilisable metal or glass vessel that is placed on the base assembly.

A peristaltic homogenizer (stomacher) with suitable plastic bags can be used for the suspension of sewage sludge matrices. Digested sludge can be homogenized easily but sludge cake and derivatives may need to be multi bagged to prevent perforation and homogenized for a longer time to achieve homogeneity. Typical operating times are 1-3 minutes.

The Pulsifier is a patented type of mixer, widely used in food sample preparation, employing high frequency shock waves to the material in a plastic bag. It is reported to be less destructive of the sample with less risk of bag burst. Operation should be according to the manufacturers' instructions.

Preparation devices should be kept clean and any spillages removed immediately. They should be disinfected regularly and particularly after potential contamination for example due to bag leakage. Servicing and calibration should be undertaken in accordance with manufacturers' instructions.

5.24 Spiral platers

Spiral platers can be fully or semi-automated and a spiral plate method can make rapid colony enumeration possible while avoiding all or some of the intermediary dilutions that would otherwise be required. The principle is that a logarithmically decreasing volume of sample is dispensed on the surface of a rotating Petri dish in an Archimedes spiral. After incubation colonies develop along the lines where the liquid was deposited. The volume is calibrated and known at every point of the Petri dish. Bacterial concentration is determined by dividing the number of colonies found by the volume dispensed in the same sector of the dish. A micro-processor in some units allows rapid calculation of bacterial numbers.

The dispensing system should be sterilised and rinsed and the sterility of the unit should be verified by plating sterile water at the start of each run. The dispensing pattern can be verified using washable ink. The ink should be densest near the centre of the plate. A gravimetric check of the volume dispensed should be performed using water. The weight obtained should be within 5% of the expected weight for the volume dispensed.

The equipment should be kept clean, any spillages being removed immediately and serviced and calibrated according to manufacturers' instructions.

5.25 Steamers and boiling baths

Steamers and boiling water baths may be used for melting agar and decontamination of small items of equipment such as filter funnels between uses. Steamers generate steam at atmospheric pressure and boiling baths heat a body of water to boiling point in a small vessel with a lid. In both cases, if permissible in the manufacturers' instructions, distilled or deionised water should be used for preference otherwise regular descaling may be necessary depending upon the hardness of the water used.

It is necessary to ensure that there is an adequate volume of water present in the unit so that it does not boil dry and for boiling baths that items to be decontaminated are fully immersed. Care should be taken in the operation of these units to prevent scalding.

5.26 Temperature recording devices, (thermometers and thermocouples), and temperature control

Thermometers may be of the mercury-in-glass or alcohol-in-glass type and are available for a wide variety of temperature ranges and in sizes appropriate for monitoring temperature in diverse laboratory applications. They are available calibrated to national standards and un-calibrated. Certified and calibrated thermometers require re-calibration and certification at pre-determined intervals typically every 5 years. Certified thermometers can be used to calibrate laboratory reference thermometers that may subsequently be used to calibrate working thermometers used to measure temperatures within the laboratory.

Electronic temperature recording devices include thermocouples and platinum resistance thermometers. The temperature reading is transferred to a display or recorder by wire or radio wave transmission. Depending on the system a visual, hardcopy or electronic record of temperature observations or data at set time intervals is made. Some units can initiate alarms that alert users to out of range temperatures both in the laboratory and via telecommunication networks. Digital thermometers are also available.

When thermometers, or temperature recording devices, are used, they should be capable of measuring a given temperature within a specified tolerance. For temperatures between 20 - 40 °C, for example in incubators, the maximum fluctuation around the given temperature should be ± 1 °C. In these circumstances, thermometers, or temperature recording devices capable of measuring within ± 0.5 °C can be used, but those measuring to within ± 0.2 °C provide more accuracy. For regulatory drinking water compliance purposes, it may be more appropriate to use thermometers, or temperature recording devices 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, or temperature recording devices should measure to within ± 0.1 °C. For dual temperature incubation (for example, incubation at 30 °C and 44 °C) two thermometers may be required due to available ranges of thermometers.

With such sensitive equipment and in order to prevent heat loss when the temperature is measured, thermometers, or temperature recording devices can be placed in suitable plastic or glass containers filled with an inert liquid. Suitable liquids comprise glycerol, liquid paraffin or propylene glycol. These liquids stabilise temperature measurements when the thermometer or items 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, usually on an annual basis, and any errors compared with the reference thermometers and should be no greater than the tolerance of the reference thermometer. It is essential that electronic temperature recording devices be regularly calibrated against certified thermometers or other calibrated temperature recording devices certified to national standards.

No measurement is perfect it has an associated uncertainty arising from many factors including errors and imperfect reproducibility. Ideally each measurement should be quoted with an indication of the uncertainty, often as a ± figure, so that decisions based on the measurement are fully informed[17]. The uncertainty must be within the tolerance for the

method, i.e. a reading of 30.9°C using a thermometer with a discrepancy of +0.2°C would be outside 30°C± 1°C. Where more than one uncertainty is known to apply the uncertainties must be added together.

5.27 Timing devices

Timers and integral timing devices may be analogue or digital and are used in applications where a specified time interval is required. They must be kept clean and be capable of achieving the degree of accuracy required and verified depending upon application and usage against the national time signal.

Timers which are integral to equipment such as autoclaves and incubators, etc. should be operated according to manufacturer's instructions and checked / calibrated periodically during servicing.

Replacement batteries and suitable arrangements for backup power should be available.

5.28 Ultra violet steriliser cabinets

Ultraviolet (UV) steriliser cabinets can be used as an alternative means of disinfecting some equipment, for example membrane filtration funnels, between uses. However, this approach may not be suitable for all types of filter funnel base. The wavelength and intensity of UV irradiation and the length of time of exposure are critical to the success of this approach. The specifications of commercial UV sterilisers may vary and the user will need to verify the conditions suitable to achieve disinfection for their intended application. This approach may be effective to disinfect the units for example by inactivating coliforms, *E. coli* and Enterococci but more stringent conditions may be required for spore forming indicator organisms such as *Clostridium perfringens*. A record of the verification data and conditions used should be kept along with periodic checks on equipment performance. The timing device used to judge length of exposure should be calibrated regularly (section 5.27).

There are particular health and safety risks when using UV and these should be assessed and suitable precautions taken. The equipment should be used in accordance with the manufacturer's instructions. Daily checks should be made on performance and bulbs should be replaced annually and as necessary in between. Records should be maintained of performance checks, bulb replacements and any faults encountered.

5.29 Vortex mixers

A vortex mixer is used for mixing the contents of tubes or bottle preparations such as decimal dilutions of a suspension of bacterial cells in a liquid. The base of the tube or container is pressed against the mixer head and a vortex forms in the liquid mixing the contents. The speed can be controlled on some models. Care should be taken that the container is large enough so that liquid does not spill out of the tube during mixing. Equipment should be kept clean and if spillage does occur the unit must be disinfected. Excessive use of hand held applications should be avoided due to the possibility of adverse vibrational health effects.

5.30 Water baths

Thermostatically controlled 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. A sloping lid is usually fitted to minimise loss of water by evaporation. 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 is at the recommended level. When in continuous use over long periods they should be drained and cleaned at regular intervals and wiped out with a suitable disinfectant (for example 70 % ethanol or 2-propanol) before being refilled. When not in use, water baths should be drained and cleaned before storing according to manufacturer's instructions. When in use, the temperature of the water should be measured at regular intervals. The minimum number of readings that should be taken includes one at the beginning of the working day, before cultures have been removed, and one at the end of the working day or when samples are placed in the bath, using a calibrated thermometer or temperature measuring device. An integral temperature display is usually for visual guidance and can only be used as a sole temperature reference if its accuracy has been verified. Continuous temperature monitoring may be considered, as for incubators.

Care should be taken when loading the bath that the level of contents of a bottle or tube is below that of the water. Suitable racks or stabilising devices should be used to prevent water ingress or spillage of the contents of tubes or bottles. Spillages must be dealt with immediately as they can result in serious contamination of the water and bath contents. Even traces of culture media can promote significant growth of bacteria in the water.

5.31 Water purifiers - distillation units, deionisers, and Reverse Osmosis (RO) devices

Water produced for preparing microbiological culture media, reagents and other laboratory applications must be of a suitable quality (see section 6.5).

The choice of system depends on the quality requirements of the application, the volume required and the mineral content of source water to be treated. Apparatus varies from simple stills that produce distilled water to more complex units that may have a number of processes including pre-filters, deioniser columns and reverse osmosis units. Some systems including storage reservoirs may include re-circulation pumps and UV irradiation to preserve the quality of the treated water ready for use. Purified water left standing may deteriorate over time due to exposure to air, by dissolution of gases and trace organic chemicals which may result in changes in pH and conductivity or promote microbial growth. All equipment should be installed, maintained and used in accordance with manufacturer's instructions. Stills should be de-scaled and cleaned as required depending upon the hardness of the water in the area used. Other units require filter changes depending upon usage. Some components may require replacing at intervals to ensure continued performance to specification.

All water produced should be checked at regular intervals and after replacement of cartridges or cleaning. It is advisable to keep comprehensive records of maintenance.

6 Materials and techniques used in a microbiology laboratory

Chemicals used in a microbiology laboratory should be of analytical grade quality where these are available. Where appropriate, reagents and chemicals should be stored and used in accordance with the manufacturer's instructions. They should be discarded if the expiry date, i.e. the date by which the reagent should be used, has passed. Reagents and chemicals are usually supplied with a safety data sheet and toxicity data. Records of these data should be kept, and any specific hazards assessed and documented [(8)]. Chemicals and reagents should always be handled with care and any spillages that occur should be cleaned up immediately.

6.1 Media

Media have been formulated for the culture of micro-organisms including a wide range of bacteria but also yeasts and other microfungi. Most media are available commercially in powder form. Manufacturers may supply media and materials in a number of different formats which include:

- Complete medium containing all the ingredients, for example membrane lauryl sulphate broth (MLSB) for the enumeration of *E. coli* and coliform bacteria from a water matrix

- As an incomplete medium which contains most of the ingredients but requires the addition of a supplement, for example the antibiotic kanamycin, to complete the medium as in kanamycin aesculin azide agar (KAAA) for the confirmation of enterococci

- Individual ingredients to be used in the preparation of a medium where the complete medium is not available, for example yeast extract and skimmed milk powder used in the preparation of cetrimide milk agar for the confirmation of *Ps. aeruginosa*

- Supplementary ingredients to be added to a medium either to

    - enhance microbial growth, for example horse blood

    - a diagnostic supplement, for example urea for urea hydrolysis

    - as a selective supplement, for example kanamycin

- Complete ready prepared medium, either as sterile broth or agar in tubes or bottles or Petri dishes containing pre-poured agar.

Liquid media are often given the term "broth". A broth may be either non-selective i.e. enabling most bacteria to grow in it, for example nutrient broth or selective allowing only certain bacteria to grow. For more fastidious bacteria, nutrient broth No. 2 or brain heart infusion broth may be used. Non-selective broth is used for the general cultivation of bacteria. Specific ingredients may be added to demonstrate particular characteristics, for example lactose and a pH indicator for lactose fermentation or to make the broth selective for the isolation of specific types of bacteria. Reagents and powders used to prepare broths should dissolve readily in water at room temperature to produce a clear solution.

Solid media are often given the term “agar”. An agar may be either non-selective i.e. enabling most bacteria to grow on it, for example nutrient agar or selective, enabling only certain bacteria to grow, for example M-enterococcus agar (MEA) for enterococci. Non-selective agar is used for the general cultivation of bacteria. For selective agars, specific ingredients may be added, for example sodium lauryl sulphate in membrane lactose glucuronide agar (MLGA) for the isolation of *E. coli* and coliforms. Reagents and powders used to prepare agar media should dissolve readily in water when boiled to produce a clear solution.

Further guidance on the general requirements for the preparation, production, storage and performance testing can be found in EN ISO 11133[20].

6.1.1 Different types of media

Nutrient media, either as broth or agar are designed to enable a wide range of bacteria to grow, including those routinely sought in water analysis. Broths or agars may be non-selective in the types of bacteria that can grow on or in them, for example yeast extract agar for the enumeration of heterotrophic bacteria. Chemicals or supplements can be added to non-selective media to make them selective for specific bacteria or to enable the differentiation of one bacterial species from another. Different types of media have been classified with their own terminologies and definition[20] for example:

- A differential medium is one which enables the testing of one or more physiological or biochemical characteristics of a micro-organism to be determined, for example the fermentation of lactose

- An enrichment medium, usually a liquid medium, contains chemicals which suppress the growth of unwanted or non-target organisms whilst allowing target organisms to grow. Once incubation is complete, the number of target organisms usually exceeds those of unwanted or non-target organisms for example Rappaport broth for the isolation of *Salmonella*

- A selective medium, whether in solid or liquid form, enables target organisms to grow whilst suppressing the growth of unwanted, non-target organisms. The medium may also contain chemicals which permit the differentiation of organisms, for example XLD agar for the isolation of *Salmonella*. As a solid medium, target organisms can grow on the surface of the medium and be identified and sub-cultured either to another medium or used for further biochemical or serological testing.

6.1.2 Basic constituents of media

Most routine media, whether nutrient, enrichment or selective, comprise a basic set of ingredients which provide carbon, nitrogen, vitamins and minerals to support microbial growth. The commonest ingredients include peptones, either as an aqueous extract or an enzyme digest of meat. Other ingredients include yeast extract (an acid hydrolysis of yeast), meat extract and casein hydrolysate (an acid extract of casein). Tryptone (an enzyme digest of casein) is rich in the amino acid tryptophan, a pre-cursor for the production of indole. Tryptophan is, therefore, one of the basic constituents of the medium tryptone nutrient agar (TNA) used for demonstrating the production of indole for the confirmation of *E. coli*. Mycological peptone is a special peptone used for the growth

of fungi. For further information on the quality of ingredients that should be used for media preparation reference can be made to EN ISO 11133[20].

6.1.3 Agar

Agar is a polysaccharide which is extracted from seaweed. It is commercially available as a powder and is usually added to a broth at a concentration of between 1 – 1.5% m/v depending on the purity of the agar. The addition of agar creates a gel which, when cooled to below approximately 42 °C, provides a solid surface, suitable for the support of growth and colony development. Colonies may then be counted and, for example by careful study of the morphology or colour, different species may be recognised and cultures identified as pure (i.e. of one colony type only) or mixed. Single isolated colonies can also be selected for sub-culture to new agar plates to provide pure cultures.

Different manufacturers supply agar of different levels of purity. Agar is routinely used at a concentration of between 1.2 and 1.5% m/v in order to provide a gel of a suitable strength for agar plates. Lower concentrations, typically about 0.7 – 0.8% m/v may be used to produce a semi-solid agar which may be used, for example, for the assay of bacteriophages. A more purified agar will produce a gel of suitable strength at a lower concentration, usually about 1%. In addition, these agar plates will generally be clearer.

Agar will only dissolve completely when heated in water to boiling point. On cooling agar solutions solidify or set, at approximately 42 °C. It is this property of agar that makes it particularly useful in microbiology for enumerating micro-organisms by either direct spread plate (on the solid surface) or as pour plates (within the agar). Failure to dissolve agar completely or to mix the solution adequately once the agar has dissolved, or melted, may result in an incorrectly formed gel, having weak gel strength, when transferred to a Petri dish. Following inversion of the Petri dish the medium may fall out. Agar should set to give a smooth, even, surface. Incorrectly prepared agar may give an uneven surface or lumpy appearance.

6.2 Storage of dry media

Most manufacturers supply media as dehydrated formulated powders. They also provide data on batch numbers, expiry dates and details of the preparation of media. Whilst details of the preparation and sterilisation of media may be provided, these should also be documented in the analytical method (see section 7.1). Containers of media should be stored in a cool dry place, and labelled clearly with the date of receipt and the date when the container is 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. Most powdered media are hygroscopic. After the container of medium has been opened and powder removed, the lid should be replaced and tightly secured to reduce the potential for absorption of moisture. Over a period of time, some media may absorb excessive amounts of water and may solidify. This usually results in discolouration of the media and deterioration of their nutrient or selective properties. Such media should be discarded even if the expiry date of the medium has not passed.

Supplements for media can also be purchased from manufacturers. Most are in freeze-dried form and should be stored and used according to the manufacturer's instructions. Supplements should also be labelled with the date of receipt and discarded when the expiry date has passed.

6.3 Preparation of media

Media should be prepared by weighing out the appropriate amounts of the individual ingredients or the amount of material required for the formulated product and adding the appropriate volume of distilled, deionised or similar grade water (see section 6.5). Many media contain selective chemicals and where these are supplied as powders, appropriate containment measures should be taken for example, the use of respiratory protection to prevent powders being inhaled. Autoclaving may change the pH of the medium and whilst it is often not essential, the pH of the medium may require adjustment before sterilisation. The pH cannot be adjusted after sterilisation is complete. Adjustment of the pH should be carried out by the addition of small volumes of an appropriate concentration of hydrochloric acid or sodium hydroxide (for example 1 M) until the required pH value is achieved. 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 the breakdown of lactose in the medium and a lowering of the pH. It may therefore be necessary to raise the initial pH of the medium by 0.2 - 0.4 pH units to ensure that, after sterilisation, the final pH of the medium is 7.4 ± 0.2. The pH of any prepared medium should be measured, using a specially kept sub-sample or sacrificial poured plate, 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 as any un-dissolved powder in the bottom of containers may char and degrade during the sterilisation process. Broths, once dissolved, can be dispensed into suitable containers for autoclaving. Any medium which contains agar should be brought to its boiling point before it is dispensed. Un-dissolved agar is granular and quickly settles out from suspension. It is therefore impossible to dispense the correct amount of agar into containers unless the medium has been dissolved by boiling. Alternatively, powders containing agar may be dispensed into containers directly and thoroughly re-suspended before autoclaving. The agar will settle to the bottom of the bottle during autoclaving and, whilst it will dissolve, the concentration of agar in the bottom of the bottle will be much higher than the concentration at or near the surface (see white colouration in Figure 6.3.1). Bottles of media autoclaved in this way should be carefully mixed to distribute the agar after autoclaving and whilst the medium is still molten.

Figure 6.3.1 A bottle of agar where the concentrated agar is at the bottom (white layer)

Bottles of medium prepared for sterilisation must always have sufficient headspace to minimise the risk during sterilisation of a build-up of pressure within the bottle or medium being lost through vigorous boiling. The headspace will also facilitate thorough mixing of the medium during cooling and dispensing. The procedure used for filling the bottles and for their sterilisation should be fully described to ensure consistency in the medium's production and in its quality control. A suitable default starting point would be for bottles to be filled to no more than two thirds of the capacity of the bottle for example, 300 ml of medium in a 500 ml bottle. The maximum volume of medium sterilised in a single bottle or flask should normally be no more than 500 ml in, for example, a one litre bottle or flask. Larger volumes of medium will take much longer to warm up during the autoclave cycle and may fail to reach the correct temperature for the appropriate length of time during the sterilisation cycle. Some media constituents may be denatured if the sterilisation temperature or the holding time is increased. Autoclave and media preparator cycles should be validated taking account of the media volumes being sterilised to ensure that the correct conditions are being achieved.

6.4 Preparation using media preparators

Operation of media preparators depends on the model purchased, but in general the sterilisation chamber is part -filled with distilled or deionised water and the correct weight of dehydrated medium added. The remaining water is added to make the final volume required. This helps to mix the powder and avoid clumping. An atomizer spray containing deionised water is useful to damp down any powder that may become airborne, and also to wet the seal to aid closure. Capacities for volume of medium prepared in a single cycle vary between preparators.

6.5 Water

The quality of water used for the preparation of culture media is critical. Tap water should never be used because it may contain relatively high concentrations of ions such as calcium or phosphate causing cloudiness or precipitation to occur in the medium. In addition traces of toxic metals from plumbing materials, such as copper, may be present or the water may contain significant amounts of chlorine, both of which are inhibitory to the growth of micro-organisms. Pure water suitable for culture media may be produced by distillation, deionisation or reverse osmosis. Whichever process or combination of processes is used, the water should have the following properties:

- It should not contain toxic metals or chlorine
- It should have a low conductivity, ideally less than 10 micro-Siemens/cm (10 μS/cm)
- It should have a low microbial load when examined by a heterotrophic plate count (HPC) at 22 °C. Counts should ideally be less than 1000 cfu/ml and should not exceed 10,000 cfu/ml

Pure water should be stored in containers made from inert materials, for example glass or polyethylene. Pure water should be checked at regular intervals to ensure a constant water quality and where the quality fails to meet the above standards, an investigation should be undertaken to identify and remediate the problem. If, for example, the HPC exceeds 10^4 cfu/ml, consideration should be given to draining the container and cleaning

thoroughly before re-filling. More information on water quality for microbiological media can be found in ISO 11133[20].

6.6 Sterilisation of media

Media should be sterilised, usually by autoclaving, within 2 hours of initial preparation. Leaving unsterilised media in a warm place for periods longer than 2 hours can lead to microbial growth and potentially to changes in the properties of the medium. Sufficient prepared medium should be kept in suitable separate portions to check its final pH and to determine its growth and selectivity characteristics before the remaining bulk of the medium is used. Once a batch of medium has been prepared, a batch number should be allocated to this medium before it is autoclaved. This batch number may then be used for quality control and analytical test recording purposes.

Once prepared for sterilisation the caps, stoppers or screw tops of media containers should be loosened, for example by a quarter turn for a screw cap bottle, before loading into the autoclave or steamer. This prevents dangerous pressurisation of the container during sterilisation, which could otherwise cause rapid boiling when the container is moved, possibly resulting in explosion and/or the violent discharge of hot liquid.

Typically, media are sterilised by autoclaving at 115 °C for 10 minutes, for example for MLSB, or 121 °C for 15 minutes, for example for nutrient agar. In a few instances, for example MEA, where the medium is highly selective, it is sufficient to bring the medium to the boil to dissolve the agar. The manufacturers' instructions should be followed. It is important that media are not over-heated during sterilisation as this may lead to a breakdown of nutrient, selective or supplement properties. A medium should not be autoclaved more than once, even to melt it for use. Equivalent sterilisation cycles are used in a media preparator but in this case the medium is mixed during the cycle.

Whilst sterilised media should be removed from the autoclave as soon as practicable after sterilisation is complete, care should be taken in handling media as it may be super-heated and boil rapidly once removed from the autoclave. The tops on the containers may be tightened and agar-containing media mixed carefully and allowed to set. Alternatively, once cooled, media may be dispensed into Petri dishes or tubes for use. A portion of these Petri dishes or tubes should undergo quality control tests to demonstrate that the medium is satisfactory for routine use (see section 6.10).

After sterilisation is complete in a media preparator, the instrument will enter the cooling phase and quickly bring the contents down to around 50°C. The media preparator will then hold this temperature for the duration of the dispensing phase. At this stage additives or supplements may be aseptically added through the filling port. Addition at this stage ensures that heat-labile supplements are not deactivated. Since the machine continues to mix, this also ensures homogeneity in the finished medium. The finished medium is usually dispensed by fitting a clean sterile dispensing tube to the integral peristaltic pump. Spare bagged sterile tubing sets should be available in case contamination is suspected. Wrapping the connectors and dispensing nozzle suitably, for example in foil, helps prevent contamination when fitting to the pump and stacker module.

To dispense the medium the tubing must first be primed and then calibrated to deliver the required volume per plate, bottle or tube. Once dispensed media may be allowed to

remain on the stacker carousel until solidified; after which it should be removed promptly and stored as described in section 6.8.

Solidified and liquid media prepared in bottles as a bulk batch may be stored in the dark at room temperature (ideally, not more than 25 °C). Whenever practical these should be subjected to quality control tests and only used when the tests have shown that the medium gives satisfactory performance. Each batch of medium should be allocated a storage period, indicating the maximum period up to which the medium may be stored. This period should be stated in the method and confirmed through suitable testing in the laboratory to establish the shelf-life of the medium. Agar containing media can be melted by heating in a boiling water bath, in steam at normal atmospheric pressure or in a microwave oven at low power, for example, 300 watts. In each case a period of time just sufficient to ensure that the agar is thoroughly molten should be used.

6.7 Petri dishes

Petri dishes may be made of glass or clear plastic and are available in a variety of diameters from 50 to 90 mm. Typically 50 to 60 mm dishes are used for membrane filtration and 90 mm dishes are used for colony counts, sub-culture and confirmation of bacteria. Glass Petri dishes can be re-used by sterilising, washing and re-sterilising after each use. Plastic Petri dishes are provided, typically as batches of 10 or 20 units, in sterile packages. They are used once, autoclaved and discarded. Plastic Petri dishes are available un-vented or as single or multiple vented dishes with vents on the underside of the lids. Multiple vented Petri dishes should be used when circulation of air or gases is required to create the correct atmosphere for micro-aerobic or anaerobic cultivation.

