SCOTTISH HOSPITALS INQUIRY

Bundle of documents for Oral hearings commencing from 19 August 2025 in relation to the Queen Elizabeth University Hospital and the Royal Hospital for Children, Glasgow

Bundle 44 Volume 3 - Substantive Core Participants' Direction 5 Responses to GGC Expert (HAD) Report & Supplementary Report/Comments on Chapter 8

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SCOTTISH HOSPITALS INQUIRY

RESPONSE TO INQUIRY EXPERT PANEL REVIEW OF EXPERT REPORT OF PROFESSOR HAWKEY, DR AGRAWAL AND DR DRUMRIGHT

ON BEHALF OF NHS GREATER GLASGOW AND CLYDE

1. INTRODUCTION

1.1. The Inquiry has requested that core participants provide comments on the following documents:

1.1.1. Review by NHS NSS of Report by Prof Hawkey, Dr Agrawal and Dr Drumright

1.1.2. Review By Dr Mumford in response to Report by Prof Hawkey, Dr Agrawal and Dr Drumright

1.1.3. Joint report by Dr Mumford and Mr Mookerjee on Aspergillus

1.1.4. CNR Panel Rebuttal of Report by Prof Hawkey, Dr Agrawal and Dr Drumright

1.1.5. Review by Mr Mookerjee of Report by Professor Hawkey, Dr Agrawal and Dr Drumright.

(together the "Review Reports"). This document is NHSGGC's response to that request.

1.2. NHSGGC submits that it is for Professor Hawkey, Dr Agrawal and Dr Drumright to provide a response to the Review Reports. They are independent expert witnesses. Their duty is to assist the Chair and their independent expert opinions must not be influenced by any core participant. They were asked to undertake an independent analysis of the evidence of risk of infection from the water and ventilation systems at QEUH and RHC. The means of carrying out that analysis, and the methodology chosen, was a matter for them. However, their analysis was data driven allowing for comparison with other hospitals. This, it is submitted, is appropriate given the expected background level of infection in a hospital and the fact that infection prevention, control and treatment is multifactorial.

1.3. As independent experts, the authors must take into account all relevant information [*Kennedy v Cordia (Services) LLP [2016] UKSC 6; 2016 S.C. (U.K.S.C.) 59*]. NHSGGC has provided general comments, correction of factual inaccuracies in the Review Reports and further factual information on the basis that it may assist Professor Hawkey, Dr Agrawal and Dr Drumright in providing a response to the questions raised in the Review Reports.

1.4. NHSGGC has provided more detailed commentary, restricted to factual issues, in **appendix 1** of this document. **Appendix 2** contains a paper prepared by Dr Dominique Chaput with input from IPC doctors. It is submitted that Dr Chaput can provide relevant evidence on these issues and NHSGGC repeats its submission that the Inquiry ought to hear evidence from Dr Chaput in the Glasgow IV hearings.

2. INQUIRY EXPERT PANEL

2.1. NHSGGC notes that Mr Mookerjee frequently relies on the conclusions in his previous report in his Review Reports. Those conclusions are, in NHSGGC's submission, fundamentally flawed. As previously set out in detail, Mr Mookerjee applies the incorrect denominator in his calculations, by failing to take into account day cases, meaning that he grossly overstates the infection rate in the QEUH/RHC. Dr Mumford's report is similarly flawed for reasons including, but not limited to, her reliance on Mr Mookerjee's flawed analysis.

2.2. Reference in this regard is made to NHSGGC's Direction 5 responses, and its written submissions dated 31 January 2025 and supplementary written submissions dated 26 June 2025 provided in respect of the Glasgow III hearings. The criticisms of the Inquiry Expert Panel are not repeated in full here. NHSGGC invites the Chair to have regard to these documents in assessing the evidence of the various experts.

2.3. It is noted that Mr Mookerjee in his review of 'Chapter 7' refers to a supplementary statement of Dr Christine Peters. That statement has since been provided to core participants. However, a second supplementary statement is referred to within Dr Peters' first supplementary statement. That has not been provided to NHSGGC, or to NHSGGC's knowledge any other core participant. Given the requirement that an independent expert take into account all relevant information, it is essential that Professor Hawkey, Dr Agrawal and Dr Drumright be provided with a copy of all of Dr Peters' statements as soon as they are available. It is for the authors to comment on Dr Peters' statements and so no further comment is made here.

2.4. It is further noted that Dr Mumford's report indicates that the Inquiry asked her a series of questions. She does not say what these questions are. NHSGGC submits that, in order to ensure transparency, the questions asked of each of the authors of the Review Reports should be made available to core participants.

APPENDIX 1 - TABLE

Bullet/ Page	Comment/Question
Review by NHS NSS of Report by Prof Hawkey, Dr Agrawal and Dr Drumright	
Summary	Within this report there are a number of areas for the HAD report authors to respond to including the interpretation of data and the conclusions reached. GGC will not comment on the specifics as it is for the HAD authors to respond. The overall report appears to be a critique of the HAD report rather than an independent view of a national health organisation. As a general point in relation to all of the Review Reports, it is not clear what the request was from the Inquiry to the Review Reports authors. It would be helpful it the requests were shared with all Core Participants to ensure full transparency. The following are high level comments.
2.4.1	If there was 'limited time to consider these files in any detail' as stated, then how much confidence can be placed in the contents/conclusions of the NSS report?
3.4.3	"To find this location as a risk for developing BSI with a potentially environmental source an epidemiological timeline is required for each patient to describe their admission history and identify links in time and place." This is something that did not happen in the HPS analysis of the outbreak.
3.4.4	Clusters being defined as two cases does not include single cases of unusual organisms or potential for polymicrobial episodes and limits interpretation. HPS did not include this within their own analysis.
3.5.1	When Yorkhill moved to RHC there was an intended reduction in activity to allow the service to orientate itself before commencing to a full operational status - this would have been the reason for the decrease in rates in the first 6 months.

Bullet/ Page	Comment/Question
3.5.4	Ms Cairns raises concerns about duration of time for which linear trend lines are fitted. Ms Cairns suggests that HAD authors could have segmented times between moves to see if there was an effect on smaller time scales. ARHAI did not include this in their own analysis.
3.5.5	Unless the 'environment' did not play a part in either hospital then the rates were due to intrinsic risk factors in the patient population.
3.5.6	There are differences in methodology between ARHAI and the HAD report including denominator and definitions.
4.5	The small numbers and the analysis makes assessment difficult. The HAD authors have by necessity due to the small numbers made descriptive comments. Ms Cairns suggests root cause analysis for each patient to explore a possibility of links. This was not something ARHAI did in their own analysis.
CNR Panel Rebuttal of Report by Prof Hawkey, Dr Agrawal and Dr Drumright	
Page 18, last para	Re design of sinks – statement "*the taps installed in the paediatric haematology oncology wards at NHSGGC were not of approved design*" is not factually correct.
Page 24, Bullet1	GGC challenges the assertion of less than adequate IPC measures.
Page 27, Last para	On a number of occasions there is reference to poor IPC practice. The suggested evidence is "poor quality data and lack of governance and assurance relating to improvement to IPC based on audit submissions". However, this is not evidence of poor practice. The CLABSI work was bespoke to RHC and was groundbreaking. Multidisciplinary team weekly visits were also in place and a report went to the Board Medical director weekly during a large part of 2017. Refer to Response by Dr Chaput with input from Infection Control Doctors in Appendix 2 of this response requesting CNR evidence of poor IPC practice.
Page 47, 2nd last para	The CNR authors seem to suggest that the movement of the patients from 2A to 6A in September 2018 stopped the cases of *S. maltophilia.* However, both wards are supplied from the same water system, and both had the same controls in place i.e. POUF.
Joint report by Dr Mumford and Mr Mookerjee on Aspergillus	
General	Refer to comments within the Response by Dr Chaput with input from Infection Control Doctors in Appendix 2 of this response.

Bullet/ Page	Comment/Question
2.17.4 & 2.17.5	There is no reference to the fact that this was a general ward and not a specially ventilated area. There is no guidance for air sampling in specialist areas let alone general wards.
Review By Dr Mumford in response to Report by Prof Hawkey, Dr Agrawal and Dr Drumright	
General	The term environment is used interchangeably. Stating patients are colonised from organisms in the environment is widely accepted. However, there is no evidence that there is a direct link to water. From early in 2018 all water in Schiehallion was filtered, mitigating risk. There is also reference to IPC practice and line care which has been previously identified as an issue. This is different to the implication that the building itself led to infection.
2.8	General point: it is not clear if the Review Authors were provided with the terms of reference of the HAD Report. Inquiry to confirm position.
3.11	States that WGS is not available for outbreak investigations. For an "outbreak" lasting over 18months or longer then WGS does have a role to play. Agreed not in the immediate first few hours or days but it is valued over the longer term and for retrospective look back.
3.12	Disagrees with proscriptive outbreak identification which is not compatible with infection control practice. This was not part of the remit of the HAD authors.
3.14	Disagrees that infections can be translocated from the gut to the bloodstream. Dr Mumford states that there is no evidence of gut carriage of rarer organisms and so other sources need to be considered. However numerically the main organisms that were looked at and by HPS (ARHAI) were Stenotrophomonas, Enterobacter, Klebsiella. The last 2 organisms are well recognised gut organisms. Stenotrophomonas have been shown to colonise the oropharynx as quoted in the HAD report. There is no data on carriage in the gut of many of the pure environmental organisms. If Stenotrophomonas, Enterobacter, Klebsiella were excluded then there is no increase in infection rates and therefore no outbreak.
3.17	Quotes MRSA contamination as an environmental organism that causes infection and suggest HPV should have been used in QEUH. Not aware of any Scottish hospital using HPV routinely. Also, MRSA very different from environmental gram negatives.

Bullet/ Page	Comment/Question
4.2	States colonisation of patients from the hospital environment before infection will make infection look like it is endogenous. However, Dr Mumford does not consider that there is the possibility that normal organism carriage as a result of colonisation outwith the hospital can occur as well.
4.4	Cites MRSA data as evidence of colonisation prior to infection. This is not relevant.
5.4	Claim that the rates of BSI reduced when the unit was transferred. The unit activity was deliberately reduced for several months in order to move so it could be hypothesised that less patients/less invasive treatments equals a reduction in BSI and not the move to the new hospital as hypothesised here.
5.7	States that not all samples were cultured for environmental organisms. All gram negatives from spring 2018 were identified and saved. As many as possible were collected and recultured. Unfortunately, not all organisms survive extended storage at -80C. Therefore, no retrospective analysis will have 100% of all organisms from patients of interest. Dr Mumford claims that different strains of organism were identified and unclear how many were saved. Prof Leanord's work shows that many organisms on a plate are clonal and so despite this evidence and no evidence from Dr Mumford to support her statement still repeats the above. Additionally, Dr Mumford states WGS was not done on all clinical isolates. She argues that if WGS evidence does not exist then no claim can be made that there was no link to the Hospital environment. This is stating that you must prove a negative before you can believe WGS, which is not possible.
5.11	Dr Mumford makes a case that meropenem would not have been used if meropenem resistant organisms were identified. That is correct. However, Dr Mumford does not address the point that meropenem selects out the resistant organisms after treatment of other organisms or because it was used as empirical treatment before any organisms were grown. In many cases no organisms are cultured. Meropenem therefore is used appropriately but selects out organisms that can either infect in real time

Bullet/ Page	Comment/Question
	or can cause and infection on re-admission as a result of an already carried meropenem resistant organism.
5.12	States that numbers of Stenotrophomonas were low and were not the main drivers of environmental gram-negative inventions. However, stenos were numerically the most numerous organism within patient infections. This statement is incorrect.
6.4	States that acquisition of environmental bacteria will result in skin or mucosal rather than Enteric carriage citing no evidence. It is just as likely that an organism could be ingested and reside in the gut.
6.8	Neurosurgical ITU is not within the QEUH/RHC building.
6.10	As discussed in paragraph 3.13 and 4.2 environmental acquisition in hospital leads to increased incidents of colonisation and carriage. This contradicts point raised at 6.4.
6.12	Filters were in place from early 2018. No unfiltered water in RHC Ward 2A after March 2018.
6.13	Cites Dr Walker's report as evidence for 6.12. Dr Walker said in evidence that there was no agreed level of bacterial numbers that distinguish normal levels of organisms from "contaminated" levels of organisms.
6.30	GGC did have typing. What was possible at the time was done to see if a link could be determined. Below is a section of the Hot Debrief document prepared by Dr Inkster in 2018 post the first IMT. *"One historical case (2016) of Cupriavidus bacteraemia linked to current incident by typing.* *Typing reveals at least 5 different strains of Cupriavidus in patients and water* *Different strains of Stenotrophomonas identified in patients and water.* *Typing of historical isolates of other organisms from patient and water has revealed no link so far"*
8	Chapter 8 gives a critique of analysis of the data. States that analysing the data on a consultant basis rather than a geographical basis is flawed.