6.8 Cooling, storage and dispensing sterile media

Molten media containing agar should be cooled, for example in an incubator or water bath, to approximately 50 °C before being dispensed into Petri dishes or sterile tubes. Media should not be dispensed at temperatures above 50 °C as this may lead to excessive condensation in the Petri dish during cooling and subsequent storage. Sterile supplements can be added at this point before the medium is dispensed. Media should not be left standing at 50 °C for long periods of time as to do so may impair their nutrient or selective properties. Media should be dispensed into Petri dishes on a flat, freshly cleaned and disinfected, work surface.

Approximately 20 – 25 ml of medium should be poured into each 90 mm Petri dish or about 10 ml of medium into each 50 – 60 mm Petri dish to give a minimum depth of 3 mm and no more than 7 mm. Smaller volumes of medium may result in the medium drying out during storage or incubation. Once poured, the medium should be left to solidify, the dish then being inverted and the medium stored at 5 ± 3 °C[17] in such a way as to prevent excessive drying of the medium. Individual Petri dishes should be labelled with the medium reference, for example NA for nutrient agar, the batch number and an expiry date. If stored in an airtight container, this container may be labelled with the same information. When medium contained in a Petri dish shows signs of excessive dehydration such as thinning, deepening in colour or detachment, the dishes should be discarded. It would be prudent to ensure the preparation of media is planned to make sure that adequate supplies are available, and that the need to discard un-used media is kept to a minimum.

It is good practice not to leave plates of freshly prepared medium on the bench for long once poured and cooled. They should not be subject to exposure to sunlight and should not be left out for more than two hours. The action of sunlight on media produces superoxide radicals such as peroxides and other toxic substances which may inhibit the growth of certain bacteria. When a medium has been prepared, it should be transferred as quickly as possible to a dark environment. Media that show obvious signs of contamination or deterioration should be discarded.

Where small volumes (for example 9 ml) of diluent, for example Ringer's solution, are required to dilute samples, these volumes should ideally be dispensed aseptically into sterile containers after the diluent has been sterilised. Diluents containing nutrients, for example MRD, should ideally be used immediately. They may be stored, for example at 5 ± 3 °C, but should then be used as soon as possible due to the risk of contaminant growth and deterioration. In some circumstances it may be preferable for a laboratory to dispense volumes of diluent prior to sterilisation, for example to minimise potential contamination in diluents containing nutrients, when for logistical reasons they will not be used immediately.

Dispensing the diluent into containers prior to autoclaving may result in changes during sterilisation and subsequently inaccurate strength and volume of diluent in the containers. This will lead to inaccuracies occurring in subsequent serial ten-fold dilutions. It is therefore essential, when diluent is dispensed before sterilisation, to verify that the correct volume is present before using it for performing test dilutions. In addition, a consistent approach is required to sterilisation conditions including, for example load size and distribution within the autoclave. The volume of diluent that needs to be dispensed prior to autoclaving to achieve the correct volume in the cooled sterile product must be established. A verification process should be applied to every batch prepared, before releasing it for use, to demonstrate within an acceptable tolerance that the diluent volumes are correct. The results of the verification should be retained with the batch preparation record.

Media in Petri dishes that have been stored at 5 ± 3 °C should be dried before use. This may be achieved by leaving the media at room temperature, that is no greater than 25 °C, for 2 hours. Alternatively, dishes may be placed in an incubator at 37 °C for 30 minutes to assist drying but this should be carefully controlled to prevent contamination and excessive drying. During the pouring and subsequent cooling of media in a Petri dish, a thin 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 growth of bacteria spreading across the agar surface. The use of unvented dishes and presence of motile bacteria are particular factors associated with the spreading of growth across the agar surface when there is a film of moisture. This may lead to no colonies being separated on the agar (see Figure 6.8) making subsequent sub-culture of individual colonies for purity impossible. This could mean that a sample may need to be sub-cultured twice before isolated colonies of a pure culture are obtained with subsequent delay in reporting of results. When large numbers of 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 adsorb excess moisture.

Figure 6.8 Spreading growth on MacConkey agar through failure to dry the plate

6.9 Sterilisation of solutions by membrane filtration

Antibiotic solutions, growth supplements and some sugar solutions may be heat sensitive and are denatured by autoclaving. These supplements may be purchased from commercial manufacturers as sterile solutions or lyophilised powders. However, a laboratory may wish to prepare its own supplements. Solutions are usually sterilised by filtration through a 0.2 µm membrane filter. Small volumes of solution are best filtered through a sterile syringe filter. These are small disposable sterile filters which can be attached to a syringe (see Figure 6.9.1). A sterile syringe is loaded with solution and this is pushed through the filter and collected in a sterile container. The syringe filter is ideal for solutions up to 100 ml. Solutions sterilised in this way can then be dispensed aseptically into sterile containers in appropriate volumes for storage either at 5 ± 3 °C or frozen at - 20 ± 5 °C or lower if appropriate.

Figure 6.9.1 Syringe filter

For larger volumes of solution, conventional membrane filtration equipment and a vacuum flask can be sterilised in an autoclave (see Figure 6.9.2). Once the equipment is cool a

sterile 0.2 µm membrane can be placed into the filter and the flask connected to a vacuum source. The solution to be sterilised is poured into the filter funnel and the vacuum applied to the flask. The sterile solution can then be dispensed into suitable containers for storage. Pre-sterilised single use plastic disposable filter units can be purchased from manufacturers with a membrane already in place.

Figure 6.9.2 Disposable plastic filter units

As with autoclaved media, each filtered solution should be given a batch number and an aliquot tested for sterility, the selectivity of antibiotic solutions, appropriate growth for growth supplements and appropriate biochemical reactions for sugars and other differential reagents, for example urea solutions.

6.10 Quality control of culture media

Microbiological media used for the analysis of samples of water and associated materials are designed to recover stressed organisms. Quality control is therefore important to ensure that there are no inhibitory substances in the medium that might adversely affect its properties and that selectivity is effective in enabling only target organisms to grow. All batches of prepared media should have quality control checks carried out and records of these should be kept. For some applications it may be appropriate to include checks on new batches of powdered media and ingredients, particularly when sourced from a different supplier, used for selective enumeration tests, for example MLGA or MEA, soon after receipt. The purpose being to demonstrate continuity by comparing performance characteristics against an ‘in use’ batch before being introduced to routine use. The records of this testing should also be kept. Freshly prepared media should, where

practical, be quarantined until such time as it has been demonstrated that the medium is fit for purpose.

Each batch of prepared medium should be uniquely identifiable, for example, by a batch number. For complex media requiring the addition of supplements after sterilisation, each bottle of medium may need to be treated as a separate batch. The batch numbers of all constituent products of the medium batch should be recorded, for example where commercial media are used this should include the manufacturer's batch number. Where the medium is made in-house from different constituents, prepared constituents should also be given a unique batch number and this recorded in the batch record when used to make the complete medium. When a medium has been prepared, it should be labelled with its batch number and expiry date. Details of sterilisation should be available for all media that require autoclaving and these should be recorded together with the results of any time cycle checks, for example autoclave temperature charts. The signatures of appropriate staff should also be included with these records at each stage of preparation to provide a suitable audit trail to demonstrate the correct preparation of media.

6.10.1 pH check

Once preparation of the medium is complete, a small aliquot of each medium should be checked for pH. The pH of the medium should be within the tolerance stated in the method, typically ± 0.2 pH units. If the medium is outside of the stated pH range 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.

6.10.2 Microbial check

Where agar media have been dispensed into Petri dishes, a representative number should be checked to ensure that they are satisfactory. Liquid media should also be dispensed aseptically into suitable containers for the same purpose. Media should be incubated at an appropriate temperature and for an appropriate time to demonstrate sterility and that they support the growth of the target organisms for which they are intended and differentiate or are selective against non-target organisms. In addition, where appropriate, quality control checks should record whether growth of target organisms displayed typical morphology.

6.10.2.1 Purchase and storage of reference cultures

Reference materials for quality control can be obtained from recognised culture collections, for example, the National Collection of Type Cultures (NCTC) or the National Collection of Industrial and Marine Bacteria (NCIMB). Cultures may be supplied as freeze-dried suspensions in sealed ampoules or for example as Lenticules®, Vitroids™, 'tablets' or other equivalent products. For each type of reference material cultures are revived by addition to or addition of a small volume of sterile broth and re-suspension of the bacteria. Some of these can also be revived directly on solid media. The suppliers' instructions should be followed carefully. The suspension can be inoculated onto Petri dishes containing a suitable nutrient agar and incubated at the appropriate temperature. Reference cultures should be sub-cultured only once[20]. The resultant growth can be preserved as a stock culture by one of several means (see following bullet points) and then removed as required to prepare working cultures.

- The bacteria can be suspended in a suitable medium contained in an ampoule and freeze-dried. A number of ampoules should be prepared to enable fresh working cultures to be prepared over subsequent years. To obtain a working culture, an ampoule should be opened and inoculated onto a suitable nutrient medium.

- The bacteria can be suspended and inoculated onto commercially available beads according to the bead manufacturer's instructions. Several vials may be prepared from one reference culture. These are labelled and should then be stored at a temperature for example below -20 °C in line with manufacturers' recommendations. To obtain a working culture, a vial should be removed from cold storage, one bead quickly removed with sterile forceps or a loop and inoculated onto a nutrient medium. The vial should then be returned to cold storage as quickly as possible.

- Stock cultures may be preserved in liquid nitrogen if this is available. Alternatively, an ultra-low temperature freezer, operated at - 150ºC, may be used. Reference cultures are suspended in a cryo-protectant medium and immersed in liquid nitrogen or stored in an ultra-low temperature freezer. To prepare a working culture, one ampoule should be removed, allowed to warm to room temperature and inoculated onto a suitable nutrient medium.

Whichever method is used for maintaining bacterial cultures, the preserved cultures should be checked for purity after storage and to ensure that they retain the phenotypic features for which they have been selected, for example *E. coli* retains the ability to ferment lactose at 37 and 44 °C.

Environmental samples known to contain the organisms being sought can also be used for quality control, particularly during routine and investigative monitoring of environments. Environmental samples offer more of a challenge for the isolation procedure because they will contain competing organisms as well as the target bacteria. A disadvantage of using environmental samples is that the presence and number of environmental bacteria is unknown and this may result in quality control results being unacceptable because no target organisms were isolated. A laboratory may wish to add bacterial strains that have been isolated from previous environmental samples during routine or investigative water monitoring. Such isolates may exhibit unusual phenotypic characteristics and can be used as part of quality control or training programmes. These isolates may be stored and maintained in the same way described above for reference cultures.

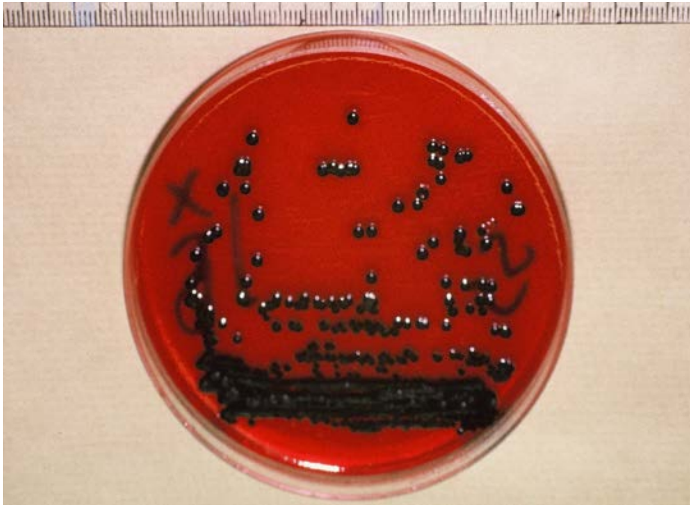
6.10.2.2 Performance testing

Control tests can be undertaken in a number of ways. Descriptions of suitable tests are also detailed in EN ISO 11133[20]:

- Qualitative control tests seek to demonstrate that a particular organism will or will not grow on a particular medium. The test does not seek to demonstrate that the number of organisms that will grow from a given suspension or environmental sample may be enumerated. The tests may be carried out on media that have been purchased as ready prepared from a manufacturer who is able to supply evidence of sterility, microbial growth and, where required, selectivity. A scoring system may prove helpful to demonstrate where growth was absent, or where growth was assessed as weak or

where growth was assessed as being good. For example, a score of 0 can be used to represent no growth, 1 for weak growth and 2 for good growth (see Figure 6.10.1).

Figure 6.10.1 Example of qualitative microbial growth – *Salmonella* on XLD agar demonstrating a score of 2



- Semi-quantitative control tests can provide differentiation of the quality of growth on a medium using a numeric basis for a suspension applied using a streak and dilution approach. This type of quality control test can be used to assess growth in liquid media. For example, a Petri dish containing agar is divided into four quarters and each quarter is inoculated from a broth culture using a 1 µl loop. Each quarter is streaked four times without re-charging or flaming the loop producing 16 lines for potential growth. Growth on a line is scored as 1 giving a maximum score of 16 if each line produces growth. To demonstrate that growth in a medium is satisfactory a minimum score may be established for example 8 out of the 16 lines for a medium to be satisfactory (see Figures 6.10.2 and 3). Membrane lauryl sulphate broth is a typical example where the broth is incubated at 37 °C for 18 hours and then inoculated onto a suitable nutrient agar. A growth control would have a score of greater than 8 and a sterility check should have a score of 0. This type of quality control is ideal for liquid and non-selective media.

Figure 6.10.2 Semi-quantitative control test for microbial growth – *E. coli* on MacConkey agar giving a score of 16

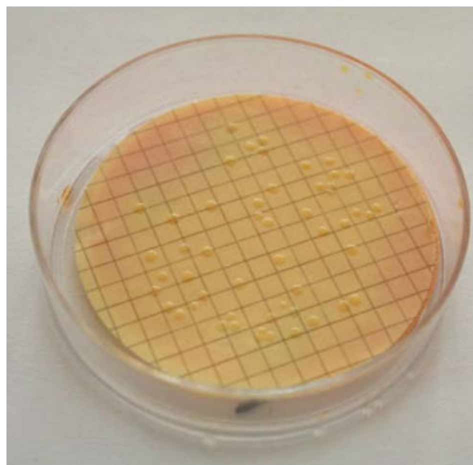
Figure 6.10.3 Semi-quantitative control test for microbial growth – *E. coli* on MacConkey agar giving a score of 2

- Quantitative control tests use a bacterial suspension of a known number of cells to determine the number of colonies that will develop on a medium using a spread plate method (see Figure 6.10.4) or membrane filtration (see Figure 6.10.5). The suspension may be obtained from commercially prepared reference materials. Alternatively, it may be possible to use broth cultures stored in a refrigerator to provide suspensions for qualitative, semi-quantitative and quantitative reference materials.

Figure 6.10.4 Quantitative quality control – *E. coli* growing on nutrient agar giving a direct count

Quantitative quality control will work with fixed numbers and over a period of time, limits can be set to accept or reject media. Performance of a medium may require 50% of the target organisms to be recovered for acceptance with no growth for non-target organisms. Alternatively, control charts can be prepared with limits beyond which batches of media or routine tests are not acceptable.

Figure 6.10.5 Quantitative quality control using membrane filtration



To enable meaningful comparisons to be made between different batches of media or membranes over a period of time, suspensions used for quantitative quality control must have reasonably stable numbers. Commercially prepared suspensions are available and these should be used according to the manufacturer's instructions. As an alternative, for some applications, broth cultures may provide suspensions suitable for quantitative quality control provided their preparation is specifically documented and usage is supported by appropriate performance data.

Working cultures, inoculated into broth and incubated for a standardised fixed time period under the same conditions should have a consistent number of countable organisms at a given dilution. At the end of the culture period, the organisms will be entering the stationary phase. Storage for a short period of time at 5 ± 3 °C, for example over a weekend, should ensure that all cells are from the stationary phase of the culture growth curve. Providing that the numbers are stable, such a suspension can be used for both semi-quantitative and quantitative quality control of media. Preliminary tests would suggest that the use of broth cultures would be an acceptable alternative for laboratories who would wish to use them. It permits the construction of quality guidance charts demonstrating acceptability of media quality control.

The following quality control procedures are therefore suggested:

- Selective broths and agar media should be assessed quantitatively either by inoculation with a preserved commercial culture or a suitable dilution of a reference broth culture stored at 5 ± 3 °C. Providing that the count on the selective medium is within a defined target of the count on a non-selective medium, for example 50%, or as established by the laboratory, the medium is deemed satisfactory for use. Where the count on the selective medium is less than 50%, the medium should be discarded. Similarly, the target recovery, when for example using Lenticules® or Vitroids™ where there may be variability in batch performance, should be set based on the suppliers' data and previous experience, ideally with the expectation of at least 50%. Alternatively, the Productivity Ratio (PR) approach described in ISO 11133[20] may be more appropriate than recovery. In some circumstances, for example selective media such as GVPC for Legionella, where a comparison between the test batch and a previously validated batch is used, the PR must be ≥0.7 (or 70% recovery). An upper limit for PR or recovery acceptability should also be specified.

- It is not necessary with nutrient media to demonstrate recovery quantitatively. However, nutrient agars can be assessed semi-quantitatively in the manner described above. Nutrient broths can also be assessed as described above by inoculating a target organism and assessing microbial growth using a semi-quantitative method.

6.11 Additional media and reagents

A number of widely used additional media and reagents are referred to in The Microbiology of Drinking Water, The Microbiology of Recreational and Environmental Water and The Microbiology of Sewage Sludge where they are not described in detail. These are included here for reference. Where reference is made to pH adjustment this is not usually necessary when using complete commercial media. Media prepared from ingredients may require adjustment to a little above the required final pH to compensate for changes occurring during autoclaving. Unless otherwise stated, the accepted range of any measured value is the stated value ± 5%[17].

6.11.1 *Nutrient broth*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Sodium chloride	5 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.4 ± 0.2. Dispense into suitable containers and sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile medium may be kept for one month. Test tubes or universal containers containing the medium may be stored at temperatures between 5 ± 3 °C for up to one month.

6.11.2 *Nutrient agar*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Sodium chloride	5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.4 ± 0.2. Sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile medium may be kept for one month. Alternatively, allow the solution to cool, distribute in Petri dishes and allow it to solidify. Petri dishes containing the agar medium may be stored at a temperature of 5 ± 3 °C for up to one month, protected against dehydration.

6.11.3 *MacConkey agar*

Peptone	20 g
Lactose	10 g
Bile salts	5 g
Sodium chloride	5 g

Neutral red	0.075 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.4 ± 0.2. Sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile medium may be kept for one month. Alternatively, allow the solution to cool, distribute in Petri dishes and allow to solidify. Petri dishes containing the agar medium may be stored at a temperature of 5 ± 3 °C for up to one month, protected against dehydration.

6.11.4 *Blood agar*

Beef extract powder	10 g
Peptone	10 g
Sodium chloride	5 g
Agar	15 g
Defibrinated horse or sheep blood	50 – 100 ml
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.3 ± 0.2. Sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile medium may be kept for one month. Alternatively, allow the solution to cool to 45 – 50°C and add the horse blood warmed to room temperature. Mix carefully avoiding bubbles, distribute in Petri dishes and allow the agar to solidify. Petri dishes containing the agar medium may be stored at a temperature of 5 ± 3 °C for up to one month, protected against dehydration.

Note 1: The basal medium without the blood is known as blood agar base and may be used as an alternative to nutrient agar for the general cultivation of bacteria. Columbia agar base may also be used as a base for blood agar.

Note 2: Haemolysis may be easier to see if blood agar plates are ‘layered’. A thin layer of blood agar base is poured into each Petri dish and allowed to set. A second thin layer of blood agar is then poured onto the base.

6.11.5 *Brain heart infusion broth*

Calf brain infusion solids	12.5 g
Beef heart infusion solids	5 g
Proteose peptone	10 g
Glucose	2 g
Sodium chloride	5 g
Di-sodium phosphate	2.5 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.4 ± 0.2. Dispense into suitable containers and sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile medium may be kept for one month. Test tubes or universal containers containing the medium may be stored at a temperature of 5 ± 3 °C for up to one month, protected against dehydration.

Note The medium may be solidified by the addition of 15 g/l agar.

6.11.6 *Quarter strength Ringer's solution*

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride (hexahydrate)	0.12 g
Sodium bicarbonate	0.05 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.0 ± 0.2. Dispense into suitable containers and sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile solution may be kept for three months at ambient temperature in the dark. See section 6.8 for guidance on dispensing the solution for serial dilutions and the storage of dispensed solution.

6.11.7 *Maximum recovery diluent*

Peptone	1 g
Sodium chloride	8.5 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.0 ± 0.2. Dispense into suitable containers and sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile diluent may be kept for three months at ambient temperature in the dark. See section 6.8 for guidance on dispensing the solution for serial dilutions and the storage of dispensed solution. Once opened, any unused diluent should be discarded as it will support microbial growth.

6.11.8 *Saline solution*

Sodium chloride	8.5 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.0 ± 0.2. Dispense into suitable containers and sterilise by autoclaving at 121 ± 3 °C for 15 minutes. Sterile solution may be kept for three months. See section 6.8 for guidance on dispensing the solution for serial dilutions and the storage of dispensed solution. Saline solutions required for slide agglutination tests or the preparation of Gram stain smears need not be sterile.

6.11.9 *Oxidase reagent*

N,N,N',N' -Tetramethyl-p-phenylene diamine dihydrochloride	0.1 g
Water	10 ml

Dissolve the ingredient in the water and use immediately. Dry powder may be dispensed into suitable containers and stored at a temperature of 5 ± 3 °C for up to one month. The reagent should be prepared fresh daily and discarded when it becomes purple in colour.

6.11.10 Catalase reagent

Hydrogen peroxide 30% w/v	1 ml
Water	9 ml

Mix the ingredients and use immediately. The reagent (3% w/v) should be prepared fresh daily and discarded once it has been used. Hydrogen peroxide solution should be stored at a temperature of 5 ± 3 °C.

Note: Hydrogen peroxide solution will cause burns and should be handled with appropriate precautions.

6.12 Gram stain

Grams' stain is a traditional and widely used means of differentiating bacteria into two distinct groups on the basis of staining characteristics visualised by microscopy. Bacteria are generally described as Gram positive or Gram negative. Gram positive bacteria possess a thick peptidoglycan layer as part of their cell wall structure which stains permanently blue/violet when exposed to stain. Gram negative bacteria have cell walls comprised of a thinner peptidoglycan layer with high lipid content which fail to retain the stain when challenged with a decolourising agent.

When viewing slides prepared for microscopy Gram negative bacteria are made visible by the use of a red/pink counterstain. Bacteria are usually further differentiated during microscopy on the basis of their morphology being either rod (bacillus) or round (coccal) shaped.

There are a number of method varieties used, the details given below are based on the modified Hucker method and are provided as an example only. The staining process is often performed manually however, for health and safety reasons and for consistency it may be automated, particularly where larger numbers of slides are being prepared. (See also Section 5.16 Microscopes). In addition to the reagents listed below the following general laboratory equipment is required: Glass microscope slides, pipettes, inoculating loops, forceps and Bunsen burner.

6.12.1 Reagents

Ready to use staining reagents are commercially available. Laboratories wishing to prepare the reagents themselves should refer to a standard textbook, for example Cowan and Steel's 'Manual for the identification of medical bacteria.'

Sterile distilled water
Crystal violet stain (1% m/v solution, alternatively, Methyl or Gentian Violet may be used). When freshly prepared the stain should be filtered before use
Grams' or Lugols' iodine solution
Decolourising agent, Ethanol (96%) or Acetone
Counterstain, for example Safranin (0.5% m/v solution)
Immersion oil

6.12.2 Slide preparation

A slide is labelled with the sample details, for example using the frosted end of the slide if present or a glass marking pen. The surface of the slide is clean and dry. A small drop of water or saline solution is delivered onto the slide. Using a sterile microbiological loop a small portion of bacterial growth from a single colony on a plate is picked off and transferred to the water drop.

Using the loop the colony material is gently emulsified in the water or saline avoiding the creation of aerosols. The resulting smear should be slightly cloudy and homogeneous. The drop size, area of smear, and/or inoculum can be adjusted to achieve optimum results.

Ideally colony material from fresh cultures, grown for example on Nutrient agar or similar, should be used since older cultures can give ambiguous results.

The slides should be allowed to air dry on a flat surface, for example on an incubator shelf, before fixing. To fix the smear the slide should be held using forceps and the underside of the slide then passed once carefully through a Bunsen flame. A second passage may be required if the smear was not completely dry however, excessive heating should be avoided as this may damage the cells.

The objective is to produce a monolayer of bacteria in a smear on the slide, sufficiently dense for visualisation but sparse enough to reveal characteristic morphology. Fixed slides should be allowed to cool thoroughly before staining.

6.12.3 Staining Procedure

The staining process should be performed close to a suitable sink or waste disposal area, and appropriate gloves worn throughout. A calibrated timer should be used to monitor time periods during the staining process.

- Flood the slide with Crystal Violet stain and leave for 30 – 60 seconds.

- Decant the Crystal Violet and gently rinse under running tap water. Excessive flow and prolonged rinsing should be avoided as these may disrupt the smear and stain. In some instances, it may be preferable to skip the water rinse and rinse the Crystal Violet off directly with Gram's or Lugol's iodine solution.

- Rinse off residual water with Gram's or Lugol's Iodine solution and flood the slide with iodine solution, leaving for 30 – 60 seconds.

- Rinse the slide briefly under running tap water.

- Hold the slide at an angle over the sink and carefully decolourise with a few drops of decolourising agent. Allow the decolourising agent to run down the surface of the slide, washing away the stain. Decolourisation occurs very quickly and the solution should not be left on the slide.

- Immediately wash the slide gently but thoroughly under running tap water to remove residual decolourising agent.

- Flood the slide with counterstain and leave for 30 – 60 seconds.
- Rinse briefly under a gentle flow of running tap water.
- Drain the slide and blot gently or air dry in a vertical position.

Examine the slide under the microscope using bright field illumination and a X100 oil immersion objective lens.

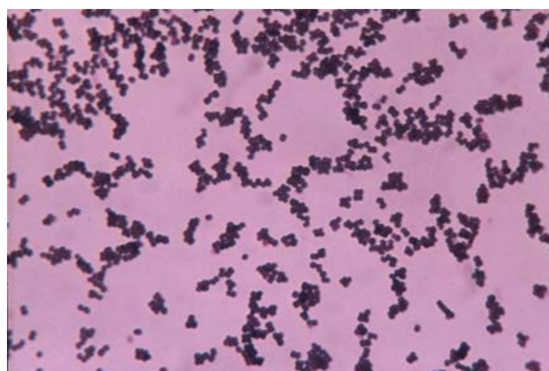
6.12.4 Quality control

Commercial stains should be stored and used according to the manufacturers' instructions and should not be used beyond their stated expiry date.

With each batch of slides undergoing Gram staining the laboratory should check reagents to ensure correct staining characteristics are being obtained. As an example slides of *Escherichia coli* (Gram-negative rods) and *Staphylococcus aureus* (Gram-positive cocci) can be stained and included with each batch as in Figures 6.12.1 and 2 below. Details of the quality controls performed should be recorded.

Figure 6.12.1 Gram stain showing *E. coli* - Gram negative rods

Figure 6.12.2 Gram stain showing Staphylococcus aureus - Gram positive cocci



6.13 Microbial identification by MALDI-TOF

In the context of microbiological analysis MALDI-TOF is a diagnostic mass spectrometer (MS) technique for the rapid identification of bacteria starting from colonies cultured from samples. It is increasingly being used in conjunction with other laboratory processes to aid in the identification of bacteria[(21)], often as an alternative confirmatory technique.

Isolates are cultured to achieve discreet colonies which may be prepared and fixed to a target plate using a protective matrix. These are then analysed on the MALDI-TOF instrument which bombards the fixed colony with a laser, “vaporising” it and freeing protein molecules to become charged. The charged molecules are propelled through the MS tube to a detector. The mass of each protein molecule produced influences its “time of flight” along the MS tube. The make-up of protein molecules within the sample is then analysed by the instrument software, using the measured “times of flight”, and expressed as a profile or spectrum. Algorithms are then used to match the profile to a database of spectra, using proprietary software. The software determines an identity for the organism based on matches to profiles within the database. Different software platforms are used by manufacturers of these instruments using proprietary algorithms and databases with their own criteria for microbial identification

A score is usually given indicating a confidence level for the identification. Typically, identifications to the species level can be achieved with lower confidence scores indicating identifications suitable to the genus level. An advantage of the technique is that strains presenting as atypical by traditional biochemical typing methods may be recognised by MALDI-TOF providing greater scope for identification, particularly as database libraries are extended over time.

As with any new technique its performance should be verified and its performance assessed over the range of variables encountered in the course of the laboratories testing routine (including for example sample matrices and growth media) to identify any adverse impacts on the identifications achieved. Guidance on appropriate verification can be found in section 9.4 and 9.5 and further information on the technique, its application and verification for use in the laboratory, in Cook N., D'Agostino M. & Thompson K. C[(22)].

7 Analytical techniques

There should be appropriate documentation of all analytical procedures in current use. Such documentation should be controlled, including for example with an issue number, date of issue, pagination and known document circulation. Where methods are revised, the original method should be stored for potential future reference. A master copy of all methods should be kept in a secure place and designated 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 or environmental microbiology section).

Documented analytical methods should include detailed descriptions of the micro-organisms being detected by the method, 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 and verification of performance purposes) and the practical details of how validation and verification trials are conducted should be recorded. Methods should include reference to organisms used as positive and negative controls for isolation procedures and confirmatory tests, as well as the quality control tests for assessing media and the day-to-day operation of the method.

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. A new test procedure should only be adopted after it has been shown that it is equivalent to, or better than, the old test procedure. The new test procedures should be fully documented and a complete record kept of all validation and verification data generated. Details on how to validate and verify performance of new test procedures are given in section 9.

7.1 Standard operating procedures

Laboratory methods should be described in detail 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. Some laboratories may choose to keep their media and reagent activities separate and have a separate set of documented operating procedures. A suitable format for a standard operating procedure is given in section 7.1.1. This format has been used for the description of analytical methods published in this and related series.

7.1.1 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, environmental waters or sewage sludge in terms of water quality, indicator value, pathogenicity and occurrence are also, generally, given.

2. Scope - Details of the sample matrix[23], for example type of water or sewage sludge, 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[8] 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) is given in general guidance to laboratories section 5.

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 or masses, special storage conditions prior to analysis, and pre-treatment or dilution preparations.

9.2 *Sample processing* - Details are given on the technique (i.e. 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.

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 along with method specific routine QC requirements.

13. References - Technical and allied references relevant to the method are given.

7.2 Methods for the isolation and enumeration of indicator and other organisms

Two principal procedures for isolating and enumerating organisms are commonly used in water microbiology. These are the membrane filtration and the multiple tube most probable number (MPN) techniques. The media and incubation conditions differ with both methods according to the organism being sought. In practice, for most conventional

testing of clean and environmental 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. For highly turbid samples, for example some wastewaters and semi-solid or solid materials, however, the MPN procedure may be a more appropriate technique. In addition to these two techniques, some analyses are performed by direct plating (for example, pour plate or spread plate) methods, heterotrophic plate counts in drinking water would be one example. Direct plating is also a method option for *E. coli* analyses in sewage sludge microbiology.

7.2.1 Preparation of samples

The volume or mass 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 and provided it is within 24 hours of sampling and has been stored appropriately, used for additional or repeat tests in the event of unexpected high counts or possible mishaps. The enumerated counts obtained for additional or repeat tests performed on stored samples older than 24 hours 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 of water samples, 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 water or sewage sludge samples are required, they can be prepared at this stage. Sterile solutions of quarter-strength Ringer's solution or maximum recovery diluent (see sections 6.11.6 and 6.11.7) 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. The bottle cap is then replaced without touching the inside of the cap or the neck of the bottle. 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 the next dilution is prepared. Tolerances for pipette performance are set out in section 5.20. A sufficient quantity of each dilution should be prepared to enable all tests to be carried out.

A minimum of two dilutions should be used for environmental samples where dilutions are required. Where samples have not previously been tested, and the likely concentration of organisms is unknown, three dilutions may be necessary.

7.2.2 The multiple tube most probable number (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 (i.e. the results are negative) and some of the tubes exhibit characteristic growth (i.e. 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 of sample. Confirmation, that positive results are due to the growth of the targeted organism sought, can be obtained by sub-culture to appropriate confirmation media. There are commercially available MPN systems based on addition of the sample to reaction pouches which, when sealed, divide into 50 or more “wells”. The greater number of wells available for inoculation, compared to the traditional tube method, results in a more accurate MPN estimation over a wider MPN range.

The multiple tube method is particularly suitable for the examination of sludges and waters containing sediment.

7.2.3 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 usually inhibited, but if they are present, they can be readily distinguished by their colonial appearance. The colonies of the target organism sought are counted and the result, taking into account any dilutions made, for water samples 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 and if contamination is suspected. Spare funnels as required can be disinfected for example 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. Disinfection of funnels by immersion in boiling water may not be sufficient when spore forming bacteria, for example *Clostridium perfringens*, are sought. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should be processed after non-polluted samples. For recreational waters, sewage sludges and similar polluted samples always process the highest dilution first and then process sequentially the series of dilutions down to the lowest dilution to be analysed.

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, Legionella* 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 have a shelf life and should not be used beyond their expiry 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 quality paper fibres, and be uniformly absorbent and free from any toxic substances that may 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.

7.2.4 Advantages and limitations of the membrane filtration method

The key advantage of the membrane filtration technique, compared to the multiple tube MPN technique, is the speed with which results can be obtained. For example, presumptive coliform bacteria 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, however, 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 similar principle applies when testing dilutions of recreational waters and sludge. Whilst high dilutions may filter well, lower dilutions may contain significant particulate material. 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.

7.2.5 Alternative confirmation techniques

The individual test methods in this series, each describe the confirmation requirements of the target organism being sought. Confirmation methods have traditionally been based on sub-culture to selective and/or non-selective broth or solid media, staining and slide examination (such as Gram stain) and biochemical tests or serological analysis.

Novel techniques, for example MALDI-TOF-MS (Section 6.13), are becoming more commonplace in laboratories and have been demonstrated to be beneficial and effective as alternatives to traditional approaches. As with all new methodologies, laboratories using them should be able to demonstrate the accuracy of application and interpretation in their hands.

7.3 Statistical considerations

Statistical analysis of microbiological results must start with a clear understanding of the methods used to obtain the data and the context of the water sampling. This requires an appraisal of all aspects of the accuracy of the results. The following discussion primarily considers accuracy with respect to water samples. Additional aspects may need to be considered for other matrices (for example, sewage sludge and environmental sediments). For example, consideration may need to be given to the ability to achieve a dispersion of organisms in solid or semisolid matrices (i.e. effective homogenisation) prior to analysis.

7.3.1 Accuracy

For the purposes of this section accuracy is the combination of both random and systematic errors to indicate the likely deviation from the true value.

The accuracy of a microbiological result is an important issue and the result cannot be interpreted without some awareness of it. Experience and understanding are needed to allow an assessment of the reliability of a result. The basic definition of accuracy is the degree of agreement of a result of a measurement process to the ‘true’ result.

Each sample yields a result which is quantitative to some degree. It may be a presence/absence test where either zero or one-or-more organisms are found; or it is a test where the result is a number. The latter, a numerical result, can be a count of organisms detected (e.g. by colony growth) or be a most probable number (MPN) derived from a series of presence/absence results from subsamples.

This section considers the accuracy of numerical results, although many of the principles have relevance to presence/absence results.

There are several aspects that contribute or influence the accuracy of a measurement:

(i) There is the accuracy in terms of how well the result answers the question that was being asked when the sample was collected (for example how many *E. coli* does this water source contain?). The variability in organism numbers at the water source can be very large. The accuracy in measuring this depends on *sampling strategy*.

(ii) The accuracy is affected by survival, without multiplication, of target organisms within the sample from the time of collection to the time of processing the sample, i.e. the *stability of the sample or organisms in the sample*.

(iii) There is the inherent accuracy of *the method chosen* for processing the sample.

(iv) There may be sources of inaccuracy introduced by *selecting a sub-portion* of the original sample, if the test procedure is not applied to the whole sample, and by confirming a sub-sample of colonies detected.

(v) There is inaccuracy during the *application and reporting of the whole test procedure* (i.e. the accuracy in applying the method, the quality of equipment and materials together with the expertise of the analyst). As will be discussed, this is the portion of inaccuracy that is equivalent to the uncertainty of measurement (see 7.3.1.5) as used in other disciplines, such as chemical testing.

7.3.1.1 How accurate is the result in representing the source material?

The examination of a single sample gives an indication of the count of relevant organisms in 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, sediment or sludge sampled may not however possess identical characteristics, with respect to microbiological quality, as those present in adjacent volumes of water, sediment or sludge. 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 microbial density in a body of water cannot, generally, be estimated from a single sample. Multiple samples are required before a range, such as a 95 % CI, can be estimated. Such a CI describes the possible range of organisms in the source but assumes that the results for each sample are accurate. CIs about the accuracy of a result itself are discussed later. The only situation where a single sample can give such an estimate is when the organisms are distributed randomly; in this case the appropriate mathematical description is the Poisson distribution which has a single statistical parameter, i.e. the mean, μ, having the same value as the variance. However, there is at present no evidence that microbes are ever randomly distributed in any part of a water system, environment or sewage sludge.

There can be enormous variation in the microbiological quality of untreated waters[24]. 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[25]. 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.

7.3.1.2 How is accuracy affected by collection, transport and storage of the sample?

These factors are largely outside the scope of this document, but careful collection of samples together with appropriate storage during transport and storage at the laboratory will minimise any effect on microbial numbers in samples. Guidance on the collection, transport and storage of microbiological samples is given elsewhere in this series[26,27]. The accuracy should be maximised by the choice of good procedures, expertise of staff and appropriate quality assurance checks.

7.3.1.3 Accuracy of the chosen method

Samples of treated drinking waters should not contain indicator organisms. Very small numbers of such organisms in samples of water are capable of being detected, and enumerated with good precision (see 9.1.2), by methods described in this series. Untreated waters, sediments and sewage sludges, 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[28] and comparison of results obtained using a reference method. For drinking waters a detailed protocol for undertaking such a comparison, together with examples, is described in section 9. A similar approach may be applied to other matrices.

Any bias or variability in the performance of the chosen method will, thereafter, affect all results, but be a hidden factor. It is important that the adequacy of the method is kept under review as part of AQC procedures (see section 8).

7.3.1.3.1 Accuracy of a 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 this particular method allows. Further, non-method related imprecisions are possible as described in following sections. These include sample dilution, selecting colonies for confirmation and uncertainty of measurement (see 7.3.1.5).

7.3.1.3.2 Accuracy of a 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 volume examined, and extrapolated to the whole sample[29,30,31]. Confidence intervals have been suggested which relate specifically to the likely accuracy of the estimated MPN and reflect the other “counts” which could have given rise to the observed combination of tubes positive and negative. These various mathematical approaches and the principles involved in the estimation of bacterial densities by dilution methods have been reviewed[31,32] and tables have been developed[33,34] which give greater detail. However, in practice, the full extent of the tables are rarely used[35,36] (for example the most probable range information which may be misconstrued as confidence limits).

Widely available computer programmes now enable the determination of the probability of counts associated with each dilution series to be quantified exactly[37,38,39]. While the latest calculation of the MPN shows little discrepancy with previously published values, these new calculations have highlighted two issues: the variability of previously published confidence intervals and, for moderate or high bacterial density, the multiple tube methods which have only 11 or 15 tubes do 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. Methods with large numbers of tubes achieve a clearer MPN, provided the dilution series gives a proportion of negative tubes.

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. This is more closely approached with recently developed multi-well MPN techniques. Apparent differences between results should, therefore, be interpreted with caution.

7.3.1.4 How is accuracy affected if only a sub-portion of the original sample is tested?

The result quoted will be a statement about the numbers estimated to be in the sample. Usually only a sub-portion of the sample is examined because of the requirements of the method and/or the sample needs to be diluted. The chosen method whether it is membrane filtration, plate count or multiple tube, will use a specified volume of water. The sample needs to be thoroughly mixed in the laboratory before the required volume is drawn off. The objective of mixing is to achieve a random distribution of the organism within the sample so that the number per 100 ml (or whatever volume is being analysed) is as close as possible to the average number per 100 ml in the whole sample. An example of random distribution is illustrated in section 9, Figure 9.1.

It would be possible, in theory, to make statistical estimates of the likely numbers present in the original whole sample when only a specified portion of the sample has been examined. This would give a specific 95% CI relating just to this aspect of imprecision. This is not usually attempted but it is accepted that with good technique the result will be as representative as possible. If a sample requires dilution then this reduces the proportion of the sample examined and attempts have been made to illustrate the likely numbers in the undiluted portion[40]. Some examples from the referenced work are given in Table 7.1. The background to these examples is described in the next three paragraphs, illustrating the potential imprecision introduced by the inherent random variation of numbers of organisms.

It is usual practice to report the bacterial count of targeted indicator bacteria, 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 50 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.

Confidence intervals of numbers present in an original volume V, given that x organisms have been observed in a sub-volume v, can be calculated on the assumption of random variation throughout V when test volume v was drawn off[40].

Heterotrophic bacteria numbers in water analysed by the pour plate or spread plate method are typically quoted per millilitre of sample, and any dilutions prepared will have a similar effect. Dilutions prepared from solid or semi-solid matrices (for example sewage

sludge) will be similarly affected and will have the added contribution of the degree to which homogenisation has been effective of the sample prior to preparation of dilutions.

Table 7.1 Illustrations of estimated count (EC) per 100 ml and 95 % confidence intervals (CI) for the number of organisms reported in 100 ml of sample, where a sub-sample is examined, following dilution

Number of organisms	10-fold dilution		100-fold dilution	
found in sub-sample	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

The variability introduced by dilution is likely to be relatively small compared with the variability in bacterial density in environmental waters, sediments and sewage sludges, where numbers are sufficiently high to require dilution of the sample before examination. Confidence intervals, as shown in Table 7.1, 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.

7.3.1.4.1 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 be sufficient to provide an acceptable level of accuracy. This usually requires 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 a high proportion of the colonies are expected to confirm as positive. The colonies selected for confirmation should also be representative of the differing morphologies present on the membrane filter.

Alternatively, if the aim is to demonstrate the presence or absence of the targeted organism, then a different approach may be chosen. 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 do not give a positive confirmation 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 50 colonies were observed on the filter, and 10 colonies were selected at random for testing, and 5 of these colonies were confirmed, then the estimated confirmed count would be 5 x 50 / 10 = 25. The 95% CI, which reflects only the confirmation uncertainty and no other imprecision, for this result of 25 is 9 to 41 (see Table 7.2). The CI is calculated as follows:

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[38]. Some examples are shown in Table 7.2.

Table 7.2 Variation in the 95 % CI with variation in the proportion of tested colonies confirmed

Colonies observed (presumptive count) i.e. N	Number tested, i.e. n	Number confirmed, i.e. 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

Wherever possible the number of colonies to be tested should be selected such that the confirmed count is a whole number. Where this is not the case the confirmed count should be rounded to the nearest whole number (for example, if there are 8 presumptive (N) colonies and 3 are tested (n) of which 1 confirmed (x) then the confirmed count is 3).

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, sediments and sludges, 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 potentially 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. For example, it may be better to take more samples analysed by a reliable presumptive test than fewer samples analysed by a less reliable test requiring a greater number of confirmation tests.

7.3.1.5 Inaccuracy introduced by the application of methods to the selected portion of water (uncertainty of measurement)

Once the water has been drawn off and processing starts with the chosen enumeration method then, at each stage, random or technical errors can occur which may affect the final result. These can be referred to as uncertainty of measurement (UM) which is defined as:

> a parameter associated with the result of a measurement, that characterises the dispersion of the values that can reasonably be attributed to the measurand[41].

These errors should be minimal in a laboratory with good practice (for example with trained staff, well controlled methods, calibrated equipment and a comprehensive quality assurance programme, see section 8). They cannot be measured for an individual sample, and can be difficult to identify, even when special studies of replicate testing are undertaken, because of random variation in numbers of organisms present in different replicates. It is suggested that a typical QC programme includes enough replicate testing to allow assessment that these errors are acceptably small by checking that the variation is not greater than random as outlined in section 8.2. Further discussion on UM and guidance on this is available in BS 8496[42].

7.3.1.6 Summary of accuracy for sample processing within the laboratory

It is now well understood that the natural random variation in microbial numbers, even in a well-mixed water, will be the dominant factor[24,36]. This makes it much more difficult for microbiologists (compared with chemists or physicists) to describe fully the attributes of a water. It makes it even more difficult to measure inaccuracies caused by laboratory procedures. Good practice should keep these inaccuracies to a minimum.

As has been described, the laboratory is responsible for storing the sample correctly, extracting the required portion for testing (which may include dilution stages), applying the chosen method and reporting the results. All stages may introduce inaccuracy. Experiments can be undertaken to measure, on average and with specially selected samples, particular affects (for example dilution process, differences between incubators, between analysts etc.) but these may not be practical in smaller laboratories.

In all laboratories it is essential that a comprehensive QA programme is in place as an ongoing check on storage conditions, mixing the sample and selecting the portion for testing. Examples of natural variation (and, therefore, one inevitable component of inaccuracy), which affects examination of sub-portions have been presented here. Sound knowledge and application of the chosen method, supplemented by continued proficiency testing, will help to minimise any inherent inaccuracy. The cumulative errors, random and systematic, during application of the method to the portions examined (the UM) cannot be

measured routinely but QC programmes of replicate testing can assist in checking that they are kept acceptably low.

7.3.2 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.

For some environmental and effluent water samples, however, prescribed limits may be set as simple pass/fail criteria. An understanding of the implications of the accuracy of a method in fairly allocating a result based on a single measurement into a pass or fail category is necessary.

For the analysis of sludge a result is often based on the average of several replicates, and any bias in a method may have a cumulative impact on the reported result. Consistency in the performance of a method is important in ensuring ongoing compliance with a standard.

7.3.3 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 within the water source, uncertainty and error due to the choice and application of methods.

Laboratories are required to be aware of accuracy. ISO 17025[2], specifies that “Testing laboratories shall have and shall apply procedures for estimating uncertainty of measurement”. This is difficult to apply to water microbiology because the distribution and behaviour of microbial cells in water is not uniform. BS 8496[42] provides practical guidance on how to interpret and implement these requirements within the context of a water microbiology laboratory.

Each laboratory should accumulate information on accuracy within the laboratory, using special studies and/or quality control results, and prepare a statement which can be made available to clients upon request.

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”.

Membrane filters or agar plates where overgrowth by competing organisms makes a count of target organisms impossible or uncertain, no count can be reported. A count should be obtained from another dilution with an acceptable count of target organisms if available otherwise the test is void.

For environmental samples analysed by membrane filtration, it is customary for laboratories to report counts exceeding the upper limit for counting as a greater than value, for example >100 at the dilution used. In some instances, where the count is just above the limit, it may be possible to estimate the count and this should be clearly identified when reporting such a result.

Where analyses are undertaken in relation to regulatory or other guidance standards the results should be reported in the units specified in the legislation or guidance. Where results have been obtained in a dilution series or MPN test they should be reported to the nearest whole integer.

8. Quality control

Quality control should be understood and applied in the context of a comprehensive quality assurance programme covering every element of the process leading to the reporting of a microbiological result. Both qualitative and quantitative controls are essential components of such a programme. They can be applied at various stages to test the integrity of individual or multiple elements of the analytical process. Quality controls can, for example, include:

- demonstrating the suitability of a prepared medium, or reagent
- use of quantitative reference materials as simulated samples and,
- participation in a scheme designed to test the whole process such as the analysis of an external quality assessment sample
- System suitability checks to verify performance of instruments and equipment

A combination of internal and external quality controls is required for a comprehensive approach.

Application of appropriate internal quality controls is necessary to systematically check each step of the process. This should ensure that a laboratory is capable of isolating, accurately identifying and enumerating target micro-organisms in a sample, while avoiding contamination of samples with extraneous micro-organisms.

8.1 Internal quality control

This consists of including quality control samples to the isolation, enumeration, identification and confirmatory procedures in use for real samples. Quality control samples should contain micro-organisms similar to those being 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 non-target organisms and sterile samples, no micro-organisms will be found. The control procedures should be undertaken with each batch of samples incubated, for each incubator used and, when reasonably practical, each analyst involved for that batch of samples. Control samples may be prepared separately but should, in every other respect, be processed in the same way as samples, being analysed as part of the batch of samples to which they relate. 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. For environmental samples, inclusion of analysis of a sample in duplicate by each analyst on each day may also be appropriate (See Section 8.2).

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 at the appropriate levels should, wherever possible, be produced from first generation cultures derived from a national collection of freeze-dried organisms. Alternatively, controls can be utilised either directly or following a rehydration procedure using commercially available reference materials. Control organisms should be derived from a pool of peer-accepted strains which exhibit typical growth patterns and biochemical reactions irrespective of their original source. A suitable list of strains can, for example, be found in EN ISO 11133[20]. Rehydration and dilution should be undertaken

with maximum recovery diluent or a similar appropriate diluent to achieve suitable numbers of organisms. 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 if possible to avoid and in any event minimise the number of sequential sub-culture operations of the chosen reference strains. The reason for this is to reduce the risk of introducing contaminating organisms and because the biochemical characteristics of some organisms 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.

8.2 Quantitative internal quality control

In addition to qualitative checks with positive, negative and blank control samples there should be checks on the enumeration procedures[43]. In principle two approaches can be considered. These are the use of appropriate reference materials and the use of split sub-samples from a source known to contain the target organism. However, it should be noted that the information derived from these approaches, and its application, is different. Reference materials are generally used as preparations, either internally generated from cultures or obtained externally as commercial products, having, within reason, a known organism count. The split sub-sample approach compares the two counts obtained from a sample of unknown count and primarily tests the reproducibility of the analysis performed (see section 8.2.2).