Bullet/ Page	Comment/Question
	These patients were at times widely dispersed and so using haem-oncology Consultants allowed them to be identified and tracked.
8.6	They were all typed, and all were found to be unique.
8.12	VRE is not a relevant infection.
8.17	Reference to the Oversight Board commenting on environmental data is not correct. The Oversight Board restated the position of the Case Note Review.
8.18	HAD report states that if environmental organisms were infecting in the same pattern as not environmentally relevant organisms, then this would indicate the environment did not play a significant role in transmitting these organisms. Dr Mumford suggests this does not take into account the complete history of infections at QUEH/ RHC. Dr Mumford does not provide the basis of her position.
8.46	No consideration is given to the reduction in planned activity post move. Please see comments at 5.4

APPENDIX 2

RESPONSE BY DR CHAPUT WITH INPUT FROM INFECTION CONTROL DOCTORS

General comments

1. Many of the criticisms voiced by the authors of the Review Reports appear to make fundamentally flawed assumptions about NHSGGC's position. To clarify:

NHSGGC is not asserting that the environment (in general) is not a possible source of infections

2. NHSGGC is not denying that the hospital environment can be a source of infection in hospitalised patients, particularly those who are immunocompromised. The difference lies in proportionality – what proportion of infections comes directly from the hospital environment versus what proportion comes from other sources, particularly the patient's own flora. It is an oversimplification to assume that in every case of an infection by an 'environmental' organism, patients have caught the infection directly from the hospital environment (keeping in mind that there is no clear definition of what constitutes an 'environmental' organism). It is more likely and increasingly recognised that infections arise from complex interactions that select for certain 'environmental' taxa that may already be present in the patient's microbiome.

NHSGGC is not asserting that meropenem use was the sole cause of patient infections with 'environmental' taxa

3. NHSGGC is not claiming a simple cause-effect, whereby individual patients are given meropenem and then develop an infection. NHSGGC are highlighting that development of infection is complex and multi-factorial, and that broad-spectrum antibiotics, including meropenem, exert selective pressures at the ward/population level that increase the likelihood of some types of infections occurring in that ward/population.

NHSGGC is not asserting that infections that occurred at the QEUH since it opened in 2015 were not from the hospital environment

4. There may have been rare instances where an infection was acquired from the hospital environment at the QEUH, but NHSGGC's position is that this was no more likely at the QEUH than anywhere else, i.e. that there is a baseline level of risk of environmental acquisition, particularly for immunosuppressed patients, regardless of where they are. If the expectation

being placed on the QEUH is that no 'environmental' HAI must ever have occurred (i.e. that the risk of environmental acquisition must have been and remained zero), then the experts must show more clearly that this expectation is also being applied to and met by other UK hospitals, many of which have published papers outlining instances of infections linked to their water systems. However, in the case of the QEUH, Dr Mumford, Mr Mookerjee and the CNR authors appear to conclude that many or most GNB and fungal infections in this patient cohort were acquired directly from the hospital environment, and it is this conclusion that is not supported by NHSGGC's data, the HAD report analysis, or the comparator hospital data in Dr Mumford and Mr Mookerjee's own analysis.

5. Both expert groups have highlighted challenges in the practical application of the outbreak definition. We believe this arises from interpretation of the phrase "same infectious agent" and the level of identification methods required to establish relatedness – whether it should be genetic methods or conventional methods identifying to species level only. We agree this is a complex issue that IPC teams face frequently and would welcome continuation of this discussion at a professional forum to clarify the view.

6. We find the oversimplification of principles that associate a microorganism with potential acquisition from surrounding environment and indiscriminatory use of term 'environmental microorganisms' unhelpful. This wrongly leads to an assumption that these microorganisms are exclusively acquired only from the hospital environment.

7. It is clear that to have confidence in data and interpretation, agreement on case definition and data collection sources and methods is absolutely essential. It needs to be recognised that there is no established surveillance system for infections in haematology/oncology patients in the UK or in other countries, and all numbers that have been proposed to be used as "expected" rate of infection are flawed and must be interpreted with caution. We would welcome a UK-wide discussion and agreement on the need for such a surveillance programme. We welcome statements of all expert groups on the importance of comparing like with like and the problems with interpreting small datasets. Strict definitions of cases and datasets will also help to avoid subjective interpretation of clinical information to either exclude or include patients described in reports of Dr Mumford and Mr Mookerjee as well as the CNR group, where they consider this being a particular strength of their work.

(i) Review By Dr Mumford in response to Report by Prof Hawkey, Dr Agrawal and Dr Drumright

Continued reliance on conclusions from Mookerjee analyses and reports

8. Dr Mumford states (par 5.3): '*It is notable that Mookerjee, in his expert report to the Inquiry found that the rate of BSI with environmental organisms was higher (for certain years) of that of comparator units in the UK.'*

9. Dr Mumford cites Mr Mookerjee's first quantitative report for this statement. However, the denominators used by Mr Mookerjee in that report were clearly different invalidating the entire analysis (a fact that was pointed out to Dr Mumford during her oral evidence). Mr Mookerjee's second report, in which he was asked to correct this fundamental error, was similarly problematic, as he deliberately excluded NHSGGC's day case wards while keeping day cases in the comparators. Only in one standalone graph, submitted on its own the evening before his oral evidence, did Mr Mookerjee finally display NHSGGC's rate with day cases included, and this showed that in general, GGC had comparable rates to the other hospitals (Bundle 27, volume 18, p.3). Furthermore, GGC have raised serious concerns about other aspects of his work, including his claims to have deduplicated all sites in the same way, his calculation of 'water positivity', and his correlation analysis.

10. It appears to be Dr Mumford's position that Mr Mookerjee's analysis is still valid. Dr Mumford claims that it is 'notable' that NHSGGC's rate was higher for certain years. But all hospitals had year-to-year variation in computed infection rate, and while NHSGGC's were higher than the comparators in 2017 and 2018, GOSH's were higher than all comparators in 2015, 2019, 2020, 2021 and 2022, and Leeds's rates were also higher than NHSGGC in 2019, 2020, 2021, and 2022. We believe this is precisely what random variation would look like.

Unjustified criticism of comparison to Yorkhill

11. The instruction from the SHI is to assess whether 'the water system […] presented an additional risk of avoidable infection'. The word 'additional' is crucial here – experts were not asked whether there was any risk, but whether the risk was greater than a baseline level. The comparison against a baseline is therefore an important part of determining whether there was an additional risk associated with the environment at the QEUH/RHC. The HAD authors and SHI expert panel both carried out comparative exercises: the HAD authors compared Yorkhill and the QEUH/RHC. The SHI expert panel compared QEUH/RHC to four other UK hospitals, with agglomerated data obtained through FOI requests. Neither approach is perfect but each can be informative, and both are valid ways to attempt to determine whether there was an

additional risk from the environment at the QEUH/RHC. Each has advantages and disadvantages.

a. The HAD report comparison with Yorkhill follows what is arguably the same cohort of patients and uses a single primary data source, but confounding variables include anything that also changed over that time period, e.g. changes in care pathways, treatments, antibiotic use, laboratory methodologies for identifying rare organisms, IPC interventions, practice and quality improvement work etc.

b. The SHI Expert Panel comparison to other hospitals provides a wider context with more sites, but has some additional confounders: differences in the patient cohorts, particularly when comparing large specialist referral centres to smaller regional hospitals whose most complex patients would likely be sent elsewhere, plus substantial differences in how the data were deduplicated and summarised by each comparator in response to the FOI requests. Since Mr Mookerjee and Dr Mumford also included a temporal element in their analyses (claiming increasing rates of infection in GGC), the other temporal confounders that apply to the Yorkhill comparison also apply to their approach. Furthermore, NHSGGC maintains that the analyses carried out by Mr Mookerjee were invalid, for reasons previously outlined in detail, and as such any conclusions drawn from his work are unsafe.

12. Dr Mumford is highly critical of the comparison carried out by the HAD authors while acknowledging none of its possible advantages nor any of the drawbacks of her own approach (the comparison against other UK hospitals). Dr Mumford and others claim that Yorkhill is not a suitable comparator given that it was an older building, but she does not take into account whether hospitals included in her comparison are also older than the RHC, and if so, why did this not invalidate her analysis? In any case, this criticism implies that older buildings are automatically worse, i.e. that patients are becoming infected directly from the environment more often in those buildings. This is too simplistic an explanation, one that relies on the environment accounting for a substantial proportion of infections overall and that does not account for other infection control measures that protect patients regardless of the age of the estate. It needs to be noted that NHSGGC applied multiple interventions to ensure patient safety in response to concerns related to increased incidence of infections. In addition to measures focussed on the built environment there was also CLABSI work, introduction of RCA for all Gram-negative bacteraemias, enhanced supervision audits and walkarounds, emphasis on compliance with SIPCs practice amongst others. Dr Mumford has chosen to ignore this

additional work and ties changes in infection incidence with changes in the built environment only.

13. Dr Mumford has criticised QEUH/RHC for insufficient water sampling and inconsistency of approach to choice of sampling sites and tests performed. We would like to point out that the water testing in the earlier years was performed according to the existing guidance and was changing to adapt to incident management requirements. NHS GGC has developed a bespoke and extensive water quality monitoring system that might not exist in other hospitals. Dr Mumford states she would expect more sampling, especially for Pseudomonas aeruginosa being done for the hospital like QEUH/RHC. It is not clear how many samples Dr Mumford would expect to be done per year and on what guidance/evidence she bases her opinion.

(ii) CNR Panel Rebuttal of Report by Prof Hawkey, Dr Agrawal and Dr Drumright

Detection and reporting of Enterobacter spp. from environmental samples

14. In their section criticising the WGS work carried out on Enterobacter isolates, the CNR authors have either misunderstood or are misrepresenting GGC's environmental testing protocols regarding Enterobacter. We request that they correct the following sections:

[Bundle 44, vol 2, p.198] 'Furthermore, it is highly probable that there were other isolates of Enterobacter spp. recovered from water samples during this period but, as pointed out by Evans, these are not one of the alert organisms specified by the National Infection Prevention and Control Manual Appendix 13, and the laboratory would typically not routinely aim to identify Enterobacter spp. in water samples.'

15. This is incorrect and betrays a lack of knowledge of microbiological water testing, and of the purpose and scope of the NIPCM Appendix 13. The NIPCM alert list is for surveillance of clinical cases, to alert IPC teams of a possible environmental link when those infections occur. The presence or absence of specific species on this list does not govern whether a water testing laboratory will report the presence of those organisms.

16. Enterobacter spp. are coliforms, and testing specifically for coliforms is one of the most fundamental water tests that are carried out. Indeed, alongside the general, non-selective, bespoke Gram-negative bacteria test that NHSGGC has carried out routinely at the QEUH/RHC, NHSGGC has also undertaken an even larger amount of potable water testing, including a test with growth media that selects for coliforms. In that sense, Enterobacter have

been given multiple opportunities to grow from water samples, both on general media and on media designed for their specific nutritional requirements. Furthermore, given the strict rules regarding presence of coliforms in potable water, there is no way that an accredited water testing laboratory would 'not typically aim to identify Enterobacter spp. in water samples.' If by this sentence, the CNR authors mean 'identify Enterobacter spp. to species level', then that might be correct in some circumstances – there is a large diversity of species within the genus Enterobacter, and identifying to species level, particularly those species that are not associated with human infection, can present challenges to routine water testing laboratories. In those instances, though, the laboratory would report as Enterobacter sp. – they would not withhold information about the presence of a coliform from the test requester.

17. To claim, without any supporting evidence, that it is highly probable that there were other isolates of Enterobacter spp. recovered from water samples during this period is a serious accusation that must be corrected or retracted. The water laboratory tested over 10,000 water samples over the period in question, with multiple microbiological tests that would have been able to detect Enterobacter spp., and it is indeed the case that Enterobacter spp. were only found in six of these samples.

[Bundle 44, vol 2, p.199]: 'Again, depending on how and why the various samples from sinks, drains, and other surfaces were processed, and the bacteria grown were/were not identified, it is highly probable that the actual total number of Enterobacter spp. isolates from these environmental sites was much higher than this.

18. This is similarly incorrect. The laboratory was asked to identify all organisms that grew from environmental sampling, and they used non-selective media that would have allowed the growth of Enterobacter spp. There is no way that the laboratory would have failed to report the isolation of Enterobacter spp. from environmental samples, so the claim that 'it is highly probable that the actual total number of Enterobacter spp. isolates […] was much higher' is a serious accusation. The CNR authors' use of the term 'highly probable' must be clarified.

Inclusion/exclusion of Enterobacter isolates from WGS work

19. The CNR authors have also misunderstood which Enterobacter isolates were included in the WGS analysis:

[Bundle 44, vol 2, p.200]: 'Almost all of the Enterobacter spp. in the CNR (n=27) were E cloacae, which is not described in Evans list, and only 2 were E. hormaechei, one from 2017

and one from 2019. At best, we conclude only these 2 patients could have been included in this WGS investigation.'