If automatic counting instruments are used these should be tested and calibrated against reference materials having known certified 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 may better be described or classified as probably out of control rather than definitely out of control. Hence, for microbiological purposes, the term “guidance chart” has often been used where response lines, rather than action or warning limits, are applied to trigger further investigation or remedial action as appropriate. In this case the use of guidance charts could be said to provide a tool for continuous improvement, rather than a rigorous check on the validity of analytical data.

The commercial development of quantitative preparations of reference organisms in stable formats, for example Lenticules® and Vitroids™, has significantly improved confidence in the reliability of the Shewhart chart approach. In the context of microbiological enumeration, particularly when using selective media, where, unlike chemistry, the ‘true’ value or count is an unknown the provision by suppliers of mean counts and confidence limits is of central importance. It is still essential however, that laboratories using such materials compare and verify the performance of these materials in their own hands.

Application of these two approaches is described below in more detail.

8.2.1 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 (See Figure 8.2.1 below). 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. For example, initially, a minimum of 20 results (two samples processed on each of ten successive days) may be required to construct a control chart, and a minimum of 60 data points to produce robust control values. However, for microbiological analysis this may not be sufficient and more data sets may be required to reliably establish the mean count and set suitable control limits. 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 may be constructed using transformed data, for example square root or log counts, where such transformation makes the data more closely conform to the normal distribution. Response lines are then drawn on the chart at appropriate intervals.

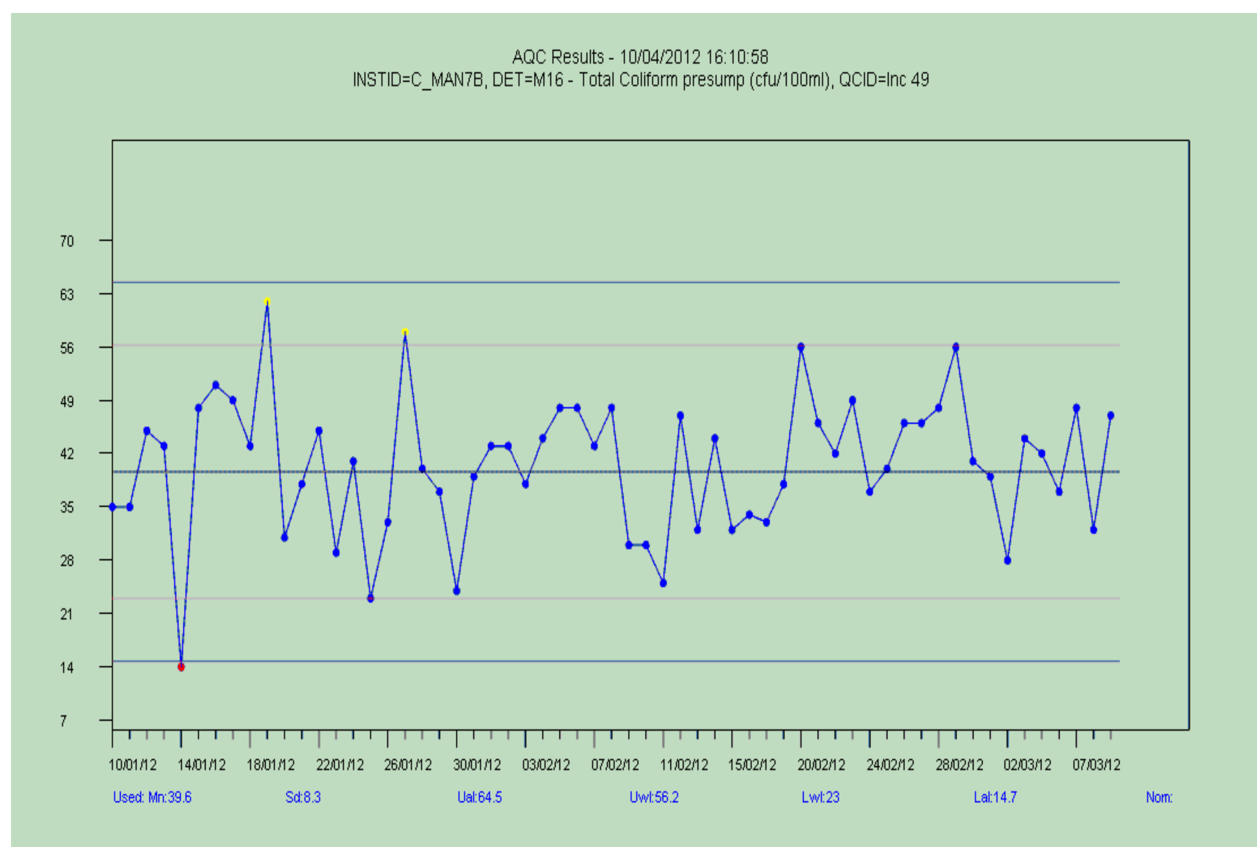
Appropriate response lines may be located at ± 2 standard deviations of the mean (equivalent to upper and lower "warning" limits) and at ± 3 standard deviations of the mean (equivalent to upper and lower "action" limits). However, appropriate response lines should be set on the basis of experience. In some cases, for example Legionella QC, greater variability in performance data may be observed resulting in a larger standard deviation. In this instance guidance charts based on percentage recovery may be more suitable.

Regular samples of the reference material are then processed with routine samples and the counts plotted sequentially. Documented investigation and remedial action when appropriate 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[43].

(i) One count falls outside an action limit: or
(ii) Two out of three successive counts exceeding a warning limit, whether the same side or different sides of the mean: or
(iii) Nine consecutive counts fall on the same side of the mean: or
(iv) Six consecutive counts show a trend that continuously rises or falls

All charts should be checked regularly for correct use and operation and the mean and limits reviewed at least annually

Figure 8.2.1 An example of a Shewhart chart for coliform bacteria showing action and warning limits is given below. In this example an exceedance of the action limit is shown as a red point and of the warning limit as a yellow point.



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 apparent 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. The performance of each batch of reference material should be reviewed regularly whilst in use and retrospectively using the whole data set afterwards and any observations, trends or deviations and lessons learnt documented.

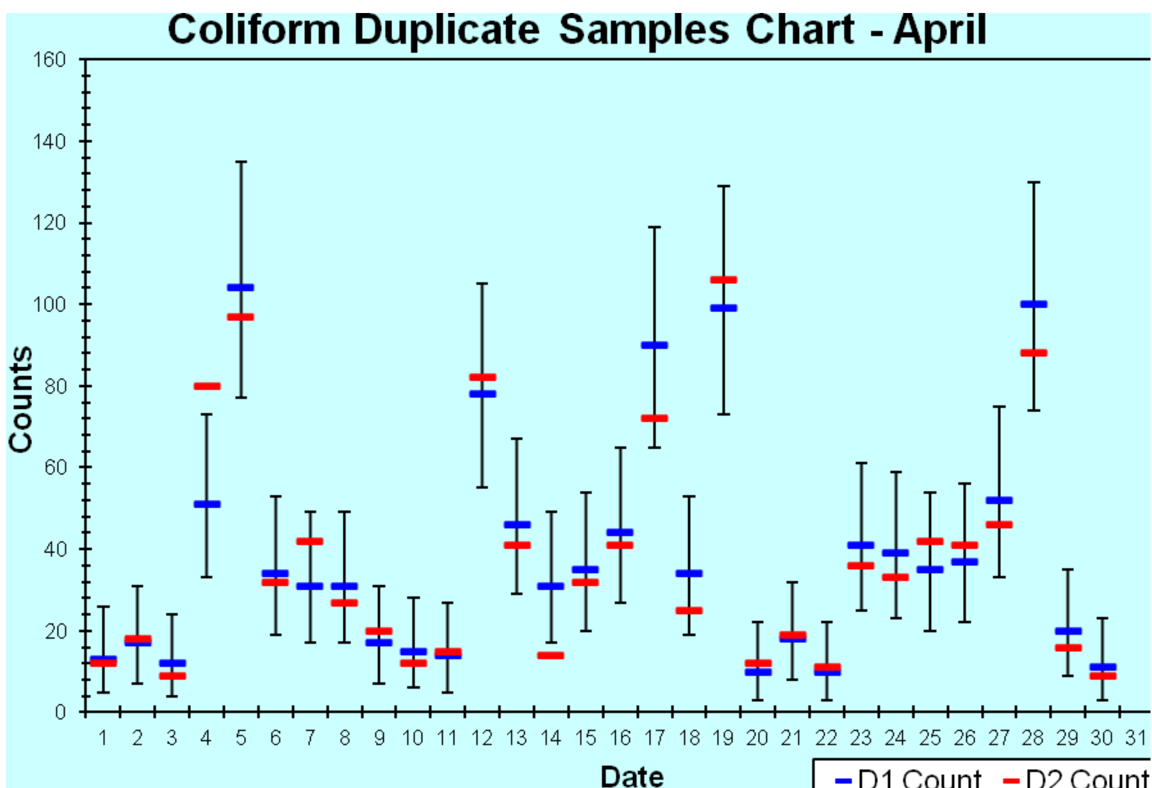
8.2.2 Split samples

Quality control checks for consistency in enumeration can also be made using split samples[44]. 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. An example chart is included below (see figure 8.2.2).

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, (i.e. once in every 20 samples). Anecdotally there is evidence that in practice exceedances often occur less frequently under laboratory conditions due to the difficulty of ensuring that quality control samples are treated in the same way as test samples. Procedures should be in place to ensure that this form of bias is minimised.

Procedures should be adopted within the laboratory to deal with situations that occur too frequently (i.e. 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[44].

Figure 8.2.2 Typical duplicate samples chart for coliforms
The figure shows a typical chart for duplicate samples examined for coliform bacteria for one month using characterised natural river water samples. Note the two occasions that the second count was outside the 95% confidence intervals for the first count on the 4th and 14th of the month. This chart demonstrates that the analytical procedure is under control.



Alternatively, a more approximate statistical approach can be used with paired counts using the Index of Dispersion chi-squared test[45,46]. For paired split samples, the formula for calculating the Index of Dispersion, D, is:

$$D^2 = (x_1 - x_2)^2 / (x_1 + x_2)$$

To construct a guidance chart, the median is plotted, as are values of the 99% and 95% confidence level limits, i.e. for p = 0.05 and p = 0.01 (i.e. 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.

Periodic checks that there is not an excess (>5%) of individual D^2 results exceeding the 3.841 level is a measurement of the repeatability of the method. Conducting reviews of the sum of D^2 over longer periods of time (for example 10-30 results or 2-4 weeks) allows reproducibility to be assessed.

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.

8.3 External quality assessment

Laboratories should participate in an appropriate inter-laboratory external quality assessment (EQA) scheme that involves the examination of samples distributed by an independent external organisation. There are a number of EQA scheme providers to choose from and the choice of scheme should be guided by the sample matrices being analysed, the organisms sought, the range of counts experienced, the frequency and the scope of the analysis performed including, for example, whether confirmatory tests are undertaken.

The laboratory's results can be compared with those intended by the scheme organisers and those obtained by other participating laboratories to provide an independent assessment on the quality of the laboratory's performance. It is essential that the instructions provided by the scheme organiser are carefully followed and that the samples distributed by them 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.

EQA scheme providers usually provide distribution interim and final reports and periodic performance assessment reports covering several distributions to assist participants in assessing their own performance. Self-assessment is essential to identifying poor performance in a timely manner and obtaining maximum benefit from participation. Care should be taken to ensure that data from EQA scheme reports includes details of the different methods used by participants as comparisons to all participants' results, and comparisons only to results for participants using a specific method, may yield different assessments of performance.

Distribution reports generally include an array of statistics derived from participants' data returns as well as scheme providers own intended and self-generated performance results. These may include the mean, median, minimum, maximum and range of participants' counts and the derivation of an 'assigned value' or estimated true count. A scoring system may be applied. In addition to visual examination of scheme reports self-assessment can be facilitated by plotting of participant results against those of the scheme organiser and the mean or median of all participants. Three styles of plot have been suggested which are applied to a parameter and use cumulated results from samples which should have contained that parameter. Each gives visual prominence to a slightly different aspect of monitoring although all three use the same information.

(i) A **line graph** with the time sequence of samples as the x-axis and the count as the y-axis (Figures 8.3.1 and 8.3.2). One symbol plots the participant's results and another the median result calculated from the results returned by all participants. A visual aid is to join up the points with lines although strictly speaking the lines, do not represent anything as there are no "results" being reported between the times of samples. A simple assessment of performance would be to monitor whether there is a consistent trend of results on one side of the median. A satisfactory performance will be when the two lines criss-cross but are seldom widely separate and that the average separation does not increase over time. A large variability of results around the distribution medians could indicate poor control of the analytical process. This type of chart shows all available information – time sequence, actual value of counts and difference between this laboratory and the median. It is, therefore, quite complex to interpret at a glance but can be supplemented by information from the other two types of charts.

Figure 8.3.1: Line graph of laboratory count (blue triangle) and EQA median count (red rectangle) for positive count samples

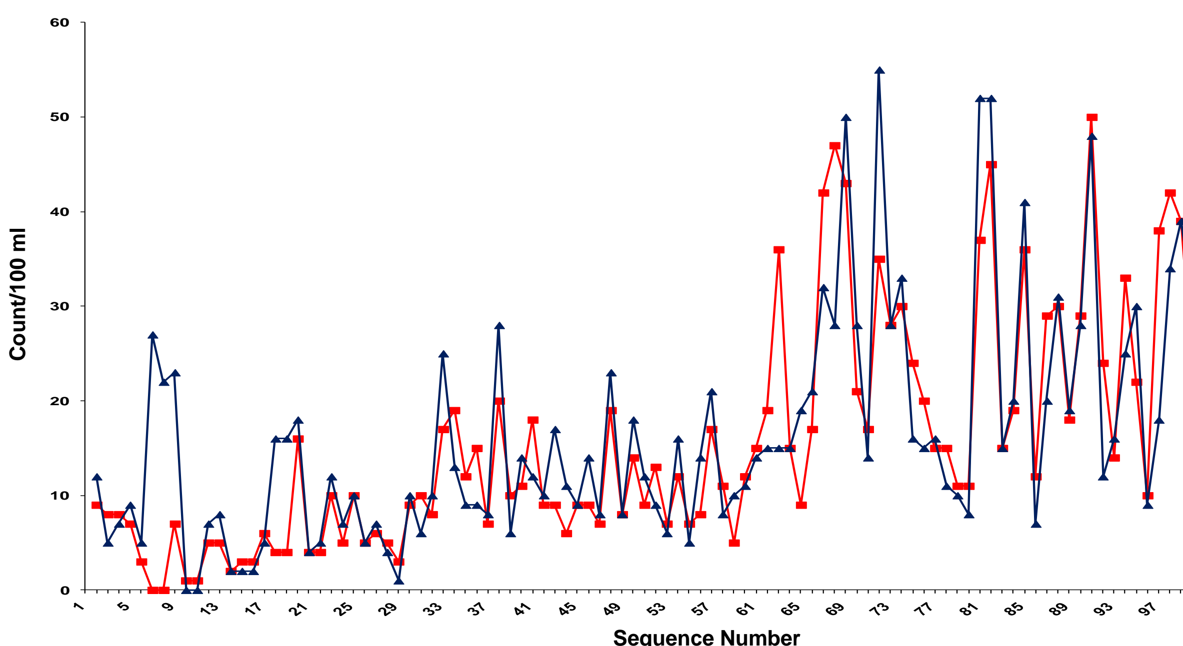
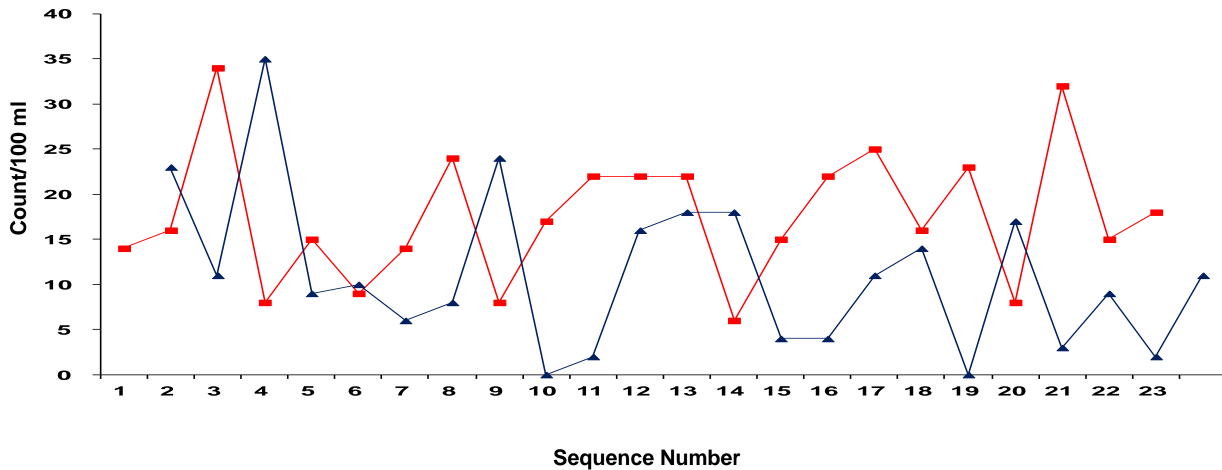


Figure 8.3.2: Line graph of laboratory count (blue triangle) and EQA median count (red rectangle) for positive samples. This graph shows a significant negative deviation from the median counts



(ii) An x/y **scatter plot** of the median count (x-axis) against the laboratory's results (y-axis) (Figures 8.3.3 and 8.3.4). Satisfactory performance is when the scatter is around the diagonal line of equality with approximately similar numbers below and above. It will also be possible to spot whether the pattern changes for higher median values, although allowance must be made for the fact that the magnitude of the scatter will inevitably increase. Random scatter is proportional to the average count (with respect to Poisson distribution). This plot does not indicate time sequence and so will not provide an early warning that performance has changed. However, a laboratory may, for example, notice that they perform adequately with moderate or higher counts but tend to record a deficit with low counts.

Figure 8.3.3: XY plot of laboratory counts compared to EQA median counts with even dispersion of values around the line of equivalence

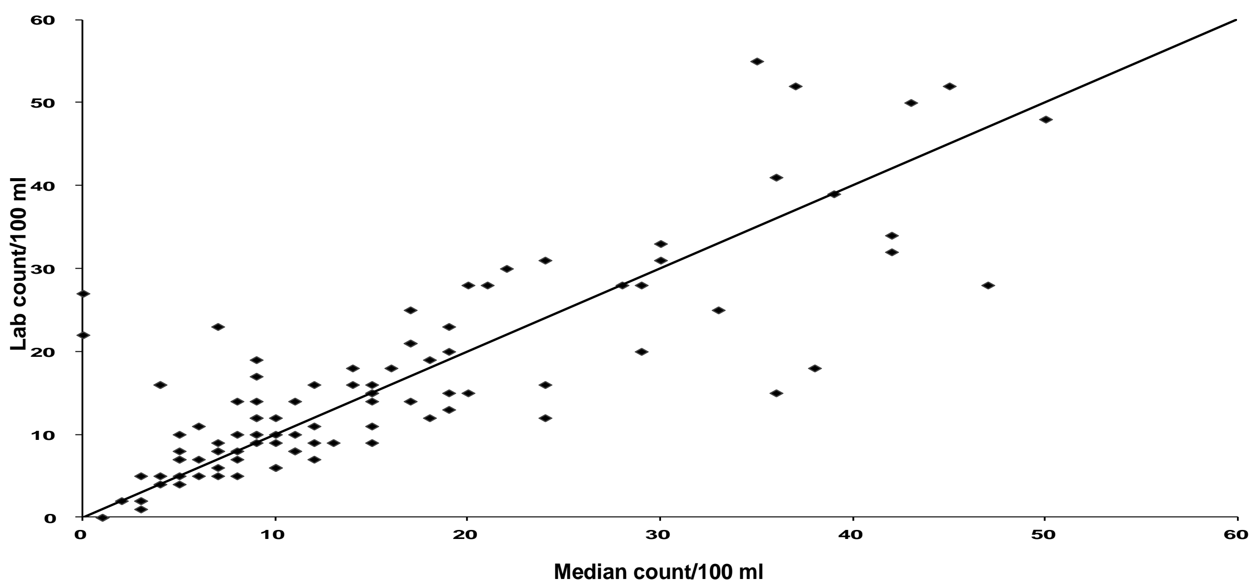
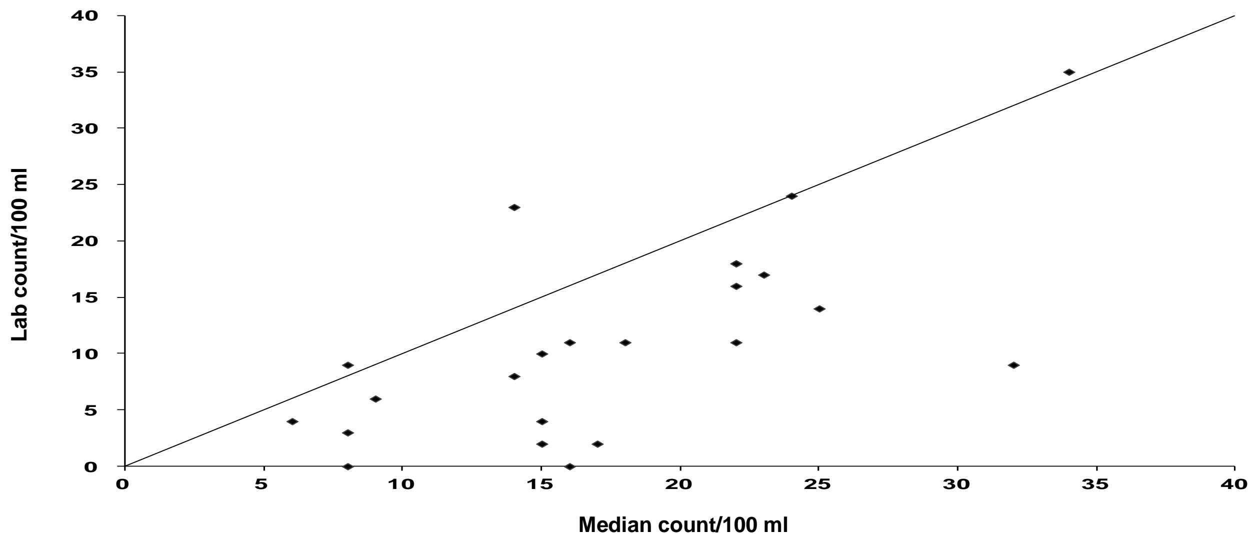


Figure 8.3.4: XY plot of laboratory counts compared to EQA median counts showing significant negative deviation from the median counts



(iii) A **bar chart of differences** (Figures 8.3.5 and 8.3.6). This plots sequentially the absolute value of the difference between the laboratory's result and the EQA median. If high average counts are involved it may be appropriate to use a different scale (for example, square root or logarithmic), but with drinking waters actual counts will be the best. It must be remembered that the choice of scale can make a major difference to the visual impact of the differences, regardless of the true facts. These plots will give a quick visual warning if a laboratory is consistently finding more or less than the average numbers (i.e. whether there is a consistent or marked trend of results on one side of the median). However, small "biases" may not be microbiologically significant and it may not be appropriate to investigate beyond routine checks.

Figure 8.3.5: Typical bar chart of difference of counts from a laboratory compared to those from EQA median counts

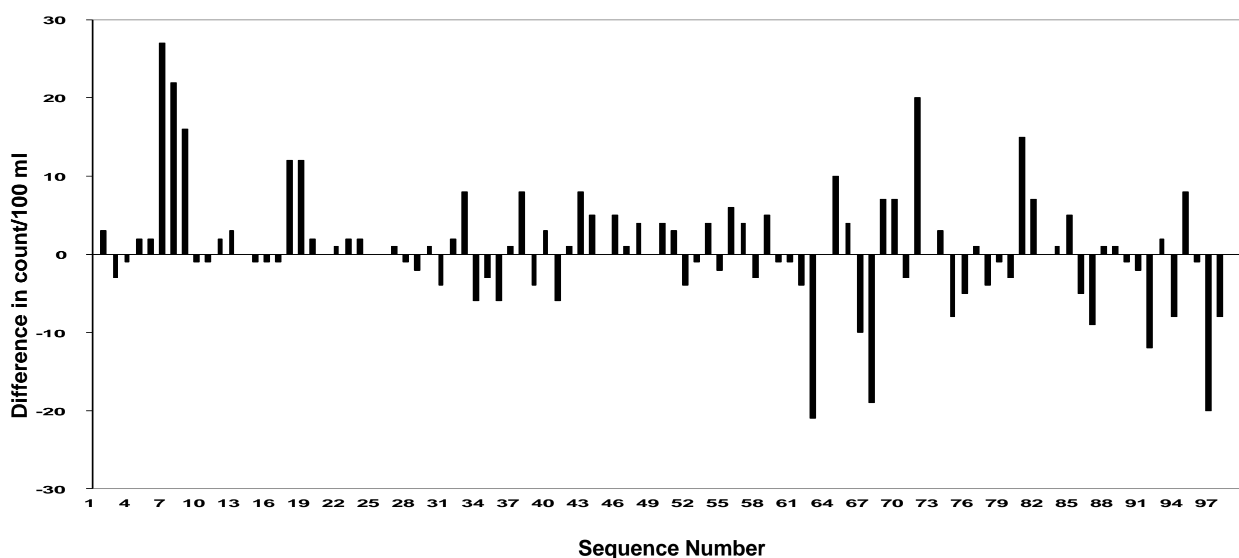
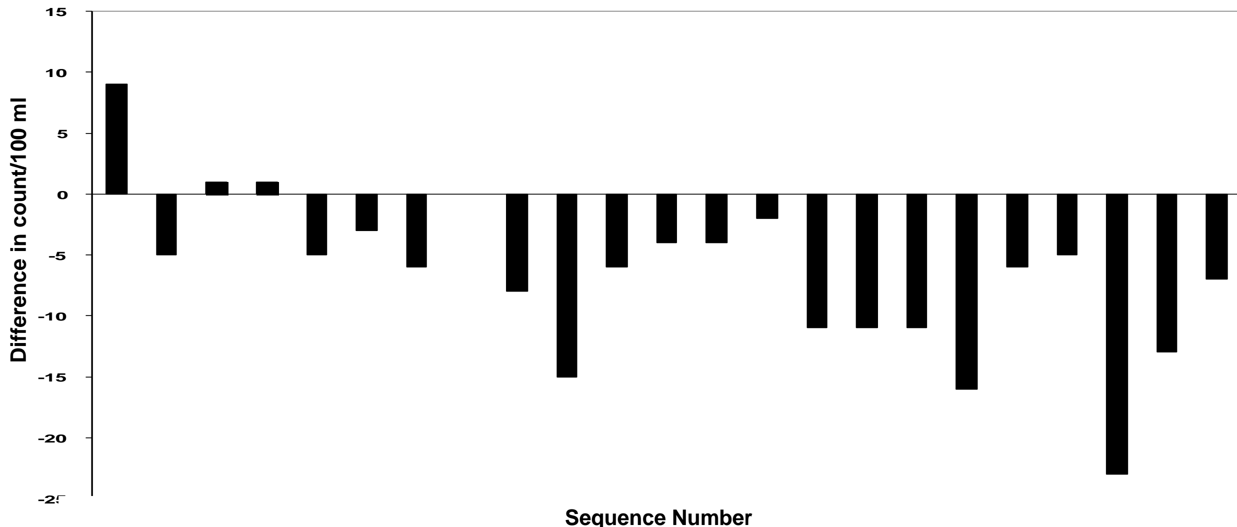


Figure 8.3.6: Bar chart of difference counts from a laboratory compared to those from EQA median counts demonstrating pronounced negative bias



In some cases scheme providers will provide a 'z' score, an approach commonly used for chemistry EQA schemes which allocates a statistically based score assuming that the results are 'normally' distributed about the mean value. It should be noted that counts may approximate to normal distribution in some schemes but this is not universally the case. It has been reported previously [47] that microbiological counts for drinking water schemes are subject to natural random variation and are better described by the Poisson distribution. In practice 'z' scores and measuring longer term trends in performance via 'J' scores[48] may provide useful information on a laboratory's EQA scheme performance. If used it should be used in conjunction with other methods of self-assessment.

The purpose of external quality assessment 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 be used for the purpose of determining whether one laboratory performs better or worse than another participating laboratory.

9 Characterisation, verification of performance and comparison of microbiological cultural methods

Methods for the bacteriological assessment of water and associated materials should be capable of serving their intended purpose, i.e. to detect and/or quantify target organisms or groups of target organisms with adequate precision and accuracy. In certain countries methods for drinking water quality assessment under legislation may be 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 by the International Organisation for Standardisation (ISO), European Committee for Standardisation (CEN), individual national standards organisations (for example BSi, DIN, AFNOR) and the Standing Committee of Analysts (SCA). These methods can be considered as reference methods.

The demonstration that new or alternative methods are at least as accurate and precise as reference methods is, however, a complex procedure. This section describes the three steps involved in the characterisation of a method, verification of its performance in a laboratory and the comparative assessment of the method compared to a statutory, regulatory or laboratory accredited method. This section is based on procedures set out in ISO/TR 13843[28] and ISO 17994[49], and describes a protocol for comparing the recoveries of confirmed target organisms by two or more methods, originally derived for the UK Drinking Water Inspectorate. Similar considerations apply to the comparison of methods for other matrices but the processing of some, such as heavily contaminated waters and sludge, may inherently present additional challenges. Sections 9.5.2, 9.5.3 and 9.5.4 describe aspects and approaches relevant for these other matrices.

9.1 Basic concepts and definitions

A laboratory considering adopting a new or alternative method to the one currently in use should obtain sufficient comparative data to demonstrate the relative performance of the two methods before adopting the new method for routine use. If appropriate, other laboratories may then undertake the process of comparison of performance and the data from all laboratories may then be pooled and reviewed to establish robustness.

9.1.1 Microbiological cultural methods

Methods are considered microbiological cultural methods when growth and multiplication of micro-organisms are the essential features for their detection and/or quantification.

9.1.2 Definitions

Alternative or trial method - Any method which is to be tested for equivalence with a reference method[49].

Characterisation - Establishment of the specifications for the performance of a new method and/or experimental verification that a method meets theoretically derived quality criteria[28].

Confirmed count - The number of the presumptive count 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.

Expanded uncertainty - quantity defining an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand[49].

Linearity – Linear dependence of the signal on concentration of the analyte[28]. In method performance evaluation this is the ability of the method to maintain a proportional response over its working range.

Measurand - Particular quantity subjected to measurement[28].

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[28], exhibiting no attraction or repulsion between micro-organisms.

Precision - The closeness of agreement between independent test results obtained under stipulated conditions[28].

Presumptive count - The number of organisms that produce a response typical of the target organism in or on a primary detection medium.

Reference method - Prescribed analytical method to analyse a given group or species of micro-organisms[49]. For example, methods published by ISO or SCA.

Relative difference – Difference between two results, a and b, measured on a relative (natural logarithmic) scale, expressed in percent, i.e. $x = [\ln(a) - \ln(b)] \times 100\ \%$[49]. This is essentially the same as $x = [2(a - b)/(a + b)] \times 100\ \%$[49] until the difference in counts become greater than three-fold.

Repeatability - Closeness of the agreement between the results of successive measurements of the same measurand carried out under the same conditions of measurement[28]. 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, materials and method.

Reproducibility - Closeness of the agreement between the results of measurements on the same measurand carried out under changed conditions of measurement[28]. For example, this can be calculated from replicate counts from sub-samples obtained from a well-mixed sample, 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[28]. For example, use of a method by different laboratories should not change the sensitivity of the method.

Standard uncertainty – uncertainty of the result of a measurement expressed as a standard deviation[28, 49].

Under-dispersion – Variation below that expected by the Poisson distribution.

Verification - Demonstration by experiment that an established method functions according to its specifications in the user's hands[28].

9.2 Characterisation of methods

Many methods used in water microbiology have not had substantial characterisation of performance, some having been developed 50 or more years ago (for example, membrane-lauryl sulphate broth for coliform bacteria and m-Enterococcus agar for enterococci). Their continued use is basically a result of their widespread (national or international) employment as well as frequent incorporation in national methods. However, many of these methods were originally adopted after only a review of data in scientific publications and limited in-house evaluation. Characterisation, validation and verification of performance of methods constitute a requirement of ISO 17025[2] for laboratories seeking accreditation. For methods in water microbiology guidance on characterisation is available in ISO/TR 13843[28], which defines (primary) validation as "an exploratory process with the aim of establishing the operational limits and performance characteristics of a new, modified or otherwise inadequately characterised method". This would also apply for when only part of a method (for example a "confirmation" step) is changed. The standard describes the information required for the derivation of the numerical and descriptive specifications of a method.

A key component in any characterisation is the unambiguous description of the target organism for the method. It is, therefore, essential to understand the analytical basis of methods (for example detection of coliforms by either lactose fermentation or the production of β-galactosidase), so that if differences are found when comparing a new method with an established one, they can be explained.

Characterisation of a method will provide information on specification of performance, not only with respect to the recovery and enumeration of the target organism(s), but also the analytical requirements of the method (for example incubation temperature and time, media preparation and storage conditions, and sample storage or pre-treatment). Key information will relate to recovery efficiency, relative recovery (against a reference medium or a non-selective medium), repeatability and reproducibility of the method and counting of colonies, upper and lower working enumeration limits, linearity, selectivity and specificity (false-positives and false-negatives), counting uncertainty (methodological and analyst) and a general estimate of precision. 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. Since these data will provide the initial assessment of performance of a new or modified method it is strongly recommended that analysts with experience in microbiological methods conduct the work.

Although it may be unreasonable to expect characterisation work based upon ISO/TR 13843[28] to be undertaken for methods that have been widely used for several decades, it is appropriate that the new methods that are being developed to replace them should have full validation. Generation of appropriate characterisation data should be the responsibility of the research team or manufacturer developing the method, and laboratories should request such information from commercial suppliers before any consideration of verification of performance and adoption in their laboratory.

Further guidance is given in ISO/TR 13843[28].

Before verifying the performance of a new method a laboratory should become familiar with the method and may wish to undertake some work to verify the characterisation data provided by the developers or suppliers. This may be a limited appraisal, typically verifying identification of target and non-target organisms and ascertainment of false-positive and false-negative results. Such an investigation can be conducted using selected strains of target and non-target organisms representative of those the method may be challenged with, as well as a selection of natural samples. This can be conducted as part of the verification of performance process (see section 9.3).

9.2.1 Identification of target and non-target organisms

Microbiological methods should be designed to detect and/or enumerate particular types of micro-organisms, i.e. target organisms. All other micro-organisms, i.e. 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 method should provide sufficient suppression of these to prevent overgrowth and competitive inhibition or obscuration of target organisms. 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.

Identification of target and non-target organisms can be achieved by challenging the method with reference strains of the target organism and selected strains of non-target organisms that may typically occur in the types of sample analysed using the method. Following this natural samples should be analysed and a selection of target colonies and non-target colonies be identified to confirm specificity.

9.2.2 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.

9.2.3 International or prescribed methods

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 is 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 a quality assurance programme.

9.3 Verification of performance

Verification of performance of a method in a laboratory is a simplified version of the characterisation process. Its purpose is to answer the basic question "Does this new method perform to its specification in my laboratory?" There is limited guidance on verification in ISO/TR 13843[28], simply that a number of natural samples should be used, analysed as split samples or replicate dilution series with duplicate counting to verify expected counting performance. A limited number of samples using an appropriate quantitative reference material can be initially used to confirm target and non-target colony morphology and colouration or reaction colour. This also allows the analysts to become acquainted with the new method without any issues of interferences associated with natural samples. Once the analysts are proficient, then natural samples appropriate to the laboratory are analysed. It should be remembered that these samples will typically contain target and non-target micro-organisms in some state of stress and probably reduced metabolic status. This may result in differing appearance or reactions compared to those using pure culture reference organisms. In addition, the species or strains in these samples are likely to be different from those encountered by the laboratory or manufacturer that undertook the original validation work. There is, therefore, the possibility of encountering atypical growth or reactions that may be specific to the laboratory. There is no recommendation on the number of natural samples that should be analysed for verification of performance, but at least 30, covering the range of water types or matrices typically analysed by the laboratory, is a reasonable starting point, more samples can be analysed if the results are equivocal. The laboratory should analyse several samples of each water type or matrix, as a single result from a sample source may not be truly reflective of how the method performed on that water type, and this may increase the total number needing to be analysed. Additionally, if the types of bacteria normally encountered by the laboratory with their current method are subject to seasonal variation, it may be appropriate to conduct the verification exercise over a period of time that would take that source of variability into account.

Verification should also be over the full range of counts for which application is anticipated. Where the method is intended to serve for both presence/absence and enumeration of low numbers of organisms particular attention is required to these aspects (see sections 9.3.1 and 9.7).

It is essential that the identity of the target organisms isolated by the method is confirmed and ISO/TR 13483[28] recommends that 100 presumptive positives should be isolated and their identity verified (using appropriate biochemical or serological protocols). A number of non-target presumptive isolates (for example, 50 isolates) should also be subject to identification to check the false-negative rate.

Situations where verification of performance of a method is needed include:-

i) adoption of a reference or statutory method, or previously validated method, by a new laboratory or by a laboratory that has not previously analysed for the target organisms (for example, by comparison against published performance data),
ii) when a validated and verified method is transferred from one laboratory to another (for example, by comparison against previous performance data), and

iii) when a laboratory wishes to adopt a validated alternative to the method currently in use.

Successful performance of a new method after the verification exercise can result in a laboratory adopting the method. If, however, the new method were to replace one already being used by a laboratory, or a statutory method, it would be appropriate to assess the new method against the current or statutory method, and to generate verification of performance data at the same time. One of the key benefits of this would be the generation of data that can be used to explain to customers why the method has been changed (for example greater recovery or specificity/selectivity, etc.), any additional benefits (for example more rapid analyses) and any potential impact it may have on the results from their future samples.

This approach should also be undertaken for any significant change in a method employed in a laboratory (for example implementation of a new confirmation procedure).

9.3.1 Verification for low number and presence/absence counts

Many of the tests performed on drinking water are directed initially at a presence/absence outcome. However, in most routine instances, for example indicator counts, once present enumeration immediately becomes important. As a consequence, membrane filtration tests for indicator organisms need to perform well at detecting low levels of bacteria when present. It is essential that laboratories performing such tests understand the performance and limitations of their methods when the numbers present may be at the borderline of their ability to recover or detect them. Similar comments apply to other matrices, within water and associated materials, where the outcome of a presence/absence test or a low count may have significant consequences.

Terminology used in this context has been, and continues to be, subject to change and controversy. It has already been pointed out for example (See section 7.3.3) that there is no equivalent in microbiology of the concept, used extensively in chemistry, of ‘limit of detection’. However, this and similar terms such as limit of determination do appear in accreditation documentation[4] and their inclusion in relevant revised ISO standards is anticipated. It is the experience of laboratories that they have been required to produce evidence of performance verification using such terminology.

Statistical considerations around accuracy of counts are described in section 7.3 and the characteristics of bacterial dispersion in water in the context of comparing methods in section 9.4. Section 9.7 specifically addresses comparison of methods at low numbers of target organisms.

The purpose of this section is to emphasise the legitimate objective of ensuring that laboratories understand the performance of their methods at low counts. Also, to provide some recommendations for good practice and consistency on suitable approaches that might be used to satisfy such requirements. All statements about the ability to detect or recover at low levels should recognise any limitations in extrapolating for example from a specific ‘laboratory culture’ to a ‘real sample’ context. Micro-organisms may vary in many ways as a result of circumstances in the test material, environmental sample or laboratory culture, and the selectivity of the test environment. They will behave differently depending for example on cell integrity, physiological state, nutritional status and dispersion within the sample.

Accreditation guidance[4] currently includes requirements for verification of methods recognising the difference between qualitative, presence/absence tests, emphasising the ability to detect the target organism (limit of detection) and quantitative tests. For quantitative tests the emphasis is on the level at which enumeration is reliable (limit of quantification). In both cases laboratories are expected to take account of matrix effects. Currently, the following definitions are given:

"***Limit of detection:-*** Applied to qualitative microbiological tests: The lowest number of micro-organisms that can be detected, but in numbers that cannot be estimated accurately.
Limit of quantification:- Applied to quantitative microbiological tests: The lowest number of micro-organisms within a defined variability that may be determined under the experimental conditions of the method under evaluation."

These definitions, and those for other terms used in this context, give little practical assistance to laboratories on what is actually required.

A limit of detection can be considered in terms either of the volume, or quantity, in which a single target micro-organism can be detected or as the smallest number of target micro-organisms detectable in a given volume, or quantity, of sample[50, 28]. Examples of practical approaches that have been applied are described below. Although, in most cases actual tests are performed only once all of these examples require sufficient replicates to support statistical evaluation of the data. The frequency required for verification of low count performance to demonstrate consistency should be considered and the reference strain(s) and preparation conditions all need to be tightly specified for reproducibility.

9.3.1.1 A direct comparison of counts obtained for appropriately diluted reference strains of selected target organisms on a non-selective medium and on a selective test medium can provide basic information on relative recovery.

9.3.1.2 Recovery from samples spiked with laboratory reference cultures of target and non-target/competing organisms of known content assayed independently by a standard non-selective method. Samples spiked at 1, 10 and 100 cfu are analysed, for example in triplicate, by the test method. This approach may not be applicable for matrices where the sample matrix may need pre-treatment, for example autoclaving sludge, altering its character and where achieving homogeneity introduces additional uncertainty.

9.3.1.3 Dilution to extinction – analysis, for example in duplicate, of each dilution in an appropriate series arranged to go beyond the dilution at which target organisms are still detected. This could be applied for example to an environmental water or associated material matrix naturally containing both target and non-target organisms.

9.3.1.4 Spiking of samples with decimal dilutions of a known laboratory culture of reference target strain with analysis seeking to identify the last dilution from which the organism can be recovered, this dilution being designated the 'limit of detection'. The result may be influenced by the number of replicates performed.

9.3.1.5 Analysis of sufficient pairs (for example 30 or more) of spiked samples having a low count, for example less than 10 per plate/membrane/MPN. The objective being to show that fewer than 5% of the pairs either has a $D^2 > 3.841$ (see section 8.2.2) or second result outside the 95% confidence interval range (see section 7.3, Annex A or, for MPN counts, appropriate tables[51]). If these criteria are met then it can be assumed that over-dispersion is unlikely and therefore the lowest result for which the lower bound of the 95% CI for the 'unobserved' count is greater than zero is a reasonable estimate of the limit of quantification. For methods using selective media, for which recovery may be significantly lower than on non-selective media, it may be appropriate to multiply this figure by the ratio of counts obtained in 9.3.1.1 above.

9.4 Comparison of methods

Adoption of alternative or new methods to replace a statutory, regulatory or laboratory accredited method should be undertaken only after a comparative assessment of their performance against the current laboratory method. This will involve analysing samples (either natural or spiked) in duplicate (one by each method) and statistically examining their respective paired results. This can be achieved for drinking waters by using the analytical protocol originally developed for the UK Drinking Water Inspectorate and the statistical procedures of ISO 17994[49].

This section describes the procedures for establishing the relative performance of microbiological cultural methods used in water and associated materials. The examples used pertain to methods for drinking water analyses. 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, preferably be thoroughly validated and its performance in the laboratory verified. The procedures compare the results of two methods using samples containing about 20 - 50 target organisms per test volume, usually 100 ml for water. 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.

9.4.1 Statistical considerations

As described in section 7.3, there are several sources of variation that may complicate the evaluation of the comparison between alternative and reference methods. These include sample variation, natural variation and systematic imprecision inherent in the methods. These may be expected to be even more significant for matrices other than drinking water.

9.4.1.1 Sample variation

A water source, sampled for monitoring purposes, may exhibit enormous variation in its microbial content over time and between sampling sites[25]. 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 8.2.2) 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.

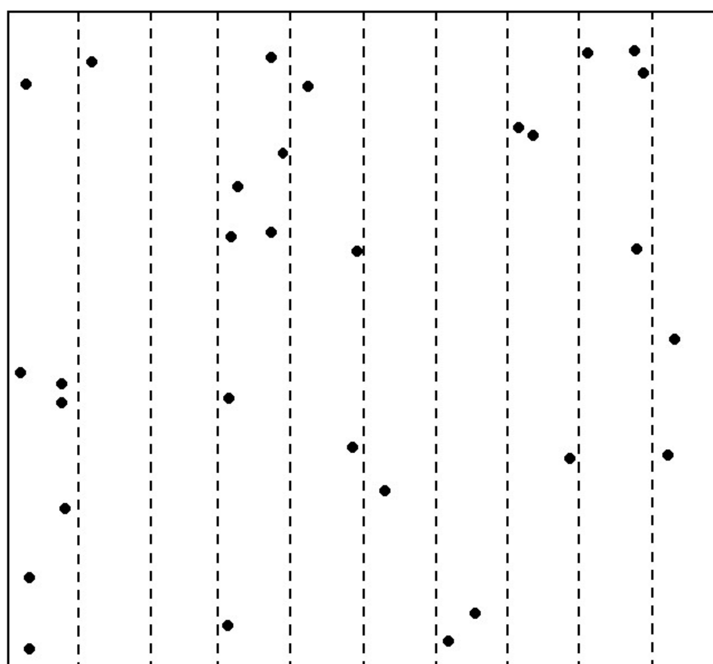
9.4.1.2 Natural (random) variation

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 drinking and environmental 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 non-target organisms that may be present.

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[44].

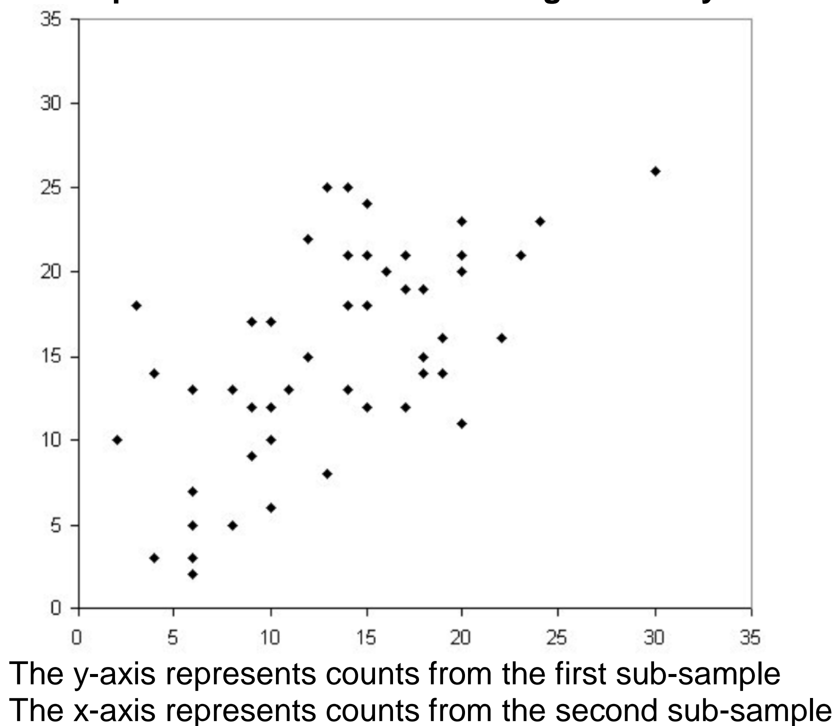
Figure 9.1 Random variation of organisms in aliquots from a sample containing 30 organisms



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. Non-parametric

correlation statistics such as Spearman R, Kendall Tau or Gamma coefficient may give more useful information than r^2.

Figure 9.2 Pairs of replicate counts of coliform organisms by the same method



The y-axis represents counts from the first sub-sample
The x-axis represents counts from the second sub-sample

9.4.1.3 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. Non-random or systematic variation, for example due to the inadequacy or difference in performance 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.

9.4.1.4 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(52). 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.

Equation (1) can be expanded to:

$$y_i = \mu_t + m_l + m_m + \varepsilon_{ti} + \varepsilon_{li} \qquad (2)$$

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 values of the laboratory effect and of the two types of error can be negative or positive. The laboratory effect plus the method effect (i.e. $m_l + m_m$) is the systematic, average difference from the true mean when that method is used in that laboratory. 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:

$$y_1 – y_2 = (\mu_t + m_l + m_{ref} + \varepsilon_{t1} + \varepsilon_{l1}) – (\mu_t + m_l + m_{new} + \varepsilon_{t2} + \varepsilon_{l2}) = (m_{ref} – m_{new}) + (\varepsilon_1 – \varepsilon_2) \qquad (3)$$

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:

$$E(y_1 – y_2) = m_{ref} – m_{new} \qquad (4)$$

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 ($m_{ref} – m_{new}$).

9.4.1.5 Limitation of errors

Errors in measurement (i.e. those for which the laboratory is responsible) 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. 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 with appropriate quality control as described elsewhere in this publication. Where used, commercially available media, reagents and membrane filters from a single batch should be used when undertaking comparisons of microbiological methods.

9.5 Practical aspects of the comparison of two methods

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 9.5.2) is very important and the waters used should be derived from several sources. Each source may be referred to as a “category of origin” or “water type”, and samples of water may be taken over different periods of time. 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 for drinking water, but may be smaller for environmental waters for example 10 and 1 ml for surface water) 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.

For environmental water samples additional comparisons may be required for each water type for example river water. This would include comparisons consisting of 10 replicates on both media at three levels of interest using real samples. These are low level, 5 – 15 cfu per membrane, medium level, 25 – 50 cfu per membrane and high level, 50 – 100 cfu per membrane.

These comparisons require a clear presentation of the data, a statistical comparison between each category of origin of samples and/or laboratories, and finally, an overall statistical comparison. The alternative or trial 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 if it is shown to be better than the reference method or it is demonstrated that there is no statistically significant difference between the methods where 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 procedure to ascertain this is the mean relative difference analysis of ISO 17994[49].