20. This is incorrect. As stated in the WGS report by Leanord/Brown, all Enterobacter isolates that underwent WGS had been identified by the routine laboratory as Enterobacter cloacae. This would have been based on MALDI-TOF MS and/or Vitek2. The fact that WGS found them to belong to other species does not show that NHSGGC neglected to include key E. cloacae isolates in its sequencing exercise, but rather points to the limitations of routine identification platforms for this genus and the much greater resolution of WGS. It also highlights the risk in ascribing clusters and assuming transmission events based on species-level identification only, which is not only too coarse for this purpose, even when correct, but further complicated by limitations of routine diagnostic laboratory identification methods that risk grouping together isolates belonging to completely different species.

21. A similar situation arose with Cupriavidus identification. While the Inkster 2021 publication outlining the 'water incident' reported a high prevalence of Cupriavidus isolates in water samples (Inkster et al. 2021, Journal of Hospital Infection 111:53), the identification methods (Vitek2 and MALDI-TOF MS) had not been independently verified, and a subsequent paper (T. Inkster et al. 2022. Journal of Hospital Infection 123:80) showed that four of the nine isolates in that study that had been identified as Cupriavidus by Vitek2 were instead found to belong to a completely different genus when they were sent to UKHSA for further typing. Similarly, the WGS report by Leanord/Brown on Cupriavidus states that of the 155 isolates identified as Cupriavidus by the routine laboratory, only 134 were confirmed as belonging to this genus, with the remaining isolates belonging to several other genera. Contrary to what Dr Mumford and others have claimed, this does not indicate failures in sample labelling, inconsistent protocols, or mix-ups by laboratory staff (i.e. they did not send the wrong isolates for WGS) – rather, it is known to occur on occasion when bacteria identified by coarser, phenotypic methods are submitted for WGS.

22. We request that the CNR authors correct their statements regarding the inclusion of Enterobacter cloacae isolates in the WGS work.

23. Regarding testing of water samples for Stenotrophomonas spp., the CNR authors are critical of the number of samples that NHSGGC collected for Gram negative testing [Bundle 44, vol 2, p.201], and dispute Prof Evans's assertion that the low rate of detection of Stenotrophomonas

in these samples is likely representative. The CNR authors claim that because approximately 40% of water samples were not specifically tested for Gram negative bacteria, that NHSGGC's water testing was therefore 'sporadic/not systematic'. This is incorrect. Over the period covered by Prof Evans's WGS report (2018-2020), NHSGGC collected over 10,000 water samples, and of these, over 6,000 were tested specifically for Gram negative bacteria, including Stenotrophomonas, with the others undergoing tests for a range of other organisms. The percentage of total samples undergoing each microbiological test is irrelevant, especially when the total number of samples is so high. No other health board carries out such systematic, routine water testing, nor does any other health board routinely test for Gram negative bacteria (this test is bespoke to the QEUH).

24. Regarding the bacterial isolates that were available for the retrospective WGS work carried out by NHSGGC, the CNR authors state that 'it is routine practice to store blood culture isolates for years', and 'we do not understand why the bacteria causing BSIs were not stored' [Bundle 44, vol 2, p.203]. This implies that the unavailability of isolates from the earlier years (2015 to early 2018) points to deficits in NHSGGC's laboratory practices or to deliberate omission from the WGS work. This is incorrect. The retrospective WGS work was carried out beginning in 2020, so isolates from early 2018 had been stored for two years. Current guidance from the Royal College of Pathologists on the retention and storage of pathological records and specimens[1] does not specify that blood culture isolates must be retained 'for years', but only for at least seven days. Draft update guidance suggests storage for six months. NHSGGC was able to retrieve isolates that were two years old, indicating that its storage practices already exceed guidance, but it is unrealistic to suggest that all blood culture isolates obtained in a health board as large as NHSGGC should be routinely retained for longer than two years.

25. Finally, throughout their rebuttal report, the CNR authors allude to unspecified deficiencies in GGC's IPC practices and are critical of the HAD authors for not commenting on these. The authors are asked to specify which IPC deficiencies they are referring to?

(iii) Joint report by Dr Mumford and Mr Mookerjee on Aspergillus

Case definition and appropriateness of comparison to rates reported in Crassard 2008

26. The authors agree that the incidence of invasive aspergillus (IA) is increasing in children and adults (last sentence of 2.10). Newer medicines may enhance the risk of IA which the IMT also

[1] https://www.rcpath.org/static/049ea966-df5c-4a9f-9353ba24a69bb808/The-retention-and-storage-of-pathological-records-and-specimens-5th-edition.pdf

acknowledged in 2.17.2. The authors state in section 2.25 that the expected rate of IA in paediatric AML patients is 5.25% and 1.5% for ALL. This is taken from a single centre retrospective review by *Crassard et al* 2008 carried out between 1986 and 2000. The authors of this paper categorise their cases as per the EORTC criteria. Dr Mumford and Mr Mookerjee have compared NHSGGC data with this paper but they do not describe if they have undertaken the same categorisation. If not, they are asked to state what system have they used?

27. The *Crassard et al* paper also helpfully provides, in table 2, a comparison of their data with other published paediatric studies. It is of note that the *Crassard* paper is now quite old. Is it unclear whether Dr Mumford and Mr Mookerjee have similarly reviewed the literature for other studies in this area published since this time. Nevertheless Table 2 in the *Crassard et al* paper shows a range of values for the incidence which Dr Mumford and Mr Mookerjee have failed to highlight including one of 27.77% for AML patients which we would agree is extremely high. Incidence in ALL has been cited to be up to 10% (Duus RM, Moeller JB, Rathe M. Occurrence and Case Fatality Rate of IA in Children With Acute Leukemia: A Systematic Review and Meta-analysis. J Pediatric Infect Dis Soc 2024; 13 (9): 475-485). It is unclear whether Dr Mumford and Mr Mookerjee have categorised the NHSGGC cases using the EORTC criteria. If they have not, the comparison given in table 5 would be invalid.

28. It is also necessary that they are appraised of the background population for the *Crassard et al*. paper as to similarities with GGC cases in terms of treatment modalities, degree of immune suppression and diagnostic capabilities for Aspergillosis given the age of this paper (review of cases from 1986 to 2000). The authors are asked to clarify whether they have this information.

Calculation of bed days

29. In table 2, Dr Mumford and Mr Mookerjee give the bed days data from which they have calculated the rate of Aspergillus infections in Yorkhill and RHC over time, by hospital and by ward. It is unclear how these bed days data have been generated. Is this total bed days for the units for that year, bed days for all haematology patients in those units in that year, bed days of patients with Aspergillosis in those units per year? The methodology for this greatly inflates or shrinks the denominator and therefore table 3 cannot be interpreted without this. Looking at the years in which bed data is given for each particular unit it is not consistent that if there was a case in that unit the bed days are included. E.g. Schiehallion YOR and ITU YOR.

30. Table 4: It appears that the diagnosis was established predominantly by culture in the first few years but mainly by PCR and serology in later years. It should be ascertained whether PCR and

serology were used much more often in recent years when the unit moved to the RHC. Increasing use of diagnostic techniques other than culture would be expected to result in detection of more cases.

31. Table 5: As there is no upper limit of incidence of invasive aspergillosis in a given setting, why is it that the incidence as quoted in Table 5 is higher in RHC compared to Yorkhill exclusively for ALL patients? Why was this trend not seen in AML patients? In fact, the incidence of invasive aspergillosis was marginally lower in RHC compared to Yorkhill for the AML subgroup.

32. Finally, in the absence of a root cause analysis on where the cases of invasive aspergillosis were acquired i.e. community acquisition or nosocomial acquisition, the role of ventilation is impossible to interpret. Particularly when dealing with small numbers, it is imperative that distinction between community and nosocomial acquisition is made. In literature, invasive aspergillosis within seven days of hospital admission has been categorised as community acquired.

(iv) Review by NHS NSS of Report by Prof Hawkey, Dr Agrawal and Dr Drumright

33. Dr Cairns states that the HAD authors 'rely heavily on unvalidated outbreak definitions' and in section 3.4.1 'the authors have not provided a reference to support the use or validity of this method'. She states that ARHAI definitions are 'evidence based'. Ms Cairns is asked to describe the evidence base for the ARHAI definitions.

(v) Review by Mr Mookerjee of Report by Professor Hawkey, Dr Agrawal and Dr Drumright.

34. It is noted that much of Mr Mookerjee's review of the HAD report does not, in fact, review the HAD report, but rather repeats sections, often verbatim, from his own previous reports as well as those of Dr Mumford and Dr Walker. He also replicates entire paragraphs from Dr Mumford's HAD report review (e.g. 2.19 point 6, 2.21 point 1).
35. NHSGGC has previously raised serious concerns about the reports of all three of these SHI experts, which we outlined in detail in Direction 5 responses. Those concerns have not been adequately addressed in the SHI expert responses to Direction 5 nor in their oral evidence, and while we will not copy the text of our previous Direction 5 responses here, we will highlight again the most important problems with the parts of Mr Mookerjee's work that he has repeated in his HAD report review.

36. In Par. 2.4, Mr Mookerjee repeats the IRR values that he presented in his second report – comparing the infection rate in NHSGGC to the average rate across all four comparators, per year. However, in calculating the infection rate for GGC, Mr Mookerjee again excluded GGC's day case admission numbers (wards 2B and 6A day unit), repeating the error from his first report, but he continued to include day case admission numbers in the comparators' rates, rendering the comparison invalid.

37. In Par. 2.5, Mr Mookerjee has copied the text from his second report that outlines how he carried out a Pearson correlation between his computed 'water positivity' and the Schiehallion infection rate. We maintain that this analysis is fundamentally flawed:

    a. Mr Mookerjee's approach to computing 'water positivity' is invalid

    b. A Pearson correlation is not appropriate for these data

    c. The correlation that Mr Mookerjee computed shows the opposite of what he claims – it is not significant, meaning there is no correlation

    d. The exclusion of data from 2020 is not justified

38. At 2.19 point 10. Mr Mookerjee is critical of the HAD report including analyses of adult infections, stating that the questions laid out by the Inquiry are only concerned with the Schiehallion cohort. This is incorrect. As stated in his own first expert report (Par. 2.1.1), the first Key Question prioritised by the SHI is: 'From the point at which there were patients within the QEUH/RHC, was the water system (including drainage) in an unsafe condition, in the sense that it presented an additional risk of avoidable infection to patients?' This key question does not specify that it applies only to the Schiehallion cohort, and looking at the adult cohort is an appropriate analysis to carry out.

39. Regarding Pseudomonas water testing, in Par. 2.25 Mr Mookerjee conflates the overall average rate of Pseudomonas water positivity listed in the HAD report (1.1%) with some individual quarterly values that are higher than 1.1%. He specifically lists the three values that are greater than 1.1%, implying that the HAD report authors are incorrect, but he fails to list the quarterly values that are lower than 1.1%, and appears to misunderstand that when an average is computed, some of the numbers used to compute this value will be lower than the average and others will be higher. Mr Mookerjee's section on Pseudomonas water testing betrays a poor understanding of this field.

DIRECTION 5 REPONSE
TO THE HAD REPORT AND RESPONSES BY INQUIRY EXPERTS
ON BEHALF OF CUDDIHY/MACKAY FAMILIES

1. INTRODUCTION

This Direction 5 Response sets out an overview of the issues we determine to be relevant in respect of the HAD Report and the responses thereto by Sid Mookerjee; Dr Sara Mumford; Prof Stevens, Prof Wilcox and Gaynor Evans, and Dr Shona Cairns ("the Inquiry experts").

We provide the following observations in the hope that this will assist the Inquiry. Whilst not framed as direct questions, the issues that we submit require to be addressed by the HAD authors in evidence are highlighted in the following. We find that the Inquiry Experts have demonstrated a consensus in their responses that criticise the methodology employed by the HAD authors. We concur in this criticism. As noted below tranches of what can only be described as highly relevant documentation and reporting have not been considered by the HAD authors yet, they exclude an environmental link with infections. This is extraordinary. It is clearly obvious that if one does not look at a source of data or intelligence, and instead wilfully ignores same, that such data or intelligence will not inform any research findings.

Poor methodology will always serve to undermine the validity of any research, however, the actions of the HAD authors go further than undermining the validity of their findings. These authors have been asked to prepare a report to be submitted to a Public Inquiry whose terms of reference include considering the link between the environment and contracted infections. To prepare a report that wilfully ignores available data on the contemporaneous state of the environment is indefensible. Whilst the authors have stated that consideration of such documents was out with their remit this appears non-sensical. Why were these documents deemed out with their remit and why did the HAD authors not challenge the position being adopted by those instructing them.

2. RELEVANT ENVIRONMENT DOCUMENTS

The impact of the HAD authors ignoring any documentation relating to environmental factors within the hospital environment cannot be overemphasised. In the response to their questionnaire, they confirm that they have neither had sight of nor considered the following documentation:

1. 1. The DMA Canyon 2015 L8 Risk Assessment (Bundle 6, Document 29, Page 122)

2. 2. The DMA Canyon 2017 L8 Risk Assessment (Bundle 6, Document 30, Page 416)

3. 3. Minutes of the NHS GGC Water Technical Group on 13 April 2018 and 20 April 2018 (Bundle 10, Document 2, Page 9 and Document 3, Page 14)

4. 4. Notes of the Water Incident Debrief meeting on 15 May 2018 (Bundle 14, Volume 2, Page 211)

5. 5. Full Incident Management Team Report covering the IMTs from 2 March 2018 to 13 April 2018 dated 5 June 2018 (Bundle 27, Volume 5, Document 19, Page 46

6. 6. Legionella Control Authorising Engineer Audit – QUEH - 23 July 2018 (Bundle 18 Volume 2, Document112 at page 909)

7. 7. Report by Innovated Design Solutions, Feasibility Study Regarding Increasing Ventilation Air Change Rates within Ward 2B, 15 October 2018 (Bundle 6, Document 33, Page 656)

8. 8. Report by Innovated Design Solutions, Feasibility Study Regarding Increasing Ventilation Air Change Rates within Ward 2A, 24 October 2018 (Bundle 6, Document 34, Page 674)

9. 9. SBAR which was used to brief the Chair of NHS GGC, Professor Brown, on or about 13 November 2018 (Bundle 4, Document 32, Page 133)

10. 10. The NHS GGC Review of Issues Relating to the Hospital Water Systems' Risk Assessment (Bundle 8, Documents 34 to 40, Page 150)

11. 11. The HFS Water Management Issues Technical Review, March 2019 (Bundle 7, Document 4, Page 70)

12. 12. Legionella Control Authorising Engineer Audit – QUEH - 30 and 31 January 2020 (Bundle 18, Volume 2, Document 125, Page 1355)

13. 13. Legionella Control Authorising Engineer Audit – QUEH - 4 and 5 February 2021 (Bundle 18, Volume 2, Document 126, Page 1402)

14. 14. Legionella Control Authorising Engineer Audit – QUEH - 28 February and 1 March 2022 (Bundle 18, Volume 2, Document 124, Page 1335)

15. 15. Legionella Control Authorising Engineer Audit – QUEH - 11 January 2023 (Bundle 15, Document 45, Page 1226)

16. 16. Legionella Control Authorising Engineer Audit – QUEH - 11 January 2024 (Bundle 27, Volume 1, Document18 at page 252)

17 Details of the design, structure, specifications and management systems of the water system of the QEUH/RHC as it stood at the following points of time:

At handover on 26 January 2015?

At the start of the 'Water Incident' on 1 March 2018?

At the time the adult BMT patients returned to Ward 4B in the QUEH on 30 June 2018?

At the time of the 'decant' from Wards 2A/2B RHC to Wards 6A/4B in the QEUH on 26 September 2018?

At the point that Ward 6A was closed to new admissions in August 2019?

18. Details of the design, structure, specifications and management systems of the ventilation system of the QEUH/RHC as it stood at the following points of time and what were you told?

At handover on 26 January 2015?

At the start of 2018?

At the time the adult BMT patients returned to Ward 4B in the QUEH on 30 June 2018?

At the point that Ward 6A was closed to new admissions in August 2019?

19. In addition, the HAD authors have stated that they had no knowledge about:

the experience of NHS GGC haemato-oncology clinicians of potential HAIs and HCAIs in the Schiehallion Unit in Yorkhill and then RHC from 2005 to 2022?

the experience of NHS GGC ICNs, ICDs and microbiologists of potential HAIs and HCAIs in the Schiehallion Unit in Yorkhill and then RHC from 2005 to 2022

20. In respect of Contemporaneous Epidemiology, the HAD authors state that they were not instructed to review and critique the following documents :

17. The HPS Situational Assessment, Wards 2A/B, Royal Hospital for Children, NHS Greater Glasgow and Clyde (Bundle 7, Document 5, Page 194) and, in particular, Appendix 4 to that document (Bundle 7, Document 5, Page 205) – the reason being It was not in their instructions to review and critique the management of infection control and this is given in response to each of

the following.

18. The HPS SBAR: To support NHS GGC IMT Mycobacterium chelonae cases and the Incidence of gram-negative bacteraemia in the paediatric haemato-oncology, September 2019 (Bundle 3, Document 16, Page 127).

19. Draft HPS Review of NHS GG&C Infection Outbreaks in the Paediatric Haemato-oncology Data October 2019 (Bundle 7, Document 6, Page 214)

20. HPS Review of NHS GG&C Infection Outbreaks in the Paediatric Haemato-oncology Data October 2019 - 29 November 2019 (Bundle 7, Document 7, Page 250).

21. Presentation by Kathleen Harvey-Wood and Dr Christine Peters: Bacteraemia rates and Resistance Paediatric Haemato-oncology 2014-2018, 30 August 2018 (Bundle 27 Volume 6, Document 9, page 107).

22. Report by Dr Iain Kennedy: Descriptive Analysis of Trends in Bacteraemia Rates for Selected Gram-Negative Organisms, 1 October 2018 (Bundle 6, Document 27, page 95).

23. Draft report by C Peters and K Harvey-Wood: Bacteraemia rates and resistance patterns in paediatric haematology/oncology patients 2014-2018, 10 October 2018 (Bundle 19, Document 19, Page 143).

24. Report by Dr Iain Kennedy: Descriptive analysis of trends in bacteraemia rates for selected gram-negative organisms, July 2019 (Bundle 6, Document 28, page 104)

25. Presentation by Dr Iain Kennedy and Jennifer Rodgers: Paediatric Haemato-oncology RHC – Summary of Data, September 2019 - Presented at IMT meeting of 20 September 2019 (Bundle 27, Volume 13, Document 13, Page 77)

3. OVERVIEW OF COMMENTS BY INQUIRY EXPERTS

In appendix A we provide a tabulated comparison of the views expressed by experts on key issues. In appendix B we provide a similar tabulated comparison in respect of the omission of Mycobacterium Chelonae from the HAD report.

4. IMPACT OF THE OMMISSION OF MYCOBACTERIUM CHELONAE

Summary of the challenges that flow from the original HAD report following omission of the consideration of the pathogen Mycobacterium Chelonae within QEUH/RHC during the period of review (2015-2021).

Critical Analysis of the HAD Report and the Impact of Mycobacterium chelonae on Immunocompromised Patients

The HAD report—authored by Hawkey, Agrawal, and Dempster under commission by NHS Greater Glasgow and Clyde (NHS GGC)—was intended to provide a comprehensive environmental risk assessment concerning healthcare-associated infections within hospital settings. However, a significant and consequential omission within this report was the failure to include Mycobacterium chelonae, a rapidly growing nontuberculous mycobacterium with well-documented clinical relevance, particularly for immunocompromised patients.

Significance of Mycobacterium chelonae in NHS Scotland Hospitals

Multiple expert analyses, including those by Sid Mookerjee and Dr Sara Mumford, have highlighted that M. chelonae was recognised in NHS National Services Scotland (NSS) and Health Protection Scotland (HPS) surveillance reports as a key environmental pathogen linked to clinical cases. Despite this, the HAD report excluded M. chelonae from its organism list, undermining the accuracy and completeness of its environmental risk evaluation.

This omission is especially critical given the documented presence of M. chelonae in hospital water systems across multiple wards (2A, 6A, and 7D) and its direct association with severe infections in immunocompromised paediatric oncology patients. The testimonies of families affected—such as those of Molly Cuddihy and Annemarie Kirkpatrick's daughter—underscore the devastating impact of these infections, including septic shock, prolonged antibiotic regimens with life-altering side effects, and the forced withdrawal or early termination of life-saving chemotherapy.

Implications for Immunocompromised Patients

Immunocompromised individuals, particularly children undergoing treatment for rare cancers, face a dual threat when infected with M. chelonae. The pathogen's intrinsic antibiotic resistance and environmental persistence complicate infection management. Treatment requires prolonged, multi-agent antimicrobial therapy, which carries risks of toxicity and drug interactions. Furthermore, balancing effective infection control with the continuation of chemotherapy presents a profound clinical challenge. Interruptions or modifications to cancer treatment to manage infection risk can adversely affect oncologic outcomes, highlighting the need for integrated, patient-centred care strategies.

NHS Scotland Guidance on Investigation and Surveillance

NHS Scotland's guidance, as reflected in the reports by NHS NSS and expert clinicians, emphasises the necessity of:

- Routine environmental screening for atypical mycobacteria, especially in water systems servicing wards with vulnerable patients.
- Comprehensive microbiological investigations including acid-fast bacilli staining, culture, and molecular diagnostics when mycobacterial infection is suspected.

- Robust infection prevention and control measures, including water system decontamination and surveillance protocols that explicitly include M. chelonae.

Despite these recommendations, the initial failure to routinely screen for M. chelonae in hospital water systems, as noted in Dr Jairam Sastry's statement, reveals systemic gaps in outbreak detection and response.

International Best Practices and Clinical Management

International guidelines, including those from the Infectious Diseases Society of America (IDSA) and paediatric oncology infectious disease experts, advocate for a multidisciplinary approach to M. chelonae infections in immunocompromised children. Treatment involves:

- Prolonged combination antibiotic therapy tailored to susceptibility profiles.
- Close monitoring for adverse effects and drug interactions.
- Careful coordination with oncology teams to minimise disruption to chemotherapy schedules.

The complexity of treating M. chelonae infections in this population is well documented in medical literature, underscoring the delicate balance between infection control and cancer treatment efficacy.

Conclusion

The HAD report's omission of Mycobacterium chelonae represents a significant oversight that diminishes the validity of its environmental risk assessment and potentially jeopardises patient safety. Given the pathogen's established presence in hospital water systems and its severe impact on immunocompromised patients—particularly children undergoing cancer treatment—its exclusion raises critical concerns about the adequacy of infection surveillance and control measures within NHS GGC.

Moving forward, it is imperative that NHS GGC incorporate comprehensive screening and management protocols for M. chelonae, ensuring that environmental risk assessments are complete and that vulnerable patients receive the integrated care necessary to manage both their infections and underlying conditions effectively.

5. NHS Scotland Guidance on Investigation and Control of Mycobacterium Chelonae (Jan 2025)

Surveillance and Screening: NHS Scotland recommends routine environmental screening of hospital water systems, especially in wards with immunocompromised patients, such as haemato-oncology units. This includes

testing water outlets, storage tanks, and mains supply for atypical mycobacteria like M. chelonae.[1]

Microbiological Investigation: When M. chelonae infection is suspected or confirmed clinically, investigations should include acid-fast bacilli (AFB) staining, culture on specialised media, and molecular methods such as whole-genome sequencing (WGS) to identify and compare clinical and environmental isolates. WGS has proven valuable in linking patient infections to hospital water sources, supporting infection control interventions.

Infection Control Measures: NHS Scotland emphasises the importance of infection prevention and control (IPC) teams implementing measures such as water system decontamination, chlorinated filtration, and removal or treatment of contaminated outlets. IPC protocols should include M. chelonae in their surveillance and outbreak management plans.[2]

Clinical Management: For immunocompromised patients, particularly paediatric oncology patients, early diagnosis and prompt initiation of multi-drug antibiotic therapy tailored to susceptibility testing are critical. Treatment is prolonged and complex, often requiring macrolides, fluoroquinolones, and intravenous agents. Coordination between infectious disease specialists and oncology teams is essential to balance infection control with continuation of cancer therapy.[3]

The current guidance as of January 2025 reflects the critical importance of recognising M Chelonae as a significant pathogen in hospital water systems affecting immunocompromised patients and the need for integrated clinical and environmental strategies to manage infection risks effectively.

6. VENTILATION ASSOCIATED INFECTIONS – AIRBOURNE PATHOGENS - *Aspergillus*, *Cryptococcus* and tuberculosis

Appendix C contains a summary of the comments made by experts to the HAD Report. Once again criticisms of methodology feature and exclusion of relevant information/factors e.g., the use of chilled beams and the findings are criticised based on the inadequacies identified.

[1] https://www.nipcm.hps.scot.nhs.uk/media/2384/2024-07-29-water-systems-lit-review-v11-final.pdf

[2] https://www.nipcm.hps.scot.nhs.uk/media/2384/2024-07-29-water-systems-lit-review-v11-final.pdf

[3] https://pubmed.ncbi.nlm.nih.gov/33945838/

SCOTTISH HOSPITALS INQUIRY

RESPONSE SUBMITTED ON BEHALF OF DR CHRISTINE PETERS

TO

REVIEWS/REPORTS BY THE INQUIRY EXPERTS

OF THE

EXPERT REPORT BY PROFESSOR PM HAWKEY, DR SG AGRAWAL AND DR LN DRUMRIGHT

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1. INTRODUCTION

1.1 On behalf of Dr Christine Peters, and in accordance with the procedure set out in Direction 5 and the email sent by the Scottish Hospitals Inquiry Team dated 6 June 2025, this response is submitted in relation to the following reviews and reports prepared by the experts instructed by the Inquiry:

(a) the Joint Report by Dr Mumford and Mr Mookerjee on *Aspergillus* dated 4 June 2025;

(b) the Review by Dr Mumford dated 16 May 2025 in response to the Report by Prof Hawkey, Dr Agrawal and Dr Drumright;

(c) the Expert Report by Mr Mookerjee dated 10 June 2025;

(d) the Expert Report by Dr Shona Cairns dated 28 May 2025; and

(e) the CNR Panel Rebuttal dated 3 June 2025 of the Report by Prof Hawkey, Dr Agrawal and Dr Drumright.