For a trial method which is found to be acceptable, it may be appropriate to test it against the reference method with samples containing low counts of the target organism(s). This should then demonstrate that there are no major differences between the two methods when much lower counts are compared. This would be particularly appropriate for methods used for analysing drinking waters.

The approach for comparing method A (for example, an alternative method, referred to as the trial method) with method B (for example, an existing or statutory method, referred to as the reference method) is made on the basis of recording the difference in results obtained for paired sub-samples of a sample processed at the same time. This data set, when complete, is then progressively evaluated to ascertain whether there are any differences between water types or laboratories and whether the average results are comparable and the confidence intervals are acceptable.

The methods to be compared should be tested with the types of samples which it is anticipated will be routinely analysed by the two methods. For drinking water 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 drinking water samples, containing chlorine as disinfectant, are given in section 9.5.1.1 For alternative disinfectants, it will be necessary to determine by experiment those conditions appropriate for the survival of suitable numbers of target organisms.

For a method for which there is no previous comparison data available it is estimated that a minimum of 150 samples and up to about 250 samples[49] may be needed in the comparison trial, which reflect the range of source waters. If a single laboratory is undertaking the study this will involve selecting a range of sources of water or water types (usually 5 to 10) for analysis. Alternatively, a group of laboratories may undertake the study with a smaller selection of sources selected for each laboratory, but still ensuring that the range of water types expected to be analysed by the trial method are included. The methods used should be tested with the appropriate volume of sample relevant to the target organism and the prescribed limit. For drinking water this is usually 100 ml and this volume is used in this section for illustrative purposes. For environmental waters a smaller volume (for example 10 ml or 1 ml) may be more appropriate. The samples should not be diluted and should be tested over a period of several days, generally testing approximately 10 - 15 samples per day.

9.5.1 Preparation of drinking water test samples to compare one selective medium with another

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 9.5.1.1.1).

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 9.5.1.1.2).

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.

9.5.1.1 Preparation of spiked samples

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).

9.5.1.1.1 Generation of chlorine-stressed target organisms using river water

Collect approximately 10 litres of tap water from a supply that is representative of the water supplies to be tested (referred to below as the 'original source'), and cool to 5 ± 3 °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.

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 5 ± 3 °C.

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, i.e. 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.

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 5 ± 3 °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.

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 5 ± 3 °C.

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.

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 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 and mixed tap and river water.

9.5.1.1.2 Generation of chlorine-stressed target organisms using sewage effluent

Collect 10 litres of tap water from a supply that is representative of the water supplies to be tested (referred to below as the ‘original source’), and cool to 5 ± 3 °C (store overnight if necessary). Collect at least 1000 ml of sewage effluent and store for one hour at 5 ± 3 °C to ensure solid material settles.

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.

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 5 ± 3 °C. Cap the container, mix the contents thoroughly and stand the container on a magnetic stirrer and stir vigorously.

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.

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 5 ± 3 °C.

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.

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 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 and mixed tap water-sewage effluent solution.

9.5.2 Preparation of environmental and recreational water test samples to compare one selective medium with another

Environmental waters usually contain a natural flora of indicator organisms, particularly where significant wastewater or agricultural contamination occurs. These indicator organisms will already be stressed and further stressing, for example by storage at low temperature, is unnecessary.

9.5.2.1 Collect a minimum of 1 litre of water. Where the water is reasonably clear, mix it thoroughly. For turbid waters, store for one hour at 5 ± 3 °C to ensure that particulate material settles.

9.5.2.2 Process 10 ml, 1 ml and any dilutions considered necessary and analyse for the target organism. A method should be used that will yield presumptive results, ideally, within 24 hours. A guide value for Enterococci can also be obtained by reading plates at 24 hours. Store the sample at 5 ± 3 °C.

9.5.2.3 After incubation, determine the number of organisms in each volume of the sample analysed and identify the appropriate volume of sample, or dilution, found to contain 30 – 90 organisms. This number is higher than a target range of 20 – 50, in order to allow for some decay in the population of organisms during overnight storage.

9.5.2.4 The appropriate volume of sample, or dilution, can now be analysed by two or more methods used in parallel by one or more analysts. Several different environmental waters can be analysed in this way.

9.5.2.5 Where environmental waters do not contain sufficient target organisms, wastewater effluent can be used to provide sufficient numbers. Collect 100 ml of treated wastewater effluent and store for one hour at 5 ± 3 °C to ensure solid materials settle. Add 100 ml of settled effluent to 900 ml of environmental water sample and follow the steps from 9.5.2.2 to 9.5.2.4.

9.5.3 Preparation of environmental and recreational water samples to verify the performance of a selective medium using reference cultures

9.5.3.1 Verification of a reference method in, for example, a new laboratory can be undertaken using broth cultures of reference organisms. Recovery of target organisms by the reference method can be compared with recovery on a non-selective medium, for example, nutrient agar. Cultures of target organisms can be prepared by inoculating a suitable broth, for example, nutrient broth, with a reference culture, incubating at an appropriate temperature for 21 ± 3 hours and storing the broth culture at 5 ± 3 °C for several days to create 'stressed' organisms. Following storage, reference cultures can be counted using pour or spread plates or a suitable alternative counting method, for example, Miles and Misra[53]. Once the numbers of target organisms are established, suitable dilutions can be prepared to give an appropriate range of target organisms for the test.

9.5.3.2 Environmental samples will contain large numbers of non-target organisms as well as target organisms and these may well interfere with counting on nutrient agar. This problem can be overcome by filtering out non-target organisms using a sterile 0.45 µm membrane and collecting the filtrate prior to inoculation with target bacteria. Enumeration may also be aided by adding an appropriate diagnostic chromogenic substrate, for example, BCIG (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) to the nutrient agar for counting *Escherichia coli*.

9.5.3.3 Inoculate nutrient broth with the appropriate target organism and incubate at the appropriate temperature, typically 37 °C for 21 ± 3 hours. After incubation, store the broth culture at 5 ± 3 °C. After storage, count the broth culture using an appropriate enumeration method.

9.5.3.4 Collect a minimum of 1 litre of water. Where the water is reasonably clear, mix it thoroughly. For turbid waters, store for one hour at 5 ± 3 °C to ensure that particulate material settles.

9.5.3.5 Prepare dilutions of the target organism in Ringer's solution or maximum recovery diluent such that when 1 ml of diluted culture is added to the water, the final concentration of organisms will be in the range of 20 – 50 organisms per ml or per 10 ml aliquot.

9.5.3.6 Membrane filter aliquots of the water sample, either 1 ml with some Ringer's solution or 10 ml in duplicate to generate paired samples. Place one membrane on the selective medium and one membrane on the non-selective medium. Incubate the two media under the same temperature and time conditions and count the number of target organisms on each.

9.5.3.7 In this way a number of replicates, for example ten, can be prepared for each type of environmental water being examined. In addition, the reference method can be assessed with different concentrations of the target organisms, for example between 5 and 10 organisms for low level recovery and between 50 and 80 organisms for high level recovery.

9.5.4 Preparation of sewage sludge test samples

Sewage sludge comprises a diverse range of materials from mostly liquid through varying states of semi-solid to almost solid. All are derived from wastewater but the consistency of the matrix and the numbers and types of organisms present depend on the character of the waste contributing from the sewerage catchment and the nature and extent of the treatment that has been applied. Liquid and semi-solid raw sludge can contain very large numbers of indicator bacteria as well as a diverse range of non-target and potentially competing micro-organisms. By contrast, sludge that has received enhanced treatment, for example by thermal drying, will have a very high solid content and very low numbers of organisms. Treatments such as anaerobic digestion, lime addition and thermal drying are intended to reduce the number of pathogens and indicator organisms and those remaining in the sludge are likely to be stressed. Some sludge may contain substances, for example certain metals, that are toxic or inhibitory.

9.5.4.1 The most important factor when comparing methods for sludge is the homogeneity of the sample under test. In all cases a robust preparation procedure to homogenise the sample is a pre-requisite to comparing methods indeed some comparisons may entirely relate to potential improvements in preparation methodology rather than the enumeration stage. Methods for the sampling and preparation of sludge samples for analysis are described elsewhere is this series(27).

9.5.4.2 As with other matrices comparisons for sludge should include samples from all the types of sludge for which the method is intended to be applied. This should include sludge consistency and derivation as well as geographical variation. The comparison should also encompass the range of intended numerical application with sufficient samples with low numbers of organisms to verify the practical lower limit of determination.

9.5.4.3 The particulate content of sludge dictates that dilution is normally a significant but variable factor prior to enumeration. Where a most probable number method is part of the comparison and low numbers are expected this will be to a lesser extent than for a membrane or plate count method.

It should be borne in mind that practical applications may involve enumeration of sludge pre and post treatment for example for the estimation of log reduction in assessing performance or as a regulatory requirement. If the character of a sludge changes markedly during treatment and the treated sludge contains very few organisms different enumeration methods, having different uncertainties and limitations, may have been used. When comparing enumeration methods the sludge should be prepared as nearly as practical in the same manner for each method to be compared. The potential impact of differences between method conditions, such as incubation temperature, which are integral to target organism definition should be well understood. It is recommended that when log reductions are calculated these should include an uncertainty estimate.

9.5.4.4 Comparisons should identify any limitations encountered and take account of the uncertainties associated with preparation and dilution of samples when performing a statistical assessment of the data. Wherever possible these uncertainties should be quantified.

9.5.4.5 Preparation of sludge samples to compare one selective medium with another within a laboratory

Collect a representative sample of sludge, typically a minimum of 100 grams, thoroughly mix the sample and prepare a homogenised sub-sample according to a documented sample preparation protocol as the starting point for processing by the methods to be compared. The avoidance of cross contamination between samples is essential. Appropriate blank controls should be included.

Prepare the range of dilutions considered necessary and analyse for the target organism.

After incubation, determine the number of organisms in each dilution of the sample analysed and identify the appropriate sample dilution, found to contain 20 – 50 organisms for statistical analysis.

Comparisons should ideally be performed using freshly prepared sludge and analysed immediately. The microbiological content of most sludge is likely to be highly unstable either because of biological activity or aggressive conditions such as those generated by the presence of lime and other bactericidal substances that may be used in sludge treatment.

For statistical purposes comparisons should include 10 – 15 pairs of analyses for each sludge type and geographical location for which the methods are intended to be applied. In most instances it should be possible to achieve greater statistical confidence if replicate analyses, 3 or 5 replicates for example, are performed[54]. This is likely to be a suitable approach where the application will involve replicates in practice but it should be borne in mind that this may not be appropriate when this is not the case.

9.5.4.6 Verification of the performance of a method for enumerating organisms in sludge using reference cultures and preparations

Assessments of recovery efficiency provide valuable information and understanding about methods. However, the practical challenges when making quantitative additions either of freshly grown broth cultures of reference strains or commercially supplied reference materials to sludge should be well understood and taken into account when interpreting data.

In view of the large numbers of target organisms likely to be present naturally, some form of treatment is usually required to eradicate these before adding the reference material. It is generally impractical to perform an assessment by known addition and subtraction of background. However, most treatments that might be applied to sludge, usually some form of heat treatment, carry the risk of changing the character of the sludge so that it is no longer representative and does not behave like the untreated material.

Approaches that have been used include autoclaving and heating to 70°C for a defined period. Sludge that has been thermally dried may be suitable for direct addition. The sludge should be homogeneous for treatment and thoroughly mixed after addition of the reference material. The numbers of organisms added should be aimed at the range typical for the intended application.

Procedures proposed for addition of reference materials should be extensively tried and tested during development to ensure that limitations and uncertainties are well understood and optimised before application to specific sludge matrices.

9.5.4.7 Preparation of sludge samples to compare the performance of one or more methods in more than one laboratory

Comparisons between laboratories require the preparation of a homogeneous set of sub-samples. This is a key part of the preparation over and above ensuring appropriate and timely despatch, transport within an appropriate temperature range, arrival and appropriate storage of samples and suitable and consistent processing in the receiving laboratories.

There are examples of inter-laboratory comparison involving:

- despatch of sub-samples of digested sludge[(55)],
- preparation and despatch of sludge cake and compost thoroughly mixed after spiking with reference culture material[(56)] and
- despatch of sludge and commercial reference material for spiking on receipt by each laboratory[(57)].

Inter-laboratory comparisons on this scale require careful planning and the development of detailed protocols to be followed by the issuing and receiving laboratories. These protocols should be designed with the type and quantity of data required together with the methods for data handling and analysis to be used in mind.

9.5.5 Confirmation tests

If confirmation of presumptive target colonies is required, then this should be carried out according to the requirements of the method. Preferably all colonies should be selected for confirmation so as to produce the most reliable confirmed count. However, if the presumptive counts are high, it may be acceptable to select 10 presumptive colonies to 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. If there are multiple types of presumptive colony then each type must be confirmed, with colonies of each type selected randomly as above (See also section 7.3.1.4.1)

9.5.6 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 or sludge 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[(52)]. 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, 50 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 or sludge 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 sample source.

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 the sample from one source, exhibits differences from other sources then examination of the identification data may facilitate an interpretation of the differences.

9.6 Interpretation of 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 paired results are omitted where this is observed for only one of the methods. 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 trial method gives a higher or lower result than the reference method.

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 trial method gives a higher or lower result than the reference method.

9.6.1 Data collection

For drinking water samples the procedures described in sections 9.5.1.1.1 and 9.5.1.1.2 should enable aliquots of samples to be prepared that contain 20 - 50 target organisms. However, samples with lower counts may be obtained and these should still be included. 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 methods must be recorded as a “paired sample” result.

It is preferable that the prepared samples described in sections 9.5.1.1.1 and 9.5.1.1.2 are derived from a selection of sources or categories of origin (water types). 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 9.5.1.1.1 and 9.5.1.1.2.

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 question to be answered satisfactorily - is the relative performance of the two methods similar for all the sources or categories of origin used (or for each participating laboratory) in the study?

The analysis of at least 15 samples for each source or category of origin giving a total of not less than 150 sample comparisons for all sources or categories should provide sufficient information to answer the above question. 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 power of the information available from 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.

9.6.2 Preliminary data evaluation

Plot the paired results against each other, differentiating each source or laboratory. Also, plot the differences (actual or transformed data, such as logarithms) on appropriate scales. An assessment for outliers should also be conducted and this can be achieved by visual assessment of plotted difference in transformed count data. Outliers should be removed only if there is a valid technical or microbiological reason for their exclusion.

Ascertain whether the data are suitable for parametric analysis, i.e. are the count differences 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, but typically $\log_{10}$, and carry out a parametric data analysis. Alternatively, perform a non-parametric data analysis, using, for example Wilcoxon signed rank test. Whether a parametric or non-parametric data analysis is carried out, the aim is to answer the question, is the relative performance of the two methods similar for all the sources used, or for all the participating laboratories, in the study?

For a parametric data analysis, do the *t*-tests or ANOVA tests show significant differences between the sources or laboratories? If the answer to this question is no, then the data can be combined for analysis as shown in the next section. If the answer is yes, then possible technical or microbiological causes should be investigated and decisions taken whether or not the differences affect part (i.e. a particular source or laboratory) or all of the data (i.e. all sources or all laboratories). Depending upon these actions and decisions, part or all of the data may need to be rejected.

For a non-parametric data analysis, does the tabulation of frequencies of paired results by source or laboratories show differences between the sources or laboratory? If the answer to this question is no, then the data can be combined for analysis as shown in the next section. If the answer is yes, then again possible technical or microbiological causes should be investigated and decisions taken as to whether or not the differences affect part, or all, of the data. Depending upon these actions and decisions, part or all of the data may need to be rejected.

9.6.3 Combined analysis of average difference

When the preliminary data evaluation has been completed satisfactorily, and if the data are suitable for combining, then an average difference between the methods can be presented which will be a mean (for parametric analysis) or a median (for non-parametric) together with a 95% confidence interval for this average.

The method for analysing the differences in counts presented in this section is based on ISO 17994, originally published in 2004 and revised in 2014,[49] which assumes that log transformation and parametric analysis are appropriate, which is the case in the majority of situations. This method is the mean relative difference analysis[49].

In the ISO method the data are log-transformed to the base *e* (referred to as natural logarithms and abbreviated to "ln"). In the DWI examples logs were taken to the base *10* (abbreviated to "log"). The results are equivalent, apart from a constant multiplier.

$$\ln(x) = 2.3026 \log(x)$$

For example, log 10 = 1 with ln 10 = 2.3026 and log 100 = 2 with ln 100 = 4.6052. Logs to the base 10 have the advantage that when graphs are being labelled that the scale can be readily converted to the pre-transformation data scale, i.e. label 1 as 10, 2 as 100, 3 as 1000 etc.

In ISO 17994[49] the relative difference (x) of each pair of counts is calculated and tabulated using the equation $x = [\ln(a) - \ln(b)] \times 100$ %, where $\ln(a)$ is the natural logarithm of the count by the trial method and $\ln(b)$ is the natural logarithm of the count by the reference method, for each sample. Data with a zero count by one method has one added to each count prior to log normal-transformation. From these data the mean relative difference ($\bar{x}$) and standard uncertainty (standard deviation) (s) are calculated. From the standard uncertainty and the number of samples (n) the expanded uncertainty (U) is calculated using the equation:

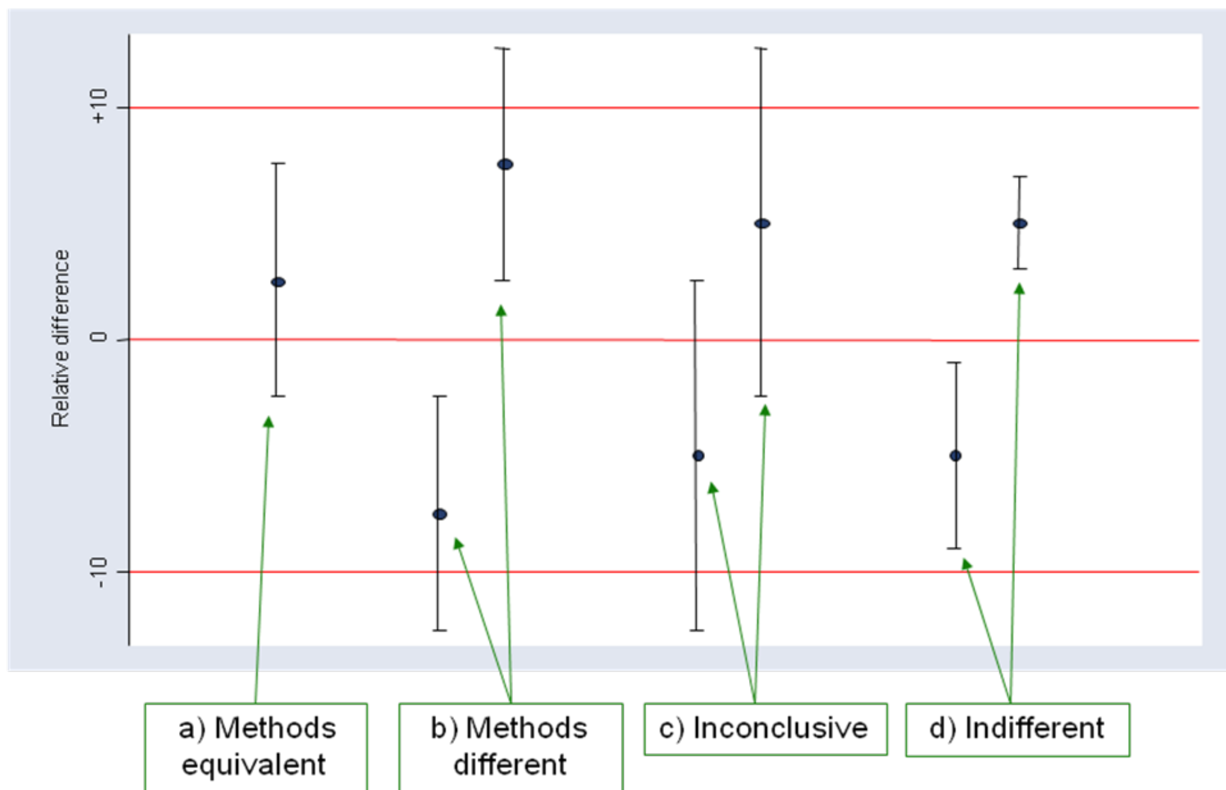
$$W = \frac{2s}{\sqrt{n}}$$

The expanded uncertainty, when added to and subtracted from the mean relative difference provides the "confidence interval" of the expanded uncertainty around the mean (X_L and X_U). The mean relative difference and its "confidence interval" are compared with a theoretical mean difference with maximum acceptable deviation limits (2L). For drinking water samples these are typically set at ± 10 %[49]. The principal potential outcomes of this analysis are:

a) $- 2L \leq X_L \leq 0$ and $0 \leq X_U \leq +2L$	methods are "not different" (i.e. equivalent)
b) $X_U < 0$ or $X_L > 0$	methods are different
c) ($X_L < - 2L$ and $X_U > 0$) or ($X_L < 0$ and $X_U > + 2L$)	inconclusive (i.e. more samples needed)
d) ($X_L > - 2L$ and $X_U < 0$) or ($X_L > 0$ and $X_U < + 2L$)	methods have a small significant difference (termed in ISO 17994 as "indifferent")

These outcomes are depicted graphically in Figure 9.3.

Figure 9.3 Graphical representation of outcomes of comparison of methods after analysis according to ISO 17994[49]



For environmental waters it has been suggested that "confidence intervals" set at ± 20 % may be more appropriate.

Where the aim is to compare a trial method with an established reference method in terms of being "at least as reliable", it is considered that the "one-sided" comparison according to ISO 17994[49] is appropriate. For drinking water in a "one-sided" comparison, only the lower 2L value is set, typically at -10 on the original scale. Similarly, for environmental waters the lower value can be set at -20.

The outcome shown in c) is where it is inconclusive and more samples need to be analysed. A method for calculating how many extra samples may be needed after an inconclusive result can be found in ISO 17994[49].

The outcome shown in d) is where a small significant difference between methods is detected and the 95% confidence interval suggests that it is unlikely to be as large as 2L.

The difference in this case is less than the equivalence criteria and the methods can be regarded as equivalent.

9.7 Low count evaluation

For some types of analyses (for example, drinking water) it may be appropriate to undertake a comparison of methods with low numbers of target organisms. This can be undertaken when satisfactory results are obtained from the main comparison. This evaluation 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. Such data may already be readily extracted from the main comparison study.

Paired results of at least 30 samples are needed, 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 9.5.1.1.1 and 9.5.1.1.2 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. In addition, samples should contain an appropriate diversity of organisms.

If the paired results obtained in the main comparison study contain at least 30 samples giving counts in this lower range for all sources, then the data from these samples can be used for this evaluation.

As for the main comparison, 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. The proportion of paired results where the count by the trial method exceeds the count given by the reference method should not be significantly lower than 50 % for the trial method to be considered to be acceptable. Thirty samples should give an estimate of the proportion, with an expanded uncertainty "confidence interval" that is not too large. For such a limited study it may be appropriate to set a maximum acceptable deviation limit of ± 20 on the count scale. If the "confidence interval" is large and there is evidence to suggest the 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.

9.8 Comparison of an MPN method with an enumeration method

The design of the study and the same procedures described in sections 9.5 and 9.6 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. However, the nature of the values obtained by the traditional 11- or 15-tube series MPN method may necessitate an alternative manipulation and statistical analysis of the data obtained.

Appropriate tables(51) 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). However, 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 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, whereas with an enumeration method all the values between the two results are theoretically available. One approach(58) to handle this difference in results obtained, 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(51) which are obtained from different conditional probabilities. Appropriate conditional probabilities have been published(58) and resulting ranges tabulated for tube combinations. For example, with the 11-tube series given above, it has been shown that counts between 69 and 110 would probably give a tube result of 1, 5, 3 and an MPN of 91. Counts between 111 and 175 would probably give a tube result of 1, 5, 4 and an MPN of 160. Enumerated counts of 69 to 110 could be interpreted as “equivalent” to an MPN of 91. Alternatively, especially with modern methods based on a greater number of tubes or wells, 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.

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.

9.9 Comparison of two MPN methods

The same procedures described in sections 9.5 and 9.6 should be used when two MPN methods are compared. The points raised in section 9.8 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.

9.10 Progression of a new method to national or international adoption

On a national or international scale, the adoption of a new method involves a sequential series of events. These are:

i) derivation or verification of validation data and comparison of the new method with a suitable reference method in one expert laboratory,
ii) subsequent comparison of the new method with the reference method in five 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 or superior 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 samples. Ideally, the comparison studies carried out in the initial five, or more, laboratories may require the replicate analysis of about 180 samples (150 samples for main comparison and, if necessary 30 samples for the low count evaluation) in each laboratory. Ideally, all procedures described in sections 9.5.1 to 9.5.3 should be used, and samples should be representative of the sources of water or sludge that the laboratory is likely to analyse by the new method. Data from the comparison studies undertaken in the different laboratories should 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 the light of the expanding database. However, for drinking waters, 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.