1.2 In this response, the expert report prepared by Professor Hawkey, Dr Agrawal and Dr Drumright will be referred to as the HAD report.

2. RESPONSE TO THE JOINT REPORT BY DR MUMFORD AND MR MOOKERJEE ON *ASPERGILLUS*

2.1 In Dr Peters' opinion, this report is a very helpful piece of work that finally provides information on *Aspergillus* rates that has been missing from the expert analysis to date. Dr Peters notes that the findings of an increased rate above what is expected is in keeping with her own understanding of the data and in keeping with concerns expressed repeatedly regarding wards 2A and 4B.

2.2 In relation to this report, the only caveat Dr Peters would make is that it is unclear if the data provided to the HAD report authors was complete as explained in Dr Peters' First Supplementary Statement.[1] As this data was also used by Dr Mumford and Mr Mookerjee in their analysis, their findings may be an underestimate. **Dr Mumford and Mr Mookerjee should be asked for their comments on this point.**

3. RESPONSE TO THE REVIEW BY DR MUMFORD

3.1 In Dr Peters' opinion, this review is a considered and excellent response.

3.2 The only point of disagreement arises at paragraph 5.8. Dr Peters' view is that *E. coli* should be considered to have potential for environmental transmission, not just endogenous infection. It is well described as having nosocomial spread both for MDR and non-MDR strains (see https://genomemedicine.biomedcentral.com/articles/10.1186/s13073-022-01150-7) and, as such, an environmental component in the chain of transmission is not ruled out simply because it is also essentially a gut coloniser.

3.3 One genomic study suggested a link between a sink isolate and a urinary site isolate - see https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000391. It does not seem likely that, of all the Enterobacteraeciae, *E. coli* would be unique in being ubiquitous in the environment and yet never causing infections from the environmental source. **Dr Mumford's comments on this observation should be sought.**

[1] Dr. Christine Peters, First Supplementary Statement, 2025.

3.4 The statements made at paragraph 8.13 ("I am not aware of any further concerns raised about infection rates in these patients whilst on 4C and 4B") and paragraph 8.14 ("To date there has not been a detailed analysis of infection rates in QEUH 4B and 4C based on the ward patients and activity and comparing them with infection rates in comparable units that I am aware of") are concerning at this stage of the Inquiry – no assurances can be given about the past or present without this level of basic data analysis. This also applies to ITU data – a site of high risk for *Pseudomonas* in which notably point of use filters (POUF) have never been applied. In Dr Peters' opinion this is an outstanding area of assessment for the Inquiry.

4. RESPONSE TO THE EXPERT REPORT BY MR MOOKERJEE

4.1 In Dr Peters' opinion this report is a helpful critique of the HAD report's epidemiological analysis.

4.2 Dr Peters observes that, in relation to paragraph 2.20, point 4, it is very concerning for confidence in any conclusions drawn that there is such discrepancy in denominator data provided by GGC at different times without an adequate explanation. This calls into question at a critical level the authenticity of GGC data that has been provided to the PI expert group.

4.3 This observation raises the following questions:

4.3.1 **Did the data given to the GGC experts predate that provided to the Inquiry experts?**

4.3.2 **What data processing occurred to explain the significant conclusion altering differences in data?**

4.4 Dr Peters observes that, in relation to paragraph 2.21, point 2, this is a useful analysis and is similar to the suggestion she makes in her First Supplementary Statement. It clearly demonstrates a statistically and clinically significant reduction in rates of infection post refurbishment. This work needs to be further examined by Dr Mumford and Mr Mookerjee.

5. RESPONSE TO THE EXPERT REPORT PREPARED BY DR CAIRNS (NSS)

5.1 Dr Peters observes that this report is a helpful commentary in which there is an attempt to assess the validity of the methodology of calculations and conclusions of the HAD report. In Dr Peters' opinion, there are major concerns identified that effectively invalidate the basis of the conclusions reached by authors of the HAD report. Dr Peters agrees with the points made in Dr Cairns' report.

5.2 Dr Peters notes the point made at paragraph 2.4.1 with concern. It does not seem fair or reasonable for there to have been a delay in the sharing of datasets once the HAD report had been admitted as evidence available to the Inquiry – the understanding that this data would be shared in a timely manner was considered as offering some mitigation to the already unsatisfactory manner in which the report was received by the Inquiry.

5.3 Dr Peters notes that at paragraphs 2.4.2 to 2.4.4, 3.4.6, 3.4.8 and 4.1, Dr Cairns indicates that certain data and workings were not made available to her. In addition, Dr Peters notes that she has also not seen the supplementary data referred to. These observations beg the following questions: **Why has this information not been provided? Will it be provided before the Inquiry experts give their oral evidence so that their opinions can be obtained?**

5.4 At paragraph 3.2.3, the discussion about denominators has been extensively explored by the Inquiry. However, a point that has not been fully delineated is that while bed days do give an indication of duration of exposure to the environment as a whole, this is not necessarily equivalent to duration of exposure to an organism given the stochastic nature of routes of transmission aligning. For example, bursts of bio film in water, mould spore release, timings of line manipulation are all unpredictable, short lived and irregular. **Dr Cairns should be asked for her comments on this observation.**

5.5 While the report is a useful consideration of the shortfalls of the HAD report analysis, there is no re-analysis of the raw data to demonstrate what alternative conclusions could be drawn from this extensive new data provided to the Inquiry.

6. RESPONSE TO THE CNR PANEL REBUTTAL

6.1 In Dr Peters‘ opinion, this report offers a comprehensive and convincing rebuttal of the HAD report’s critique of the CNR report. Dr Peters agrees that the time, place, person and individual assessment of the CNR was a more appropriate methodology for establishing likelihood of links to the environment than the retrospective look for increases in infection in large groups across long time periods, which was the approach taken in the HAD report.

6.2 In relation to paragraph 3.7, it is concerning that the CNR authors were not given sight of the data that confirmed linkage of cases to the environment. This does not seem appropriate given the importance placed on the CNR report by the CNO office.

6.3 At paragraph 4.2 there is reference to the CNR suggesting that a patient most likely acquired an infection in the home environment. It is important to note that this was also the conclusion of the IPCT when Dr Teresa Inkster was lead ICD, and is not a novel discovery by external assessors.

6.4 In relation to paragraph 4.4, while Dr Peters agrees that *E. coli* are historically considered to be largely endogenous infections, in her view, they are worth considering as having the potential to transmit from the environment.

6.5 At page 38, the CNR authors state "140 other samples from the total of 550 samples in the database for that year had incomplete location information, so any results that might have been obtained could not be accurately related to sites of patient care." Dr Peters considers that it is unfortunate that the CNR authors were not given access to the de-coded water results that Dr Inkster had at the time of the IMTs.

6.6 Page 55 of the report contains the following important sentence that Dr Peters completely agrees with:

> “It is also the case that inter-institution comparison could lead to ‘normalisation’ of higher than desirable rates if the comparison offers similar or better results than elsewhere, whereas the focus should be on searching for ways to reduce rates to the minimum and identify any source”

6.7 In relation to paragraph 7.2, Dr Peters observes that it appears that the new WGS information with CHIs and date level information on the trees has not been shared with

the CNR authors to see if it would alter their conclusions. The sharing of this information with the CNR authors would serve to inform further the comparisons of the conclusions in the HAD report and the CNR. Dr Peters considers this to be a vital step which should be undertaken. It is Dr Peters‘ position that, not only does the WGS not rule out a link, it points to, and supports, the likelihood of an environmental source. This needs to be urgently assessed by appropriate experts who should have the details of dates and CHIs in order to fulfil the requirement for a stringent scientific assessment of the links of the infections (including deaths) to the environment.

6.8 On page 85, the CNR authors state "Similarly, and surprisingly, some bacteria causing BSIs in children were not included in the WGS investigations, yet it is routine practice to store blood culture isolates for years." It appears from this observation that the CNR authors are under the impression that not all blood culture isolates were stored. This is not the case. The SOP for the lab is to store all blood culture isolates. Any lack of availability to do WGS on clinical isolates needs independent scrutiny and confirmation.

7. CONCLUSION

7.1 In relation to the above comments, Dr Peters would be happy to provide further input, information and/or clarification as required.

Helen Watts KC and Leigh Lawrie, Advocate

On behalf of Dr Christine Peters

1 July 2025

SCOTTISH HOSPITALS INQUIRY

RESPONSE SUBMITTED ON BEHALF OF DR TERESA INKSTER

TO

REVIEWS/REPORTS BY THE INQUIRY EXPERTS

OF THE

EXPERT REPORT BY PROFESSOR PM HAWKEY, DR SG AGRAWAL AND DR LN DRUMRIGHT

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1. INTRODUCTION

1.1 On behalf of Dr Teresa Inkster, and in accordance with the procedure set out in Direction 5 and the email sent by the Scottish Hospitals Inquiry Team dated 6 June 2025, this response is submitted in relation to the following reviews and reports prepared by the experts instructed by the Inquiry:

(a) the Joint Report by Dr Mumford and Mr Mookerjee on *Aspergillus* dated 4 June 2025;

(b) the Review by Dr Mumford dated 16 May 2025 in response to the Report by Prof Hawkey, Dr Agrawal and Dr Drumright; and

(c) the CNR Panel Rebuttal dated 3 June 2025 of the Report by Prof Hawkey, Dr Agrawal and Dr Drumright.

2. RESPONSE

2.1. In the Joint Report by Dr Mumford and Mr Mookerjee on Aspergillus dated 4 June 2025, there is reference to 8 cases in ward 2A in 2022 (see page 13, table 1). Looking also at the Agrawal data, Dr Inkster queries whether Dr Mumford has included cases that were Beta D-glucan positive only. In the report (at page 6), Dr Mumford and Dr Mookerjee highlight that "Beta D-glucan is not considered as mycological evidence here and and the paediatric patients who had this as their only laboratory result on the data sheet provided did not have radiological evidence of fungal infection according to

the imaging reports seen". Dr Inkster submits that it is very unlikely that there are 8 true cases of hospital acquired *Aspergillus* following the ventilation upgrade and is not aware of these being reported to ARHAI. Given the comments regarding Beta D-glucan, Dr Inkster is of the opinion that it would be misleading to include these cases and this would lead to a false conclusion. **The experts should be asked to comment on these obsevrations.**

2.2 In both the Review prepared by Dr Mumford and the CNR Panel rebuttal, there is reference to the inadequacy of environmental testing. While Dr Inkster agrees with this, she does not feel that the Inquiry has looked into why this was the case. Accordingly, provided with this response is an SBAR from ARHAI regarding the risks in Scotland posed by the lack of an environmental testing laboratory. This SBAR has been shared with PHS and Scottish Government colleagues. It is important for the Inquiry to understand that colleagues in England have access to three specialist UKHSA environmental laboratories. This is not the case in Scotland and, therefore, poses various challenges. **This is an area which should be considered by the Inquiry and the experts instructed by the Inquiry should be asked for their views.**

3. CONCLUSION

3.1 In relation to the above comments, Dr Inkster would be happy to provide further input, information and/or clarification as required.

Helen Watts KC and Leigh Lawrie, Advocate

On behalf of Dr Teresa Inkster

27 June 2025

Documents provided:

1. SBAR: Environmental Testing, November 2024, ARHAI (and related email dated 30 May 2025)

Some Examples of Polyclonal and Polymicrobial Outbreaks

(as referenced in paragraphs 76-81 of Closing Submissions for Glasgow III on behalf of Drs Peters, Inkster and Redding)

Contents:

Polyclonal outbreaks

1. Boszczowski I, do Prado GV, Dalben MF, Telles RC, Freire MP, Guimarães T, Oliveira MS, Rosa JF, Soares RE, Llacer PE, Dulley FL, Costa SF, Levin AS. Polyclonal outbreak of bloodstream infections caused by Burkholderia cepacia complex in hematology and bone marrow transplant outpatient units. Rev Inst Med Trop Sao Paulo. 2014 Jan-Feb;56(1):71-6. doi: 10.1590/S0036-46652014000100011. PMID: 24553612; PMCID: PMC4085834.

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POLYCLONAL OUTBREAK OF BLOODSTREAM INFECTIONS CAUSED BY *Burkholderia cepacia* COMPLEX IN HEMATOLOGY AND BONE MARROW TRANSPLANT OUTPATIENT UNITS

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SUMMARY

Aim: The objective was to describe an outbreak of bloodstream infections by *Burkholderia cepacia* complex (Bcc) in bone marrow transplant and hematology outpatients. **Methods:** On February 15, 2008 a Bcc outbreak was suspected. 24 cases were identified. Demographic and clinical data were evaluated. Environment and healthcare workers' (HCW) hands were cultured. Species were determined and typed. Reinforcement of hand hygiene, central venous catheter (CVC) care, infusion therapy, and maintenance of laminar flow cabinet were undertaken. 16 different HCWs had cared for the CVCs. Multi-dose heparin and saline were prepared on counter common to both units. **Findings:** 14 patients had *B. multivorans (*one patient had also *B. cenopacia),* six non-*multivorans* Bcc and one did not belong to Bcc. Clone A *B. multivorans* occurred in 12 patients (from Hematology); in 10 their CVC had been used on February 11/12. Environmental and HCW cultures were negative. All patients were treated with meropenem, and ceftazidime lock-therapy. Eight patients (30%) were hospitalized. No deaths occurred. After control measures (multidose vial for single patient; CVC lock with ceftazidime; cleaning of laminar flow cabinet; hand hygiene improvement; use of cabinet to store prepared medication), no new cases occurred. **Conclusions:** This polyclonal outbreak may be explained by a common source containing multiple species of Bcc, maybe the laminar flow cabinet common to both units. There may have been contamination by *B. multivorans* (clone A) of multi-dose vials.