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Annex A 95 % Confidence intervals for the (unobserved) count from the second half-sample for the observed count from the first half-sample (see section 8.5.2)

Observed count in first half-sample	95 % CI for unobserved count in second half-sample	Observed count in first half-sample	95 % CI for unobserved count in second half-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		

Observed count in first half-sample	95 % CI for unobserved count in second half-sample	Observed count in first half-sample	95 % CI for unobserved count in second half-sample
101	75-131	151	118-188
102	75-133	152	119-189
103	76-134	153	120-190
104	77-135	154	121-191
105	78-136	155	122-192
106	79-137	156	123-193
107	80-138	157	124-194
108	81-139	158	125-195
109	82-140	159	125-196
110	82-142	160	126-198
111	83-143	161	127-199
112	84-144	162	128-200
113	85-145	163	129-201
114	86-146	164	130-202
115	87-147	165	131-203
116	88-148	166	132-204
117	88-149	167	133-205
118	89-151	168	134-206
119	90-152	169	134-208
120	91-153	170	135-209
121	92-154	171	136-210
122	93-155	172	137-211
123	94-156	173	138-212
124	95-157	174	139-213
125	95-159	175	140-214
126	96-160	176	141-215
127	97-161	177	142-216
128	98-162	178	142-217
129	99-163	179	143-219
130	100-164	180	144-220
131	101-165	181	145-221
132	102-166	182	146-222
133	102-167	183	147-223
134	103-169	184	148-224
135	104-170	185	149-225
136	105-171	186	150-226
137	106-172	187	151-227
138	107-173	188	151-229
139	108-174	189	152-230
140	109-175	190	153-231
141	110-176	191	154-232
142	110-178	192	155-233
143	111-179	193	156-234
144	112-180	194	157-235
145	113-181	195	158-236
146	114-182	196	159-237
147	115-183	197	160-238
148	116-184	198	160-239
149	117-185	199	161-241
150	118-186	200	162-242

Observed count in first half-sample	95 % CI for unobserved count in second half-sample	Observed count in first half-sample	95 % CI for unobserved count in second half-sample
201	163-243	251	209-297
202	164-244	252	209-298
203	165-245	253	210-300
204	166-246	254	211-301
205	167-247	255	212-302
206	168-248	256	213-303
207	169-249	257	214-304
208	169-250	258	215-305
209	170-252	259	216-306
210	171-253	260	217-307
211	172-254	261	218-308
212	173-255	262	219-309
213	174-256	263	219-310
214	175-257	264	220-312
215	176-258	265	221-313
216	177-259	266	222-314
217	178-260	267	223-315
218	179-261	268	224-316
219	179-263	269	225-317
220	180-264	270	226-318
221	181-265	271	227-319
222	182-266	272	228-320
223	183-267	273	229-321
224	184-268	274	230-322
225	185-269	275	230-323
226	186-270	276	231-325
227	187-271	277	232-326
228	188-272	278	233-327
229	188-273	279	234-328
230	189-275	280	235-329
231	190-276	281	236-330
232	191-277	282	237-331
233	192-278	283	238-332
234	193-279	284	239-333
235	194-280	285	240-334
236	195-281	286	241-335
237	196-282	287	241-336
238	197-283	288	242-338
239	198-284	289	243-339
240	199-285	290	244-340
241	199-287	291	245-341
242	200-288	292	246-342
243	201-289	293	247-343
244	202-290	294	248-344
245	203-291	295	249-345
246	204-292	296	250-346
247	205-293	297	251-347
248	206-294	298	252-348
249	207-295	299	253-349
250	208-296	300	253-350

Annex B Test micro-organisms media quality control

The table gives examples of reference cultures that can be used to test media[20] (see glossary for full names) together with expected reactions. This is not intended to be a comprehensive list for all potential media. **This table should be used alongside references to control organisms given in existing methods documents.** Alternatives for these and other media should be tested and characterised before use. They will be acceptable when shown to consistently give appropriate reactions.

Medium	WDCM Culture reference[59] and (NCTC[60] equivalent)	Reaction
MLSB / MLSA	*E. coli* 00090 (9001) *Klebsiella pneumoniae* 00097 (9633) *Ps. aeruginosa* 00024 (10322)	Growth, yellow colonies or broth 37 ^{O}C and 44 ^{O}C Growth, yellow colonies or broth 37 ^{O}C and 44 ^{O}C Growth, pink colonies (dark centred) or broth 37 ^{O}C and 44 ^{O}C
MLGA	*E. coli* 00090 (9001) *Klebsiella pneumoniae* 00097 (9633) *Ps. aeruginosa* 00024 (10322)	Growth, green colonies 37 ^{O}C and 44 ^{O}C Growth, yellow colonies 37 ^{O}C and 44 ^{O}C Growth, pink colonies (dark centred) 37 ^{O}C and 44 ^{O}C
Colilert	*E. coli 00090 (9001)* *Klebsiella pneumoniae* 00097 (9633) *Ps. aeruginosa* 00024 *(10322)*	Yellow and fluorescent well at 37 ^{O}C Yellow well at 37 ^{O}C Colourless well
MEA	*Ent. faecalis* 00009 (775) *E. coli* 00090 (9001)	Magenta colonies at 37 ^{O}C and 44 ^{O}C No growth
Enterolert-DW	*Ent. faecium 00010 (7171)* *Serratia marcescens (10211)*	Green well at 41°C Blue well
TSCA	*Cl. perfringens* 00007 (8237) *E. coli* 00090 (9001)	Growth, black (or colourless) colonies anaerobic at 37 ^{O}C and 44 ^{O}C No growth
TCA	*Cl. perfringens 00007 (8237)* *E. coli 00090 (9001)*	Growth, colourless colonies anaerobic at 37 ^{O}C and 44 ^{O}C No growth
YEA	*E. coli* 00090 (9001) *Micrococcus luteus* 00111 (2665)	Growth of colonies from a diluted suspension at 22 and 37 °C
PSA	*Ps. aeruginosa* 00024 (10322) *E. coli* 00090 (9001)	Growth, fluorescent green colonies at 37 °C No growth
Pseudomonas CN	*Ps. aeruginosa* 00024 (10322) *E. coli* 00090 (9001)	Growth, fluorescent green colonies at 37 °C No growth
Pseudalert	*Ps. aeruginosa 00024 (10322)*	Positive wells/tubes fluoresce blue under UV at 38°C

	Ps. fluorescens 00115 (10038) *E. coli* 00013 (12241)	No blue fluorescence No blue fluorescence
LPW	*E. coli* NCTC 09001 *Ps. aeruginosa* NCTC 10322	Growth, yellow broth at 37 and 44 °C Growth, pink broth at 37 or 44 °C
TBXA	*E. coli* 00090 (9001) *Klebsiella pneumoniae* 00097 (9633)	Growth, blue colonies 37 ^{O}C and 44 ^{O}C Growth, colourless colonies 37 ^{O}C and 44 ^{O}C
TW	*E. coli* 00090 (9001) *Klebsiella pneumoniae* 00206	Growth, indole production at 37 and 44 °C Growth, no indole production at 37 or 44 °C
TNA	*E. coli* 00090 (9001) *Klebsiella pneumoniae* 00206 *Ps. aeruginosa* (10322)	Growth, β-galactosidase (ONPG tablets), indole at 37 and 44 °C Growth, β-galactosidase (ONPG tablets), no indole at 37 and 44 °C Growth, no β-galactosidase (ONPG tablets), no indole 37 or 44 °C
KAAA	*Ent. faecalis* 00009 (775) *E. coli* 00090 (9001)	Aesculin hydrolysis on membrane transfer within 4 hours at 44 °C, growth and aesculin hydrolysis on subculture, 18 hours at 44 °C No aesculin hydrolysis on membrane transfer within 4 hours at 44 °C, no growth or hydrolysis on subculture, 18 hours at 44 °C
BAA	*Ent. faecalis* 00009 (775) *E. coli* 00090 (9001)	Aesculin hydrolysis on membrane transfer within 4 hours at 44 °C, growth and aesculin hydrolysis on subculture, 18 hours at 44 °C No aesculin hydrolysis on membrane transfer within 4 hours at 44 °C, no growth or hydrolysis on subculture, 18 hours at 44 °C
Milk agar	*Ps. aeruginosa* (10322) *E. coli* 00090 (9001)	Growth and hydrolysis of casein at 37 °C within 24 hours Growth but no hydrolysis of casein within 24 hours.
1:10 phenanthroline	*Ps. aeruginosa* (10322) *Ps. fluorescens* 00115 (10038)	Growth of *Ps. aeruginosa* up to the disc within 24 hours at 37 °C Zone of inhibition around the disc within 24 hours at 37 °C
BPW	*Salmonella* Enteritidis 00030 (12694)	Growth 18 hours at 36 °C
Brilliant Green agar	*Salmonella* Enteritidis 00030 (12694) *E. coli* 00013 (12241) *Ps. aeruginosa* 00025 (12903)	Smooth red colonies Yellow colonies Small crenated colonies
Rapapports broth	*Salmonella* Enteritidis 00030 (12694) *E. coli* 00013 (12241) *Ps. aeruginosa* 00025 (12903)	Growth (turbidity) 24 hours at 41.5 °C No growth No growth
XLDA	*Salmonella* Enteritidis 00030 (12694) *E. coli* 00013 (12241) *Ps. aeruginosa* 00025 (12903)	Black colonies 24 hours at 37 °C Yellow colonies 24 hours at 37 °C Red or yellow colonies with grey/black centre 24 hours at 37 °C

Preston broth	*Campylobacter jejuni* 00156 (11322) *E. coli* 00013 (12241)	Good typical growth when plated on CCDA No growth when plated on CCDA
Bolton broth	*Campylobacter jejuni* 00156 (11322) *E. coli* 00013 (12241)	Good typical growth when plated on CCDA No growth when plated on CCDA
CCDA	*Campylobacter jejuni* 00156 (11322) *E. coli* 00013 (12241)	Good typical growth No growth
Vogel Johnson agar	*Staph. aureus* 00032 (10788) *E. coli* 00013 (12241)	Black or grey colonies No growth
Ampicillin dextrin agar	*Aeromonas hydrophila* 00063 (8049) *E. coli* 00013 (12241)	Good growth yellow/yellow with green edge colonies 24 hours 30 °C No or poor growth
Shread's medium	*Aeromonas hydrophila* 00063 (8049) *E. coli* 00013 (12241)	Growth of pale orange colonies 24 hours 30 °C Red colonies due to xylose fermentation
Ryan's medium	*Aeromonas hydrophila* 00063 (8049) *E. coli* 00013 (12241)	Growth of yellow/yellow with green edge colonies 24 hours 30 °C No growth
TCBS	*Vibrio paraheamolyticus 00185* *Vibrio furnissii 00186* *E. coli* 00013 (12241)	Growth of green colonies Growth of yellow colonies Inhibited, no growth

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

Secretary
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Standing Committee of Analysts
Members assisting with this method

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

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N Barr Northern Ireland Water
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Z Bickel South West Water
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J Watkins formerly CREH *Analytical* Ltd
J Yeo UKAS

06/12/18

QEUH Renal Plants Chlorine Dioxide Protocol

RO Plant Storage Tank Water Supply (Pre Tank ClO_2)

↓

If residual is greater than 0.7ppm inititate BMS Alarm (and text) to renal technician on call and estates duty manager (24/7)

↓

If greater than 1ppm close fill valve to tank, open dump valve and ensure booster ClO_2 plant in basement is suspended. BMS alarm / text to renal technician on call and estates duty manager 24/7

RO Plant Storage Tank Water Supply (Post Tank ClO_2)

↓

If residual is greater than 0.7ppm inititate BMS Alarm (and text) to renal technician on call and estates duty manager (24/7)

↓

If greater than 1ppm close fill valve to tank, open dump valve and ensure booster ClO_2 plant in basement is suspended. BMS alarm / text to renal technician on call and estates duty manager 24/7

Post Carbon Filter ClO_2 - (if <0.03ppm no action)

↓

If greater than 0.03ppm then raise alarm to renal technician on call

↓

If greater than 0.05ppm switch to standby carbon filter. If remains greater than 0.05ppm for 30 minutes shut down renal system. Raise alarm to renal technician on call and estates duty manager 24/7

Version 1.0

From: Ritchie Lisa (NATIONAL SERVICES SCOTLAND)
To: Abigail.Mullings@[redacted] Alistair.Leanord@[redacted] Allison.Wood[redacted] craig.peaston[redacted] Guy.Fey[redacted] HPSInfectionControl (NATIONAL SERVICES SCOTLAND); Rachael.Dunk[redacted] sharon.mooney[redacted]
Cc: Joannidis Pamela (NHS GREATER GLASGOW & CLYDE); Armstrong Jennifer (NHS GREATER GLASGOW & CLYDE); Margaret.Mcguire[redacted]; Inkster Teresa (NHS GREATER GLASGOW & CLYDE); Mcnamee Sandra (NHS GREATER GLASGOW & CLYDE); Walsh Thomas (NHS GREATER GLASGOW & CLYDE); Reilly Jacqui (NATIONAL SERVICES SCOTLAND)
Subject: NHS GG&C NNU Serratia marcescens Outbreak Debrief Report
Date: 04 May 2016 12:44:14
Attachments: SBAR Serratia MAIN 10.52am 0300516.docx
HPS epi report GGC serratia Final.pdf

Dear Abigail,

Please find attached:

- NHS GG&C's Report of the Outbreak of *Serratia marcescens* in the NNU; and
- HPS ICT's Report of the descriptive epidemiological investigation of this outbreak

You will note that there a number of actions/recommendations in both reports. If the HAI Policy Unit is content with the reports and the proposals therein; we (HPS ICT and NHS GG&C ICT) will arrange to further discuss and progress the recommendations highlighted for action by both organisations e.g. development of a NNU Screening protocol for NHSScotland.

Kind regards,
Lisa

Lisa Ritchie
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Health Protection Scotland

NHS National Services Scotland

Descriptive epidemiological investigation of an outbreak of *Serratia marcescens* in a neonatal unit (NICU) in NHS GG&C.

(27/04/2016)

CONFIDENTIAL

Catherine Dalziel – healthcare scientist (health protection) HPS

Elaine Glass – data manager HPS

Shona Cairns – senior epidemiologist HPS

Michael Lockhart – consultant microbiologist HPS

Lisa Ritchie – nurse consultant infection control HPS

1. Background

On Tuesday 3rd November 2015, SGHSCD contacted HPS to invoke formally the National Support Framework CNO (2015) (http://www.documents.hps.scot.nhs.uk/hai/infection-control/guidelines/cno-algorithm-v2-2015-10.pdf) *"..to address in particular the reasons for the number of cases of Serratia marcescens identified in the NICU, Queen Elizabeth University Hospital since July 2015."* Between the 15th of July 2015 and the 3rd of November 2015, 13 cases of *S. marcescens* were identified in the NICU of the QEUH.

The purpose of this brief report is to present the findings of a descriptive epidemiological investigation of this outbreak. This report has focussed on describing the presence of identified risk factors in the affected patients and identifying potential clusters or cross transmission between cases during the outbreak period.

2. Introduction

Serratia marcescens, a member of the *Enterobacteriaceae* family, is an important cause of invasive infections in neonatal intensive care units (NICUs), with significant associated morbidity and mortality.[1] *S. marcescens* often develops multidrug resistance; tends to spread rapidly in the nosocomial environment; and has been implicated in outbreaks of nosocomial infection both in neonates and adults.[1] *S. marcescens* is more likely to colonise the respiratory and urinary tracts of hospitalised adults and the gastrointestinal tract of neonates.

A rapid literature search was performed in OVID Medline and Embase to identify peer-reviewed reports of similar outbreaks. The aim of this review was to identify potential outbreak sources and/or risk factors within this patient group. Using the key words 'serratia', 'outbreak', and 'neonatal'.

Sources of *S. marcescens* in reported outbreaks

The rapid literature search identified 82 reported outbreaks of *S. marcescens,* 32 of these identified possible sources of transmission, including:

- ventilator equipment[2-5]
- milk and feed additives [6-10]
- incubators[11;12]
- suction/aspirating equipment
- contaminated analysers (Blood-gas, glucose/lactate etc.)[13-15]

The most frequently implicated source of outbreaks was healthcare worker (HCW) hands. [5;12;16-21] However, items required for hand washing such as soap (plain or antimicrobial)[8;16;22;23],soap dispensers[4;24;25] and sinks/taps[1;2;21;26;27] were also frequently reported.

Single point-sources were uncommon in the literature, most studies did not identify an environmental source and the role of the sources that were identified was not always clear e.g. contaminated sink drains were possibly a result of contaminated hands and not contributing significantly to the outbreak(s).

A50795008

CONFIDENTIAL

Risk factors for *S. marcescens* colonisation/infection in neonates

The microbial colonisation of preterm, low birth weight infants' gastrointestinal tracts is markedly different from that of full term 'healthy' infants and has been described as 'delayed', 'aberrant' and 'dysbiotic', it is characterised by limited microbial diversity and the presence of opportunistic pathogens including *Serratia* spp.[28;29] Factors such as gestational age, birth weight, type of feed, reduced exposure to maternal flora and exposure to antimicrobials influence microbial succession in the preterm infants gut.

Reported risk factors for neonatal colonisation/infection with *S. marcescens*;

- Low birth weight <1500g
- Premature delivery <30weeks
- Use of invasive devices
- Mechanical ventilation
- Prolonged hospital stay >35 days
- Prolonged use of antibiotics >3 days
- Maternal infection prior to delivery[11;28;29]

It has also been shown that preterm infants tend to develop a profile that correlates with the flora present in the neonatal unit indicating a reservoir for these microbes in the NICU.[30] Bacteria present in the hospital environment typically display resistance to multiple antimicrobials and enhanced ability to form biofilm which aids survival in the hospital environment.[1;30]

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3. Methods

A descriptive epidemiological review was undertaken in order to address the following objectives:

1. Describe the cases in time, place and person
2. Investigate the presence of identified risk factors in the affected patients
3. Identify any potential clusters or cross transmission between cases during the outbreak period

Case definition (defined by NHS GG&C)

Cases were identified as *"Any baby colonised or infected with* S. marcescens *from any sample/screen site in the NNU from 27th July 2015."*

Data collection

NHS Greater Glasgow & Clyde (NHS GG&C) retrospectively reviewed the medical record and notes and produced a line listing to support the epidemiological investigation of this outbreak. The line listing included:

- CHI number,
- date of birth,
- date of discharge (or death),
- place of birth,
- mode of delivery,
- gestational age,
- weight at birth,
- date of admission to NNU, service,
- ventilation,
- presence of vascular catheters,
- feed (total parenteral nutrition, mother/donor expressed breast milk),
- surgical procedures,
- screening data including date of first positive, site and serotype,
- cot/room/unit occupancy.

HPS sought and gained approval to support the investigation from the Caldicott guardian in NHS GG&C and data were shared from NHS GG&C. HPS then used patient CHI numbers to extract additional microbiological data from ECOSS including sample dates, serotyping results and antimicrobial susceptibility of isolates.

Definitions for identifying clusters

An epidemic curve was plotted using dates of patients' first positive samples; a cluster was defined as two or more cases with the same serotype and ≤2 weeks between first positive samples.

Room occupancy data were analysed for patients with the same serotype to identify any overlap in room occupancy and therefore any potential cross-transmission events. Clusters

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within rooms were defined as two or more cases with the same serotype, at least one of whom first tested positive for *S. marcescens* while sharing a room. Cases were considered part of a cluster while housed in separate rooms provided they had shared a room no more than 7 days prior to their first positive sample (to account for weekly screening).

Data analysis

Distribution of patient characteristics

A descriptive epidemiological analysis was carried out to describe the distribution of demographic and exposure information provided in the QEUH line listing e.g. weight, gestational age, presence of lines among the cases identified.

Analysis of patient journeys (unit occupancy, first isolate dates and serotypes)

Data on duration of stay, unit occupancy and date and serotype of first isolate were extracted from the QEUH line listing. Patient journeys were plotted from admission to discharge (or death) and unit occupancy was colour coded for the duration of stay. The date of first positive sample was highlighted for each patient and colour coded by serotype.

Analysis of room clusters

Data on room occupancy including corresponding dates were processed to find evidence of patient clashes in time and place. Clashes within the same room were plotted and matched with serotype data to find evidence of clusters within a specific room.

All data analysis was performed using Microsoft Excel 2007 and Microsoft Access 2007.

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4. Results

Between 27th of July 2015 and 25th of January 2016 eighteen cases of *S. marcescens* were identified in the Queen Elizabeth University Hospital (QEUH) neonatal unit (2 infections, 16 colonisations) including three patient deaths (1 colonised, 2 infected). Since the 25th of January 2016 no new *S. marcescens* positive screen results have been reported.

The epidemic curve (Fig. 1) of this outbreak appears to show two possible clusters, the first occurring between 20th of July 2015 and 7th of September 2015 and the second between 5th of October 2015 and the 2nd of November 2015.

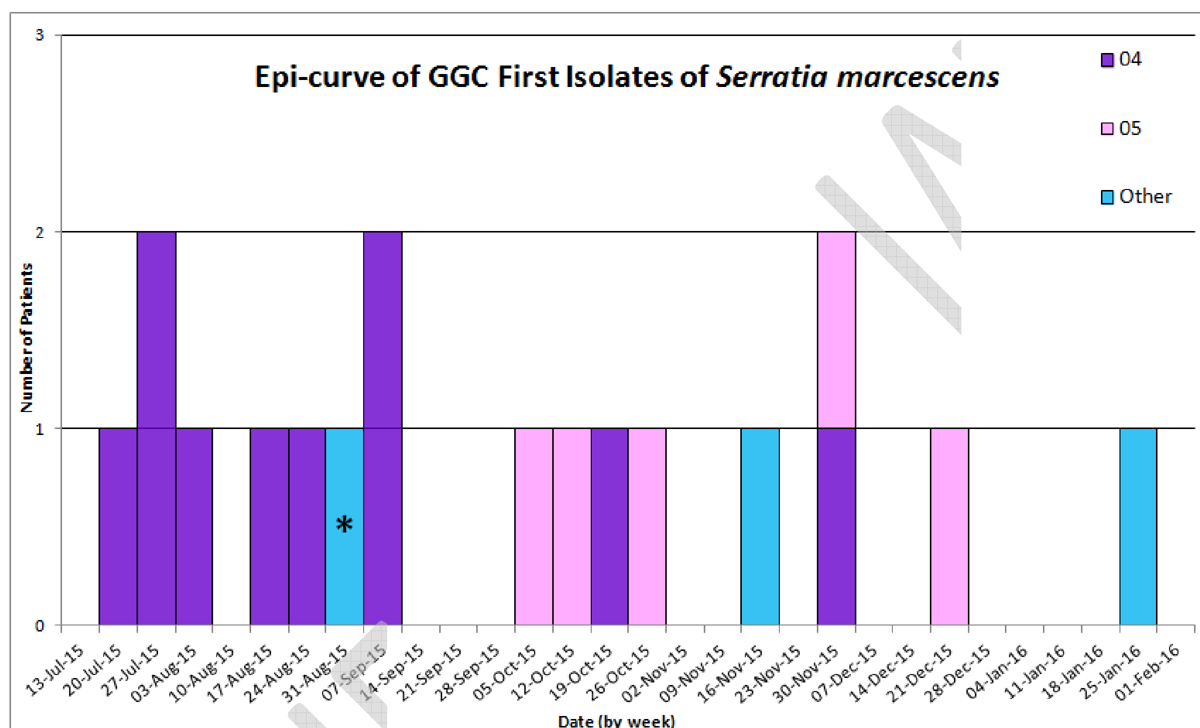

Figure 1: Epidemic curve of cases (infection or colonisation) of *S. marcescens* in neonates at the QEUH neonatal unit between July 2015 and January 2016. The epidemic curve shows the date (week) of first positive (*S. marcescens*) sample per patient and the isolate serotype (purple - serotype 04, pink – serotype 05, light blue – a unique serotype). Data were provided in the line listing by QEUH and verified against data extracted from ECOSS using patient CHI numbers. *One patient was previously identified as colonised with *S. marcescens* in April 2015 while admitted in a different board, they subsequently screened positive in September 2015 while admitted to the QEUH.

Patient characteristics/risk factors

A descriptive analysis of patient characteristics provided in the QEUH line listing was performed. All of the cases had a least one of the risk factors identified in the literature.

As shown in **table 1** the gestational age (weeks) of cases was evenly distributed between:

- full term (≥37 weeks),
- preterm (32 to 36 weeks),
- very preterm (28 to 31 weeks) and
- extremely preterm (<28 weeks).

Table 1: Distribution of gestational ages of cases

Gestational age (weeks)	≥37 weeks	32 to 36 weeks	28 to 31 weeks	<28 weeks
Number of cases	4 (22%)	6 (34%)	4 (22%)	4 (22%)

Low birth weight and premature delivery were present as risk factors for some of the cases; five cases (27.7%) had birth weights of <1500g and these cases were all ≤29 weeks gestation. However, the majority of cases (12 out of 18 (67%)) were born at >30 weeks gestation, 11 of whom had birth weights of >1500g including four weighing ≥3195g (7lbs) at birth. The median birth weight of all 18 cases was 1785g, with birth weights ranging from 540g to 3640g.