KEYWORDS: *Burkholderia cepacia* complex; Bloodstream infection; Nosocomial infection; Hematology; Bone marrow transplant.

INTRODUCTION

Burkholderia cepacia complex (Bcc) comprises at least 15 different species or genomovars based on phenotypic and genotypic analyses[16,17]. Identification to species level has been troublesome in the clinical laboratory. Automated methods seem to perform poorly in identifying species of Bcc[19,25]. Molecular methods are more appropriate[8,12,16]. Clinically, Bcc mainly *cenocepacia* and *multivorans* species are important colonizers and cause respiratory tract illness among cystic fibrosis patients[1]. In the hospital, the importance of Bcc is due to its potential to spread from person to person and to survive in moist environments. Multiple healthcare-associated outbreaks have been described involving contaminated water[11], prefabricated moist washcloths[13], contaminated medication[14], nebulization solution[15], antiseptic solution[9], heparin[24], moisturizing body milk[2], mouthwash solution[1]. We report here an outbreak involving different species of Bcc and the dissemination of a predominant clone suggesting heavy contamination by a common source.

METHODS

The outbreak occurred in Hospital das Clínicas, a 2000-bed hospital affiliated to the University of São Paulo, during the period from January 1 to February 29, 2008. The hospital is divided into six institutes and transfer of patients between institutes occurs. The outbreak involved two outpatient units: one for patients with hematological malignancies and the other for bone marrow transplant patients. These units were adjacent and independent but shared one room which contained a laminar flow cabinet used to prepare chemotherapy and a counter used to prepare intravenous medication. In these units, the patients were admitted for a few hours at a time and received medication including intravenous therapy such as chemotherapy, antimicrobial drugs and hydration through central venous catheters.

The outbreak of bacteremia caused by *B. cepacia* was suspected on February 15, 2008 and an investigation was triggered. A case was defined as a patient with *B. cepacia* bacteremia occurring in January or February, 2008 who received care or medication prepared in these units. An evaluation of all positive blood cultures in patients hospitalized in the entire hospital during these two months and during the previous 12 months was also undertaken. Visits to both affected outpatient units were made for evaluation.

The hospital records of all case patients were evaluated and the

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following factors were recorded: sex; age; underlying diseases; the presence and the type of central venous catheter (CVC), insertion date, type of dressing used (gauze or transparent), type of antiseptic used during CVC handling; the use of IV medications, including heparin, and of blood products; the use of chemotherapy; dates of the last three uses of the CVC before the first positive culture and the healthcare workers who had cared for each patient. Cases were treated with meropenem.

Infection Control Measures: Two visits on different days and meetings with all the staff of the affected units were held in order to evaluate practices in infection control and managing of infusion therapy. The main findings were the following:

- Multi-dose heparin, methylprednisolone and saline solution for intravenous infusion were routinely used. The containers and vials were not left with needles inserted in them but it was not clear how many patients used each vial nor was there a control of when they should be discarded. These multi-use items were placed on the shared counter;
- Nurses frequently prepared prescribed medications in the morning for use during the whole day in order to "save time";
- Syringes with aspirated solution without identification (patient name, type of solution or time of preparation) were placed on the counter and used for flushing catheters during the day;
- There was no exclusive sink inside the room used for the preparation of infusion therapy;
- There were empty vials of alcohol-based solution for hand hygiene;
- There was no evidence of systematic cleaning of the laminar flow cabinet and the refrigerator used for medication storage as well as controls of refrigerator temperature;
- There was no evidence of systematic cleaning of surfaces with a disinfectant product.

Based on these findings, the following control measures were implemented:

- Heparin, methylprednisolone and hydrocortisone vials were defined for a single patient and each vial received a label with the patient's name;
- Syringes with medication were left in the laminar flow cabinet until the moment of administration to patient and medication other than chemotherapy was to be prepared immediately before administration;
- Batches of antiseptic chlorhexidine (soap and alcohol-based) were replaced;
- Cleaning of laminar flow cabinet and control of temperature of refrigerators were reinforced;
- Cleaning of the areas in which medication was prepared with a chlorine-based product was reinforced;
- Hand hygiene was emphasized and the number of alcohol rub dispensers in the units was increased;
- Blood cultures of all patients with a CVC that had been handled in the affected units during the outbreak period were collected;
- Lock with ceftazidime for all long-term CVCs was instituted;
- Active surveillance for new cases was implemented.

Microbiologic study: The following items were cultured between February 21 and 28:

- Swabs from taps, sink drains, counters, laminar flow cabinet and healthcare workers hands (nurses, pharmacists) and other patients in direct contact with cases. Swabs were plated on BCSA (*Burkholderia cepacia* selective agar) and then inoculated in a 600,000 UI/L polymyxin B, vancomycin 2.5 mg/L and gentamicin 10 mg/L BHI broth (brain heart infusion). They were incubated at 10-32 °C up to seven days. Growing colonies were then submitted to Gram staining. Glucose fermenting was tested by OF (oxidation-fermentation) open and closed tubes and oxidase tests. After non-fermenting confirmation, biochemical tests for *B. cepacia* complex identification were performed: OF-glucose, OF-maltose, OF-xylose, OF-lactose, OF-sucrose and/or lysine decarboxylation;
- Water samples were collected (1 L of both cold and hot water) in sterile containers. Samples were divided into two aliquots of 500 mL each and filtered in an acetate cellulose membrane with 0.25 µm pores and then plated on BCSA and MacConkey agar.

The hospital's clinical microbiology laboratory used an automatized system for identification (Vitek, bioMerrieux). Reidentification of *B. cepacia* isolates was performed using the phenotypic method API ® 20 NE (bioMérieux SA, Lyon, France). The isolates that were phenotypically defined as belonging to the *B. cepacia* complex were submitted to molecular identification using multiplex polymerase chain reaction with the primers and technique as described elsewhere[12]. The *fur* gene was amplified for various strains using primers JD490 and JD491 and the DNA sequences were determined by direct sequencing from the amplicons using the MegaBACE 1000 DNA Sequencer. The sequences were analyzed using the software Sequence Analyzer with the Base Caller Cimarron 3.12 and compared with those in GenBank (www.ncbi.nlm.nih.gov). This method can identify the following species: *B. cepacia, B. multivorans, B. cenocepacia, B. dolosa, B. vietnamiensis, B. ambifaria, B. stabilis, B. antina*, Bcc group K and *B. pyrrocinia*. Isolates were typed using pulsed-field gel electrophoresis (PFGE)[18] using the restriction enzyme *Spe*I and interpreted according to criteria by TENOVER *et al.*[20]. Minimum inhibitory concentrations (MIC) for ceftazidime, meropenem, minocycline, levofloxacin and sulfamethoxazole-trimethoprim were determined and interpreted using the agar microdilution technique[4].

RESULTS

Based on the case definition, 27 patients were identified but we could only retrieve clinical information pertaining to 24 due to failure to retrieve complete records for three patients. Revision of the infection control database could not identify any cases of bacteremia by *B. cepacia* from January through December 2007 in our hospital (Fig. 1).

The main symptoms of cases were fever, present in 21 (88%) patients, chills in 14 (58%), coughing in two and hypotension and septic shock in one patient each (Table 1). Ten patients had their CVC used on February 11 and/or 12 and, in nine of these patients, this had been their last CVC use before the positive blood culture. None of the HCWs could be directly implicated as the source of the outbreak, especially because each HCW only worked in one of the affected units (Fig. 2).

Two patients were hospitalized in Hematology inpatient unit when they acquired the *Burkholderia* infection. However, they were included in the outbreak because they received chemotherapy prepared in the outpatient unit. Fifteen patients (56%) had their catheters removed, on average 17.8 days after the positive blood culture (range: 1-70, median: 14). Eight patients (30%) were hospitalized, and one patient died within thirty days after the positive culture of causes not related to the infection.

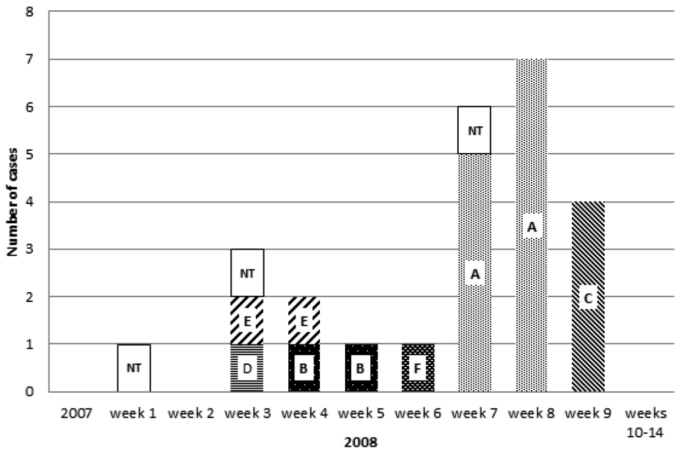

Fig. 1 - Distribution of cases over time and molecular types during an outbreak of bloodstream infections caused by *Burkholderia cepacia* in two outpatient units (Hematology and Bone Marrow Transplant). Hospital das Clínicas, University of São Paulo, Brazil. 2007 to March, 2008. (Only the first isolate from each patient is depicted).

Fig. 2 - Number of case patients cared for by each healthcare worker during an outbreak of bloodstream infections caused by *Burkholderia cepacia* in two outpatient units. (Hematology Unit: black bars; Bone Marrow Transplant Unit: grey bars). Hospital das Clínicas, University of São Paulo, Brazil. January-February, 2008.

After the implementation of the control measures no new cases occurred.

None of the environmental cultures or HCW hand swabs was positive for *Burkholderia cepacia* complex.

Twenty-three isolates from 21 patients were available for molecular identification and typing and for determination of MICs. 14 isolates were identified as *B. multivorans* and six belonged to the *B. cepacia* complex but could not be identified to the species level. One isolate did not belong to the *B. cepacia* complex, and could not be completely identified. The distribution of molecular types based on PFGE can be seen in Figure 1. PFGE types were named A through F. The predominant type, called A and identified as *B. multivorans,* was only present starting on February 12, 2008 and all infections occurred in patients belonging to the Hematology Unit. PFGE type D was *B. cenocepacia* and type E was *B. multivorans*. Isolates PFGE types B and C were the *B. cepacia* complex ones with

Table 1

Characteristics of 24 patients involved in an outbreak of bloodstream infections caused by *Burkholderia cepacia* in two outpatient units (Hematology and Bone Marrow Transplant). Hospital das Clínicas, University of São Paulo, Brazil. January-February, 2008

Characteristics	
Male sex	10 (42%)
Age (years)	
Mean (SD)	34 (16.4)
Median (range)	30 (2-64)
Outpatient unit	
Hematology	13 (54%)
Bone marrow transplant	11 (46%)
Underlying disease	
Hodgkin's lymphoma	8 (33%)
Non-Hodgkin's lymphoma	7 (29%)
Aplastic anemia	3 (13%)
Chronic myelocytic leukemia	2 (8%)
Myelofibrosis	1
Acute myelocytic leukemia	1
Acute lymphocytic leukemia	1
Primitive neuroectodermal tumor	1
Type of CVC	
Totally implanted	13 (54%)
Partially implanted	11 (46%)
Type of dressing used on CVC site	
Transparent film	17 (71%)
Gauze	7 (29%)
Use of chlorhexidine at CVC site	24 (100%)
Under chemotherapy	8 (33%)
Products administered through CVC	
Heparin	24 (100%)
Dexamethasone	7 (29%)
Teicoplanin	7 (29%)
Ondansetron	6 (25%)
Cefepime	5 (21%)
Dipirone	5 (21%)
Meropenem	5 (21%)
Filgrastim	4 (17%)
Ceftriaxone	3 (13%)
Platelet concentrate	3 (13%)
Fluconazole	3 (13%)
Levofloxacin	3 (13%)
Acyclovir	2
Red blood cell concentrate	2
Dimenidrate	2
CVC was used on February 11 and/or 12, 2008	10 (42%)
Days between CVC insertion and positive blood culture[1]	
Mean (SD)	276.5 (257)
Median (range)	189 (16-853)
Days between last use of CVC and positive blood culture[2]	
Mean (SD)	6.4 (6.6)
Median (range)	6 (1-32)

SD: standard deviation; CVC: central venous catheter; [1] data available for 20 patients; [2] data available for 21 patients.

species level not identified. They only occurred during the last three days of the outbreak. The isolate presenting PFGE type F was the one that did not belong to the *B. cepacia* complex.

Two patients presented more than one isolate. One patient had two different species, initially *B. multivorans* (PFGE type E), and then *B. cenocepacia* 25 days after the first episode (PFGE type D). The other patient had two isolates with different PFGE types (B and E) that were identified from cultures collected five days apart.

MIC_{50} was 4 µg/mL; 1; 2; 2 and 1 µg/mL for ceftazidime, levofloxacin; minocycline, meropenem and sulfamethoxazole/trimethoprim, respectively. MIC_{90} was 8 µg/mL; 16; 8; 4; and 8 µg/mL respectively for the same antimicrobial drugs.

DISCUSSION

Our study describes an outbreak of *Burkholderia cepacia* complex bloodstream infections in Hematologic and Bone Marrow Transplant outpatient units with multiple clones and species. No other cases of Bcc infections were diagnosed in the entire hospital during the outbreak period or in the previous year, fitting the definition of outbreak and strongly pointing to a localized problem in the two affected outpatient units. Although evidence points to a common source, this could not be completely proven and the fact that there are multiple clones and species makes interpretation difficult. During the entire outbreak period, inadequate practices in preparation and storage of intravenous medication probably favored contamination leading to bloodstream infections. This hypothesis is reinforced by the fact that two case patients were hospitalized and their only epidemiologic link to the other cases was that they had received medication prepared in the outpatient area. No healthcare worker could be directly implicated as the source of the outbreak especially because, although the unit shared areas, each worker belonged strictly to one of the units and there was no sharing of healthcare workers between the units.

We hypothesized that two distinct events took place. There seems to clearly have been a contamination by *B. multivorans* (molecular type A) and probably the source was the use of intravenous drugs or solutions prepared from multi-dose vials in the Hematology unit. Although we could not definitely demonstrate a common source of *B. multivorans,* the case distribution and the fact that ten patients had had their CVC manipulated on February 11 and/or 12 makes it probable that contaminated solution was administered. As there were no cases in other units of the hospital, contamination during industrial production is improbable and it must have occurred during manipulation of the multi-dose vials. Solutions under suspicion were saline used for central catheter flushing and heparin. All twelve patients who presented with type A-*B. multivorans* in blood cultures had received heparin through their catheters and it is not possible to ascertain whether they received saline from a multi-dose vial as this was not routinely registered in the patients' records. Saline flushing of the CVC was a common practice in the units. Among the recommendations on good practices in intravenous therapy, multi-dose vials should be avoided and intravenous solutions should not be stored for long periods of time after preparation. To our knowledge there have not been reports of outbreaks of *B. multivorans* due to the contamination of multi-dose vials of intravenous medication although reports of such contamination by other agents have been reported. In an outbreak of CVC associated bloodstream infection by *B. cepacia* and *Myroides odoratus,* commercial ampoules of sterile water intrinsically contaminated were found to be the common source[7]. The contamination of Ringer lactate solution used as multiple-dose vial to flush peripheral venous catheters was the cause of an outbreak of bloodstream infections by clonal *B. cepacia*[6]. The contamination of a heparin flush solution used for catheters in an oncology unit caused a *B. cepacia* (formerly *Pseudomonas cepacia*) outbreak already back in 1991[21]. In a systematic review of HAI related to contaminated substances[23], Bcc ranked first as contaminating pathogen in substances other than blood.

The contamination of multi-dose medication does not fully explain the outbreak. Although most outbreaks of Bcc described have been clonal[6,10,11], an outbreak caused by contaminated bromopride in 2006 was shown to be polyclonal[14]. Bcc are bacteria largely present in the natural environment, such as soil, water and rhizosphere, which is the environment adjacent to the roots of plants[22], in which different species may co-exist. Most species belonging to Bcc have also been shown to produce biofilm[5] which may be important in the pathogenesis of human disease. Patients with cystic fibrosis have been described to carry different species of Bcc[3]. Thus it is possible that in the hospital, environmental contamination by Bcc be polyclonal, thus causing polyclonal infections. It is possible that a contaminated environmental source, such as the laminar flow cabinet, was involved in the outbreak. This cabinet was the only common feature between the two units and inadequate maintenance of the cabinets was observed. It is possible that biofilm formed within the cabinet contained multiple species of *Burkholderia* which would explain the two cases with *B. multivorans* found in BMT patients that did not belong to the same clone, *B. cenocepacia* and the cases caused by unidentified species of *Burkholderia*. Unfortunately, this is only a hypothesis as cultures obtained from the cabinet were negative. Measures to improve cabinet maintenance and cleaning were implemented among others to control the outbreak.

Identification to the species level of *Burkholderia cepacia* poses a problem to the clinical laboratory because phenotypical tests are not reliable to differentiate between species within Bcc. Reference laboratories are usually needed for identification that depends on molecular techniques. Thus, managing clinical and epidemiological issues, such as outbreaks, at the point of care is a problem. New methods that allow the search for species-specific biomarkers such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) are promising for Bcc[16]. Molecular typing such as PFGE, used in our outbreak, matched the results of species identification. However typing was useless to explain the outbreak. In fact it could even have been misleading as finding multiple clones may have weakened the hypothesis of an outbreak.

A limitation of our study was that medications from multi-dose vials were not available for culturing and that the laminar flow cabinet was not entirely dismantled for culturing.

In summary, the outbreak of bloodstream infections caused by *Burkholderia* spp. was interrupted by reinforcing good practices, probably eliminating the contamination of multi-dose vials and the common source. The finding of different species was an original feature of this outbreak.

RESUMO

Surto policlonal de infecção de corrente sanguínea causada pelo complexo *Burkholderia cepacia* em unidades de hospital-dia de hematologia e transplante de medula óssea

O objetivo foi descrever um surto de infecções da corrente sanguínea por complexo *B. cepacia* (Bcc) nos ambulatórios de hematologia e transplante de medula óssea. Métodos: Em 15/02/2008, um surto de Bcc foi suspeitado. 24 casos foram identificados. Os dados demográficos e clínicos foram avaliados. Mãos de profissionais da saúde e ambiente foram cultivadas. Espécies foram determinadas e tipadas. Reforço da higiene das mãos, cuidados com cateteres, terapia de infusão e manutenção da câmara de fluxo laminar foram realizadas. 16 profissionais de saúde (PS) diferentes manipularam os cateteres. Heparina multidoses e soro eram preparadas em um balcão comum a ambas as unidades. Resultados: 14 pacientes tiveram *B. multivor*ans (um paciente teve também *B. cenopacia*), 6 Bcc não-multivorans e um teve um agente não pertencente a Bcc. Clone A de *B. multivorans* ocorreu em 12 pacientes (da Hematologia), em 10 o cateter havia sido utilizado nos dias 11 ou 12 de fevereiro. Culturas ambientais e de PS foram negativos. Todos os pacientes foram tratados com meropenem e selo de ceftazidima. Oito pacientes (30%) foram hospitalizados. Não ocorreram mortes. Após as medidas de controle, nenhum novo caso ocorreu. Conclusões: Este surto policlonal pode ser explicado por uma fonte comum contendo várias espécies de Bcc, talvez a câmara de fluxo laminar comum a ambas as unidades. Pode ter havido contaminação por *B. multivorans* (clone A) de frascos multi-dose.

FUNDING

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest involving this study.

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or inhibit bacterial growth,[6] and ampoules, syringes or tubes may be contaminated in a busy environment.[7] There are many known cases where contaminated drugs were responsible for serious hospital infections.[6] Even multidose ampoules of these drugs should not be administered to more than one patient and this leads to increased costs. EMLA cream may be used in many procedures that may pose an infection risk (venous or arterial cannulation, vaccination, certain surgical procedures). Bacterial colonization of skin precedes infection in a large number of cases.[8] However, our results suggest that the use of EMLA cream as a multidose tube is safe and may be beneficial as far as infection control is concerned.

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Polyclonal outbreaks—more common than you expect

Sir,

We read with interest the recent report by Magalhães *et al.*[1] on a polyclonal outbreak of *Burkholderia cepacia* infection in haemodialysis patients. We share the authors' conclusion that the polyclonal nature of such outbreaks might be an under-reported phenomenon, however, this 'under-reporting' could include many other environmental and non-environmental micro-organisms.

We have recently analysed 57 outbreaks of infection in intensive care units, which included neonatal ($N = 25$), paediatric ($N = 10$) and adult ($N = 22$) intensive care units.[2] Molecular genomic typing was used for clonal identification of outbreak isolates. Nearly 30% of outbreaks were polyclonal. The majority of clones causing outbreaks were from multiple sources, patients and/or environment.[3,4] Additionally, in a few long-lasting outbreaks a mutation from the original clone was probable.[5,6] Magalhães *et al.*[1] reported that all identified clones (three different strains of *B. cepacia*) were detected in water samples from haemodialysis equipment or rooms. However, the authors did not clarify whether all three clones were present simultaneously in one or more environmental sources, or different clones were present in different and separate sources. This information would contribute to a better understanding of the polyclonal origin when outbreaks are caused by more than one strain.

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Ralstonia pickettii outbreak associated with contaminated distilled water used for respiratory care in a paediatric intensive care unit

Sir,

Ralstonia pickettii is a non-fermentative Gram-negative bacillus that grows well in moist environments. It has been implicated in nosocomial outbreaks associated with contaminated solutions used for patient care[1-5] and pseudo-outbreaks associated with contaminated solutions used for laboratory diagnosis.[6,7] We report an outbreak involving two patients in a paediatric intensive care unit (PICU). The source of this outbreak was contaminated distilled water used for respiratory care.

Case 1 was a two-month-old girl, who had undergone surgery because of congenital heart disease. She developed ventilator-associated pneumonia on the third postoperative day. Tracheal aspirate cultures were positive for *Escherichia coli*. Five days later, after antibiotic therapy with meropenem, the patient was afebrile and her chest radiography, white blood cell count and C-reactive protein level were within normal limits. On the 10th day of antibiotic therapy, she became febrile again with an abnormal WBC count. Blood and tracheal aspirate cultures were positive for *R. pickettii* and the antibiotic treatment was changed to piperacillin–tazobactam. In spite of the fact that the patient's clinical and laboratory findings improved, piperacillin–tazobactam susceptible *R. pickettii* persisted in 17 consecutive blood cultures over 13 days.

Case 2 was a 14-year-old boy with cerebral oedema due to central nervous system Burkitt's lymphoma. The patient was mechanically ventilated, had multiple indwelling catheters (including central venous catheter, urinary catheter, external ventricular shunt and radial arterial catheter) and was treated with meropenem. He was afebrile and there were no clinical, laboratory or radiological findings of infection for 10 days. On the 12th day of antibiotic therapy (the eighth day of *R. pickettii* bacteraemia of case 1), he became febrile with a raised white cell count. Cerebrospinal fluid, urine analysis and chest X-ray were normal but peripheral blood and tracheal aspirate cultures were positive for *R. pickettii*. Antibiotic therapy was changed to piperacillin–tazobactam.

We initiated a surveillance study. Fluids used in the care of the patients (e.g. total parenteral nutrition fluids, intravenous fluids, distilled water used for respiratory therapy, antiseptic and antibiotic solutions) were cultured. *R. pickettii* with the same antibiotic susceptibility pattern as the patient strains (meropenem resistant but piperacillin–tazobactam susceptible) was isolated from distilled water used for humidification of the air in the ventilator circuit. All *R. pickettii* strains were identified by the use of the API NE identification system (bioMérieux). The distilled water had been stored in a container, and this stored distilled water was also contaminated. The container and the ventilators of two patients were sterilized. After this the patients improved, their acute phase reactants returned to normal within three days, and blood cultures were sterile. After 14 days of therapy, piperacillin–tazobactam was discontinued in case 1 who was discharged uneventfully. However, case 2 died from cerebral herniation due to his primary illness. No further cases of infection or contamination with *R. pickettii* were observed during the following year.

R. pickettii (formerly called *Pseudomonas pickettii* or *Burkholderia pickettii*) has been well described as a contaminant of solutions used for patient care. Characteristics of *R. pickettii* that enable it to contaminate sterile solutions include its ability to grow at a wide range of temperatures (15 °C to 42 °C) and in saline solution. In addition, *R. pickettii* may not be completely retained by a

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An outbreak of *Serratia marcescens* on the neonatal unit: a tale of two clones

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KEYWORDS
Serratia marcescens; Neonatal; Infection control; Outbreak

Summary *Serratia* spp. are an important cause of hospital-acquired infections and outbreaks in high-risk settings. Twenty-one patients were infected or colonized over a nine-month period during 2001-2002 on a neonatal unit. Twenty-two isolates collected were examined for antibiotic susceptibility, β-lactamase production and genotype. Random-amplified polymorphic DNA polymerase chain reaction and pulsed-field gel electrophoresis revealed that two clones were present. The first clone caused invasive clinical infection in four babies, and was subsequently replaced by a non-invasive clone that affected 14 babies. Phenotypically, the two strains also differed in their prodigiosin production; the first strain was non-pigmented whereas the second strain displayed pink-red pigmentation. Clinical features suggested a difference in their pathogenicity. No environmental source was found. The outbreak terminated following enhanced compliance with infection control measures and a change of antibiotic policy. Although *S. marcescens* continued to be isolated occasionally for another five months of follow-up, these were sporadic isolates with distinct molecular typing patterns.