In this particular outbreak, weight and gestational age of cases did not consistently meet the criteria for risk factors as identified in the literature. Of the two cases who died from blood stream infections (BSIs), one was extremely premature [redacted]) and weighed 1060g at birth while the other was born prematurely at [redacted] and weighed 2300g. Also, no correlation was found between gestational age of cases or weight at birth and the length of time from birth until first positive sample (PCC = -0.09 and 0.01, respectively). The median number of days from birth to first positive sample was 27 days (ranging from 19 to 93 days).

All of the cases except one (94.4%) had ventilation or an invasive vascular device (umbilical catheter/peripherally inserted central catheter/central vascular catheter) or both; 16/18 cases were ventilated (88.8%) and 13/18 (72.2%) cases had an invasive device at some point during their hospital stay. The median length of stay was 63.5 days (ranging from 21 to 207 days) (discharge data unavailable for 2 patients); 14 (87.5%) of the cases for whom data were available were admitted for ≥35 days.

Antimicrobial therapy data have not been made available but informal discussion with QEUH suggests that all patients received some form of antimicrobial prophylaxis/treatment during their stay, and indeed weekly screening was described as a method of guiding empirical treatment.

Laboratory testing of isolates

Serotyping of isolates from 18 cases identified 10 SERN07SE-4 isolates, 1 unique SERN07SE-4 isolates, 5 SERN07SE-5 isolates and two isolates with unique serotypes.

Antimicrobial susceptibility for all isolates was extracted from ECOSS; all isolates were susceptible to aztreonam, cefixime, cefepime, cefradine, ciprofloxacin, ertapenem, gentamicin, meropenem, tazocin and trimethoprim (data not shown). In addition, all isolates tested for sensitivity to cefuroxime, amoxicillin and co-amoxiclav were found to be resistant (*S. marcescens* is intrinsically resistant to ampicillin, to which amoxicillin and co-amoxiclav are closely related).

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Interestingly, strains isolated later in the outbreak tended to have resistance to additional antimicrobials. In some later isolates additional resistance or intermediate susceptibility to one or more of amikacin, cefoxitin, temocillin (this is unusual and as MIC data were not provided in ECOSS, HPS could not confirm this result) and tobramycin was found.

Patient admission journeys and first isolate dates

Patient admission data extracted from the QEUH line listing was plotted in chronological order and overlaid with the date of first isolate including the isolate serotype (Fig.2). With the exception of [redacted] and [redacted] (who had previously tested positive for *S. marcescens* while in another board), all of the patients first tested positive while admitted to, or immediately after leaving the NICU. [redacted] left NICU room 4 on 29/08/2015 and a positive test result was returned on 30/08/2015.

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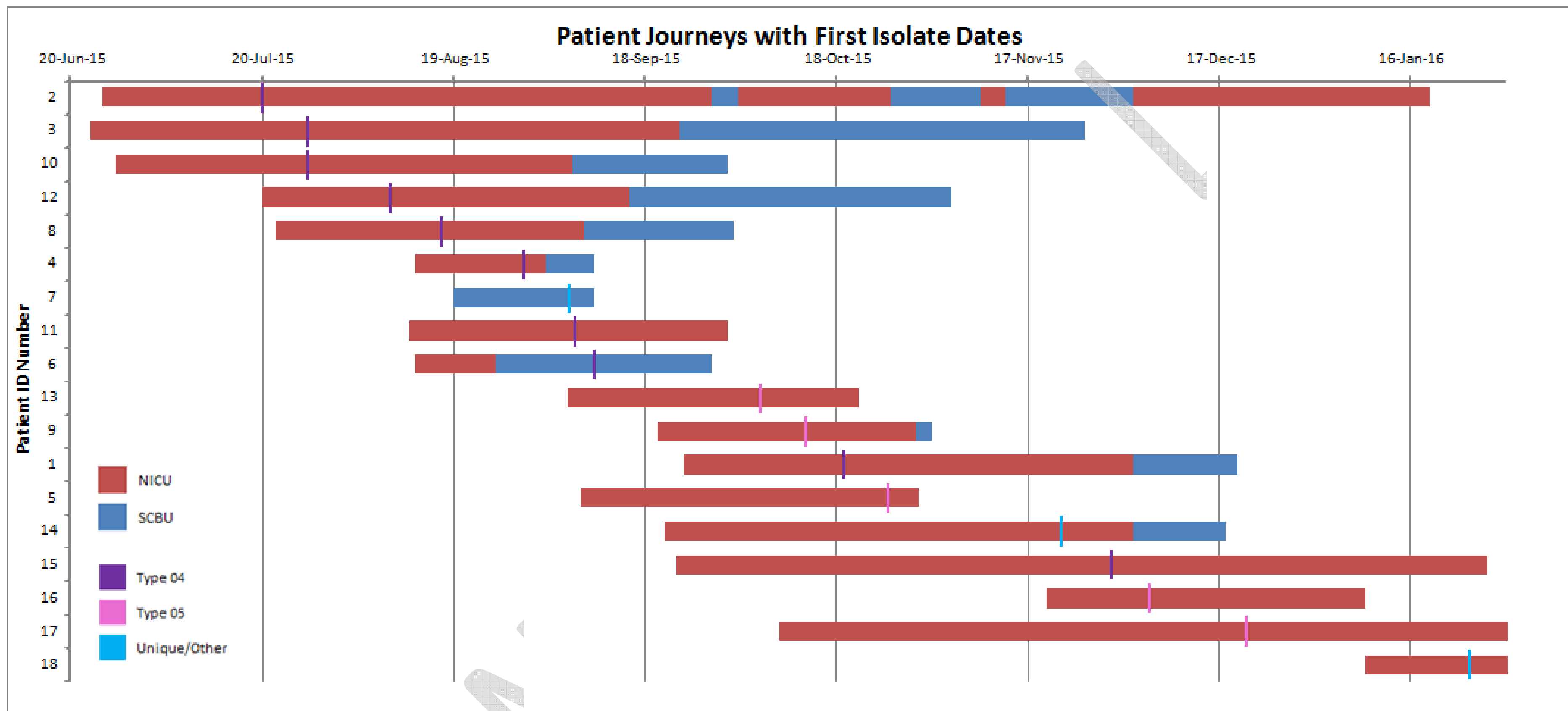

Figure 2: Patient Journeys: unit occupancy, date and serotype of first isolate. Data on duration of stay, room occupancy and date and serotype of first isolate were extracted from the QEUH line listing. Patient journeys were plotted from admission to discharge (or death), time spent admitted to the NICU and SCBU are highlighted in red and dark blue, respectively. The date of first positive sample is highlighted by a vertical bar (purple - serotype 04, pink – serotype 05, light blue – a unique serotype) for each patient.

Identification of potential clusters in rooms

Analysis of room occupancy data identified three potential clusters where cases were linked in time and place and were colonised or infected by strains with the same serotype.

- Cluster 1: patients 2, 3, 4, 11 and 15 were all colonised with a serotype 04 strain. Analysis of room occupancy data shows overlap between these patients in NICU room 4 before testing positive for *S. marcescens* (Fig. 3).
- Cluster 2: patient 4 tested positive for *S. marcescens* after occupying NICU room 6 with patient 12, both were colonised with a serotype 04 strain. (Fig. 4).
- Cluster 3: patients 5, 9 and 13 all occupied NICU room 7 at the same time before testing positive for *S. marcescens* (Fig. 5). These patients were all colonised or infected with a serotype 05 strain. Patients 5 and 13 died nine days apart, *S. marcescens* sepsis was reported on the death certificate of one patient while the other was colonised. Patients 5 and 13 were both extremely premature and had very low birth weights, the patients had similar interventions, although one patient was born by emergency caesarean section and the other by standard vaginal delivery and the patients were in different specialities (medical and surgical). The patients were born in different boards and neither was born at the QEUH. As these patients had the same serotype, this indicates that at least one of the patients was colonised while at the QEUH rather than the board they were born in.

Interestingly, isolates from patients 2 and 3 in cluster 1 and patient 12 in cluster 2 showed the same antimicrobial susceptibility profile (resistance to amoxicillin, cefuroxime and co-amoxiclav). This was also true of patients 5, 9 and 13 in cluster 3 (resistance to amoxicillin, cefuroxime, co-amoxiclav and cefoxitime). Antimicrobial susceptibility profiles were not available for patients 4 and 15.

Analysis of patient journeys to determine whether patients linked in time and place were also linked by procedures etc. has been performed, however, no other potential sources/locations of cross-transmission were identified (data not shown).

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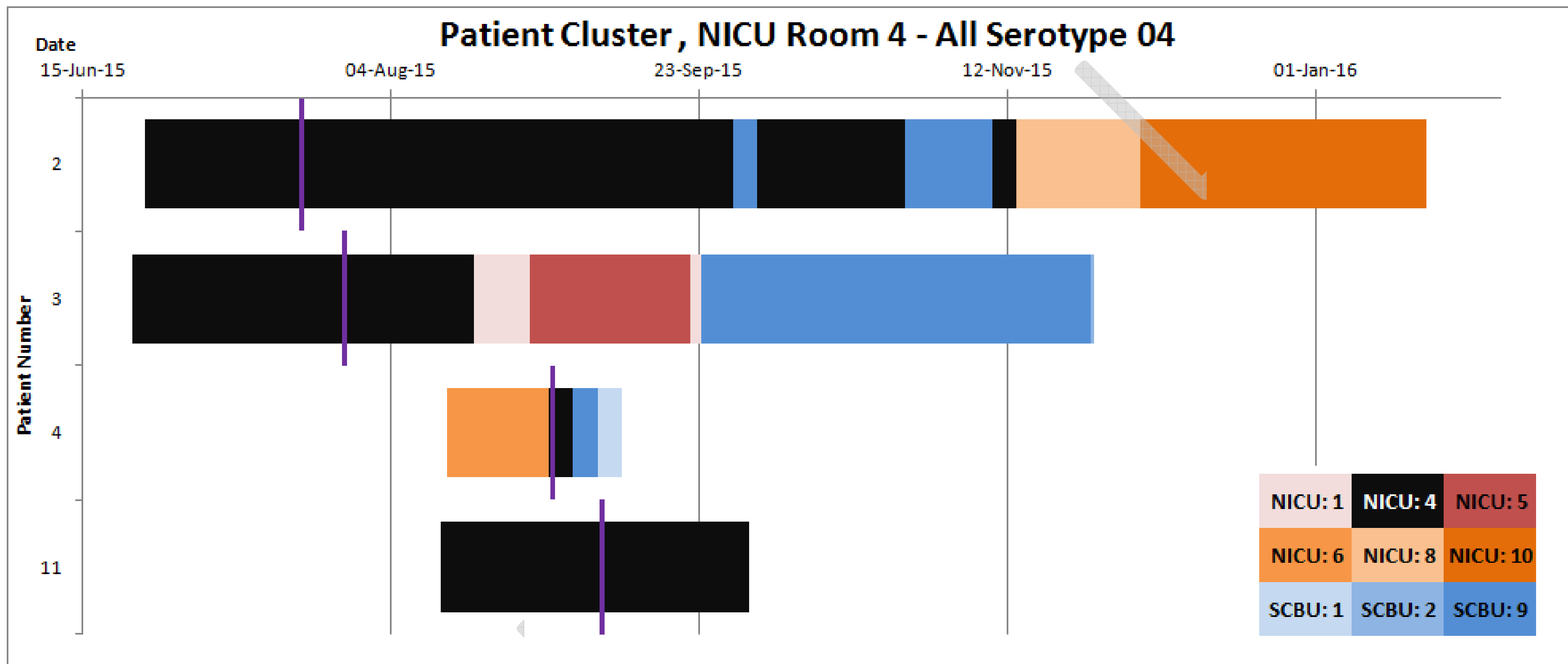

Figure 3: Potential cluster in NICU room 4 (black bars) involving patients 2, 3, 4 and 11. Room occupancy (transfer history) data was extracted from the QEUH line listing and potential clusters were identified where patients shared a room and tested positive for an identical serotype either while sharing a room or within one week of leaving a shared room. The date of first positive sample is highlighted by a vertical bar (purple - serotype 04) for each patient.

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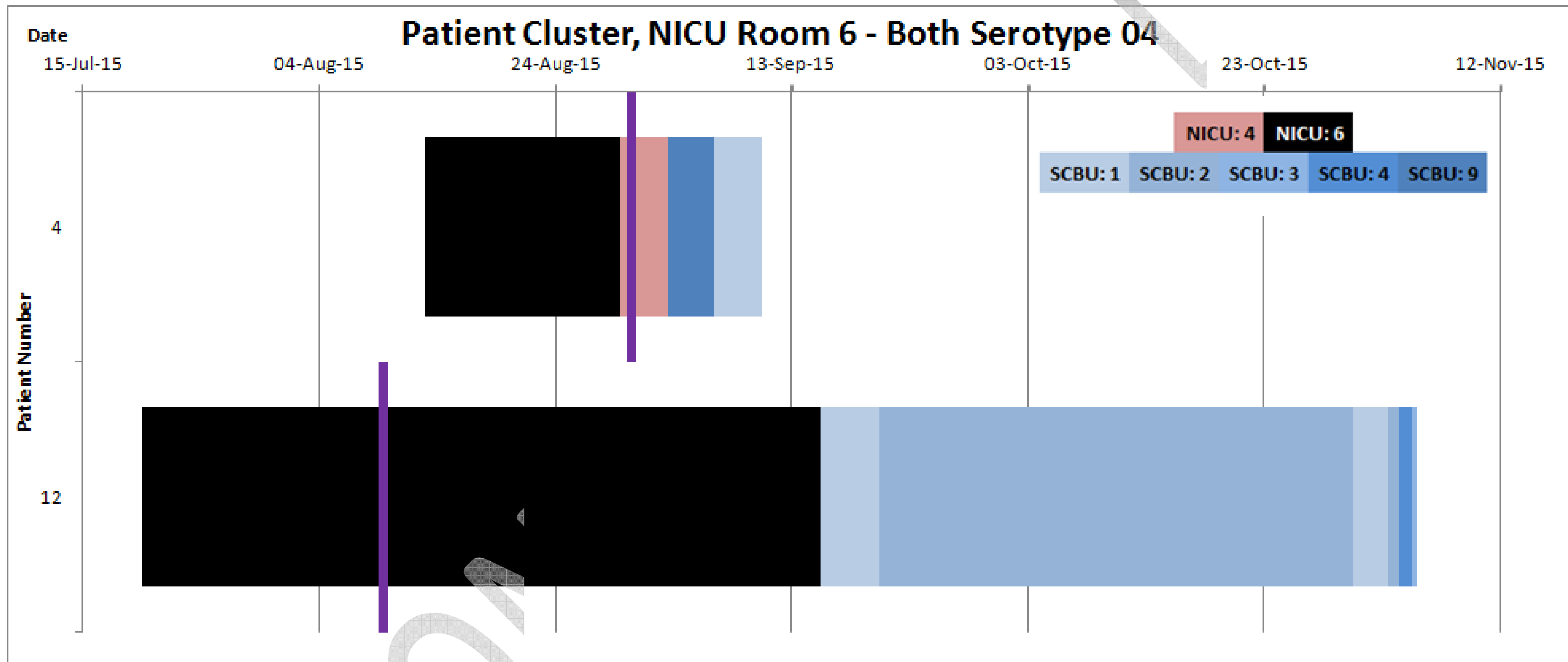

Figure 4: Potential cluster in NICU room 6 (black bars) involving patients 4 and 12. Room occupancy (transfer history) data was extracted from the QEUH line listing and potential clusters were identified where patients shared a room and tested positive for an identical serotype either while sharing a room or within one week of leaving a shared room. The date of first positive sample is highlighted by a vertical bar (purple – serotype 04)) for each patient.

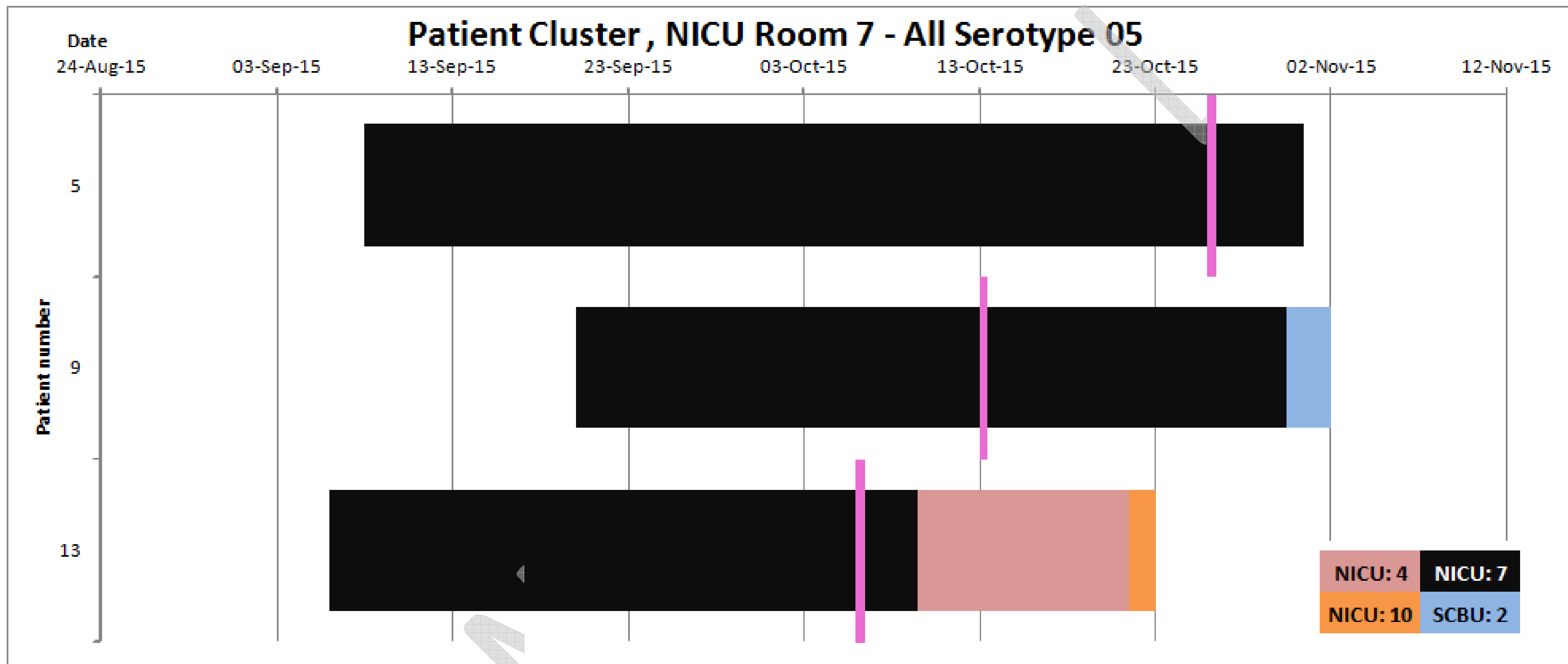

Figure 5: Potential cluster in NICU room 7 (black bars) involving patients 5, 9 and 13. Room occupancy (transfer history) data was extracted from the QEUH line listing and potential clusters were identified where patients shared a room and tested positive for an identical serotype either while sharing a room or within one week of leaving a shared room. The date of first positive sample is highlighted by a vertical bar (pink – serotype 05) for each patient.

5. Discussion

A number of risk factors for colonisation of neonates with *S. marcescens* were identified in the literature. The majority of cases in this outbreak had several of the risk factors identified in the literature including ventilation, presence of invasive vascular devices, duration of stay ≥35 days and potentially, prolonged use of antibiotics. There is a suggestion in the literature that colonisation with *Enterobacteriaceae* such as *S. marcescens* may be typical in premature or very low birth weight babies as the gut microflora develops very differently to that of full term, healthy weight babies, however, premature delivery (<30 weeks) and low birth weight (<1500g) were not commonly described in the cases in this outbreak (33% and 28%, respectively).

This outbreak primarily took place in the NICU and centred on rooms 4, 6 and 7. An analysis of room occupancy data identified three potential clusters where patients with identical serotypes were linked in time and place. It may be useful to focus on rooms 4, 6 and 7 for a more detailed analysis of staffing and/or procedures within these rooms. Several patients were not associated with room clusters based on room occupancy data but screened positive at the same time as other patients and also had identical serotypes and antimicrobial susceptibility e.g. [redacted] for room cluster 1. An assessment of the dates of various interventions e.g. line insertion or ventilation, did not highlight any potential overlap in equipment or feed that could account for cross-transmission.

As part of their own outbreak investigation the QEUH performed extensive environmental and equipment testing (including hand hygiene sinks, taps, soap dispensers, water sampling, breast pumps, milk bottles, fridges etc.) but did not find a source of *S. marcescens* on their unit; this is not unusual and most reported outbreaks in the literature were also unable to identify an outbreak source. QEUH established an extensive infection prevention and control action plan to resolve the outbreak which focussed on hand hygiene compliance and education for staff and parents, correct PPE usage, management of equipment and environmental cleaning. It should also be noted that a hand hygiene audit in November 2015 identified 60% combined hand hygiene compliance including two failures to perform hand hygiene and six failures due to technique, HCWs hands were the most identified outbreak source in the literature[5;12;16-21] and steps have been taken to improve hand hygiene compliance on this unit. It is likely that this outbreak was multifactorial; in particular the service changes as a result of the amalgamation of neonatal services in NHSGGC (completed in mid-June 2015) may also have been an outbreak provoking factor.

It is important to note that this outbreak was identified by routine screening. HPS has performed a survey into neonatal screening in NHSScotland; the responses identified a lack of consistency both within and between boards, no two boards performed screening in the same way. In addition, at the time of collating survey responses NHSGGC had a comprehensive screening protocol and was the only board routinely screening for *Serratia* spp. (other than those boards that began specifically screening neonates transferred in from NHSGGC for *S. marcescens* after this outbreak was reported). However, a baseline rate or ‘acceptable’ number of *S. marcescens* colonisations or infections had not been determined by NHSGGC and there is generally a lack of National or evidence-based guidance on this topic.

Finally, it was reported by GG&C that screening results were used to guide antimicrobial therapy. Variation in antimicrobial resistance profiles was noted among isolated strains over the course of this outbreak. However, as antimicrobial prophylaxis/treatment data has not been made available it is not possible to determine whether this variation was driven by antimicrobial prescribing practice.

6. Conclusions

While it is accepted that many of the patients involved in this outbreak would not be described as 'full term, healthy weight' and had additional risk factors as described above, the presence of three clusters epidemiologically linking the cases in time and place strongly suggests that cross-transmission did occur within the unit and that the detection of *S. marcescens* by routine screening was not simply a reflection of 'typical' colonisation of a susceptible patient group. In addition, all patients had been admitted for at least two weeks (19 to 93 days) before their first positive screening sample, clearly indicating that *S. marcescens* was healthcare associated.

Often an outbreak will start with a change in something e.g. a new process or a new product. Recent, key changes that may have increased the likelihood of this outbreak arising may have been as a result of the Neonatal services amalgamation (completed mid-June 2015); resulting in changes to/differences in staffing arrangements, visiting policy, screening protocols and decontamination practices and procedures.

There is no nationally agreed screening protocol for neonatal units (NICU, SCBU, SHDUs); there is also a lack of guidance available on acceptable rates of *S. marcescens* detected by screening on neonatal units and appropriate actions to take when patients are identified as colonised. This may have affected both the detection of this outbreak and subsequently, its reporting to HPS/SG.

Finally, it may be that antimicrobial prescribing encouraged the selection of relatively resistant organisms in this outbreak, but as prescribing information was not shared no firm conclusions can be inferred.

7. Recommendations

- Epidemiological investigation:
  - Processes should be agreed to facilitate formal epidemiological investigation in the event of an outbreak. Specifically, case definitions for colonisations and infections should be determined rapidly and reviewed regularly as should the collection of patient data into a comprehensive line listing.
  - In the event of an outbreak a systematic approach to environmental and equipment sampling should be devised which prioritises the highest risk items/areas based on a rapid review of the outbreak literature.
- Screening:
  - A clearly defined neonatal unit screening policy should be agreed which specifies sampling sites.
  - Acceptable limits for colonisations/infections should be defined and plans for appropriate actions to take in the event of a positive screening result should be agreed.

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  - HPS, in collaboration with colleagues nationally and across the UK will aim to produce guidelines on neonatal screening to improve consistency of practice across NHSScotland, determine acceptable limits for neonatal colonisations and advise on actions to be taken for positive screening results.
- Antimicrobial stewardship:
  - We would recommend for further similar incidents that antimicrobial prescribing information is shared with the Incident Management Team.
- Adherence to standard infection control precautions (SICPs):
  - SICPs audits, particularly hand hygiene should be reviewed following service changes such as amalgamation as well as during an outbreak situation.
  - SICPs audits reports should be made available to the IMT

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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 – Miscellaneous Documents

Volume 16

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