Introduction

Serratia marcescens, previously thought to be a benign saprophyte and only relatively recently well recognized as a nosocomial pathogen, causes a wide spectrum of infections such as pneumonia, wound infections, urinary tract infections,

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meningitis, endocarditis and septicaemia.[1–3] These can be difficult to treat due to multiple antibiotic resistance and have a high mortality. The organism produces virulence factors such as lipase, lecithinase and haemagglutinating activity, and a red pigment, prodigiosin, the apoptotic action and anti-cancer properties of which have been described recently.[4] In hospitalized adults, *Serratia* spp. tend to colonize the respiratory and urinary tracts, especially if foreign bodies such as indwelling catheters and endotracheal tubes are present.[5] In contrast, in neonatal intensive care units, where outbreaks have been reported, it is the gastrointestinal tract of neonates that may be an important reservoir for cross-contamination. In such circumstances, a number of epidemiological typing methods have been described including serotyping, phage typing, biotyping, bacteriocin typing, whole-cell protein fingerprinting, plasmid analysis and ribotyping.[6] Typing procedures using molecular-biology-based techniques are generally preferred for differentiating bacterial strains in epidemiological studies because of their superior reproducibility and discriminatory power.

This study describes an outbreak of *S. marcescens* that occurred over nine months in a neonatal unit (NNU), and discusses its investigation and control.

Methods

Description of the outbreak

The NNU at City Hospital, Birmingham is divided into three sections: an intensive care unit (ICU), comprising of a bay with six cots plus an isolated cot; a high-dependency unit and a low-dependency unit. The NNU admits babies born at the host hospital and also those transferred from other units in the region. The antibiotic policy at the time of the outbreak recommended benzylpenicillin and gentamicin as first-line empirical therapy for suspected sepsis, and cefuroxime with or without vancomycin as second-line antibiotics.

S. marcescens was first isolated on 22 October 2001 from the tip of an umbilical artery catheter, removed from a two-day-old baby born at 30 weeks' gestation, and transferred to the NNU from another hospital. Two days later, *S. marcescens* was also grown in a blood culture from the same patient. She received a course of intravenous benzylpenicillin and gentamicin, started empirically at birth, then continued for a total of 10 days once cultures were positive. The baby improved clinically. Within a week, microbiological samples yielded *S. marcescens* from three more babies in the same intensive care bay. Three babies were bacteraemic; two of them, who also had meningitis, were treated with intravenous meropenem but both subsequently died.

The affected babies were cohort nursed on the ICU. The entire NNU was closed to transfers from other hospitals and remained open only for admission of babies born at the maternity unit within the host hospital. Throughout the next nine months, microbiological surveillance was extended to include stool specimens from all babies on the NNU. Clinical samples continued to yield *S. marcescens* for 12 months but no serious infections occurred after the first three cases.

Following identification of the first isolates, the infection control team visited the NNU and carefully reviewed the medical records of these cases in order to identify any epidemiological associations. Use of protective clothing (i.e. aprons and gloves when handling affected babies) and improved compliance with handwashing were emphasized. Additional cleaning of the equipment and environment, with the use of a 1000 ppm chlorine-releasing agent wherever possible, was recommended.

All babies in the neonatal ICU, where the first cases were identified, were screened for *S. marcescens* from oropharyngeal secretions, urine and any skin lesions. Sampling of the environment (incubators, ventilator humidifiers, suction bottle, resuscitation table, phototherapy unit, baby scales, laryngoscope blades, floors, sinks and creams) was also carried out using swabs moistened with sterile water or impression plates, as appropriate. Staff members of all designations were screened for carriage of the organism by finger impression on to nutrient agar plates (Oxoid, Basingstoke, UK). Air sampling was also performed with a Casella Slit Sampler (Casella Ltd, Bedford, UK) that samples 0.7 m^3/min.

Laboratory studies

Organism identification

Isolates of *S. marcescens* from clinical or screening specimens were identified by standard laboratory methods, including the API 20E system (bioMerieux, Marcy l'Etoile, France). They were stored at −70°C for further studies.

Antibiotic susceptibility

Isolates were tested according to the British Society of Antimicrobial Chemotherapy (BSAC)

standardized disk diffusion method with ampicillin, trimethoprim and cefuroxime as well as cefoxitin to detect chromosomal AmpC enzyme production.[7] In addition, minimum inhibitory concentrations (MICs) for gentamicin (Sigma, Poole, UK), tobramycin (Sigma), amikacin (Sigma), ceftazidime (Smithkline Beecham, Harlow, UK), meropenem (Astra Zeneca, Macclesfield, UK), ciprofloxacin (Bayer, Leverkusen, Germany), ofloxacin (Sigma), ticarcillin (Smithkline Beecham) and piperacillin/tazobactam (Wyeth, Maidenhead, UK) were determined by the BSAC agar dilution method on Isosensitest agar (Oxoid) with an inoculum of 10^4 colony-forming units (cfu)/spot.[7]

β-lactamase production

The iso-electric point of the β-lactamase produced was determined using the method described by Matthew *et al*.[8] Isolates were cultured overnight in 10 mL nutrient broth both without and with ampicillin as a β-lactamase inducer. β-lactamases were extracted by three cycles of freeze thawing. Supernatant (5 μL) was applied to an Ampholine PAG plate, pH range 3.5-9.5 (Amersham Biosciences, Little Chalfont, UK). Iso-electric point standards, methyl red dye (pI 3.75) and horse myoglobin (pI 6.8 and 7) were applied together with the samples. The gel was run on an LKB 2117 Multiphor II Electrophoresis Unit (Amersham Biosciences) at a constant voltage of 500 V for 2.5 h at a controlled constant temperature of 10°C. The gel was stained with nitrocefin (Oxoid, Basingstoke, UK) and photographs were taken with a Fuji Finepix 6800Zoom digital camera (Fujifilm, Japan).

Molecular typing

Genomic DNA was extracted with the Wizard® Genomic DNA purification kit (Promega Madison, Wisconsin, USA), and genotyping by random-amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was performed with primers described previously by Hejazi.[9] DNA products obtained were separated on an agarose gel and stained with ethidium bromide.

For pulsed-field gel electrophoresis (PFGE), isolates were inoculated into nutrient broth and incubated overnight at 37°C. Next day, 1.5 mL of the broth culture was spun at 3000 revolutions/min for 15 min at 4°C. The pellet was washed with SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5) and, after repeat centrifugation, was resuspended in 0.3 mL SE buffer. An equal amount of warm low-gelling agarose was added and mixed gently. The mixture was dispensed into the slots of the Bio-Rad PFGE mould (Bio-Rad, Hemel Hempstead, UK), giving a final concentration of organisms of approximately 1×10^9 cfu/mL (1 μg DNA/100 μL).

The plugs were lysed for 48 h with lysis buffer (1% w/v N-lauryl sarcosine, 0.5 M EDTA, pH 9.5) to which proteinase K (Sigma, Poole, UK) was added at a final concentration of 0.5 mg/mL. Chromosomal DNA was digested overnight at 37°C with 20 U of *Xba*I (Promega) in 200 μL reaction buffer and the restriction fragments were separated with CHEF-DRII apparatus (Bio-Rad) with a pulse time of 5-25 s and a 22-h run time at 6 V/cm. Gels were stained with ethidium bromide and viewed by ultraviolet transillumination. Patterns were compared visually and band differences were noted.

Results

Five isolates from the initial four cases of infection and 14 isolates of *S. marcescens* from other clinical and screening samples (Figure 1) were collected between October 2001 and June 2002. They were cultured from a variety of sites, including blood, cerebrospinal fluid, umbilical artery tip, eye swab, nasopharyngeal aspirate, oropharyngeal secretions, groin, Broviac line exit site and faeces (Table I). Environmental sampling yielded *S. marcescens* from only one site; a sink drain (Isolate 20). No isolates were obtained from any of the 45 staff members screened. Three further isolates (Isolates 21-23) of *S. marcescens* obtained from babies in the NNU after the end of the outbreak and

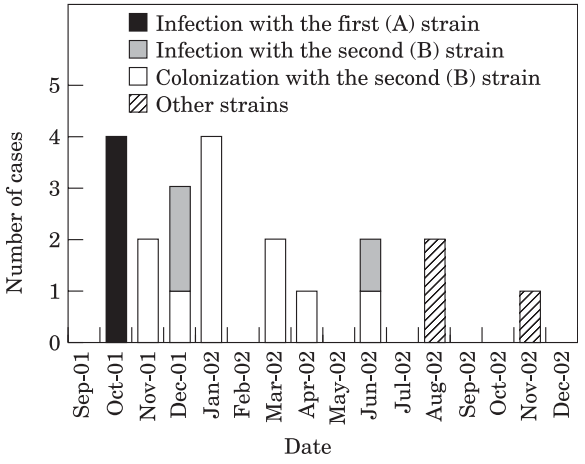

Figure 1 Monthly incidence of *Serratia marcescens* infection or colonization cases identified in the neonatal unit.

Table I Characteristics of epidemiologically related and control isolates of *Serratia marcescens*

Isolate no.	Source of the strain	Antibiogram	RAPD pattern	PFGE pattern
1	Nasopharyngeal aspirate	ApCxmTcTnCfx	A	A
2	Blood	ApCxmTcTnCfx	A	A
3	Cerebrospinal fluid	ApCxmTcTnCfx	A	A
4	Cerebrospinal fluid	ApCxmTcTnCfxGAk	A	A
5	Blood	ApCxmTcTnCfx	A	A
6	Umbilical swab	ApCxmTn	B	B
7	Screen stool	ApCxmTn	B	B
8	Screen umbilical swab	ApCxmTn	B	B
9	Catheter tip	ApCxmTn	B	B
10	Eye swab	ApCxmTn	B	B
11	Groin swab	ApCxmTn	B	B
12	Screen groin	ApCxmTn	B	B
13	Screen groin	ApCxmTn	B	B
14	Screen nose	ApCxmTn	B	B
15	Endotracheal secretions	ApCxmTn	B	B
16	Eye swab	ApCxmTn	B	B
17	Oropharyngeal secretions	ApCxmTn	B	B
18	Eye swab	ApCxmTn	B	B
19	Nasal secretions	ApCxmTn	B	B
20	Environmental	ApCxmTn	C	C
21	Eye swab	ApCxmTcTn	D	D
22	Eye swab	ApCxm	D	D
23	Eye swab	ApCxm	E	E
24	Unrelated (blood)	ApCxmCipOf	Distinct	Distinct
25	Unrelated (blood)	ApCxmTnTic	Distinct	Distinct
26	Unrelated (blood)	ApCxm	Distinct	Distinct
27	Unrelated (blood)	ApCxm	Distinct	Distinct
28	*S. marcescens* NCTC	ApCxm	Distinct	Distinct

RAPD, random-amplified polymorphic DNA; PFGE, pulsed-field gel electrophoresis; Ap, ampicillin; Cxm, cefuroxime; Tc, ticarcillin; Tn, tobramycin; G, gentamicin; Ak, amikacin; Cip, ciprofloxacin; Of, ofloxacin; Cfx, cefoxitin.

four blood culture isolates from other wards were also included in the study.

Both molecular typing methods revealed that two clones were present during the outbreak (Figure 2). The first clone (Type A), which caused invasive clinical infection in four babies (Isolates 1-5), was replaced by a non-invasive clone (Type B) that affected 14 babies (Isolates 6-19). Although *S. marcescens* continued to be isolated occasionally for another six months, the end of the outbreak was signalled by the replacement of the original strains with sporadic strains with other molecular typing patterns. The environmental isolate (Isolate 20), sporadic isolates and clinical isolates from other wards were genetically distinct.

Phenotypically, the two strains also differed in their prodigiosin production. Type A isolates were non-pigmented whereas Type B isolates displayed pink-red pigmentation. The environmental isolate was also strongly pigmented, with a deep red colour.

All isolates were resistant to ampicillin and cefuroxime and were susceptible to meropenem, ciprofloxacin, ofloxacin and ceftazidime. Both Type A and Type B isolates also displayed low-level resistance to tobramycin. Clear cefoxitin resistance was only detected in Type A isolates, although Type B isolates appeared borderline sensitive. Two morphologically different *S. marcescens* (Isolates 3 and 4) were cultured simultaneously from the cerebrospinal fluid of one baby who had previously received gentamicin. Both isolates were Type A by PFGE and RAPD, but the susceptibility pattern of Isolate 4 was distinct. Whereas Isolate 3 displayed the same sensitivity profile as other Type A organisms, Isolate 4 showed overall increased antibiotic resistance with low-level resistance to amikacin (MIC 64 mg/L), gentamicin (MIC 4 mg/L) and cefotaxime (MIC 2 mg/L).

Iso-electric focusing of a few representative isolates of Type A and Type B confirmed the presence of an inducible beta-lactamase with a high iso-electric point (pI 7.7), consistent with the presence of an expressed chromosomal AmpC enzyme. In the Type B isolates, AmpC presence was only detected in those in which ampicillin was added to the broth culture, thus inducing its expression.

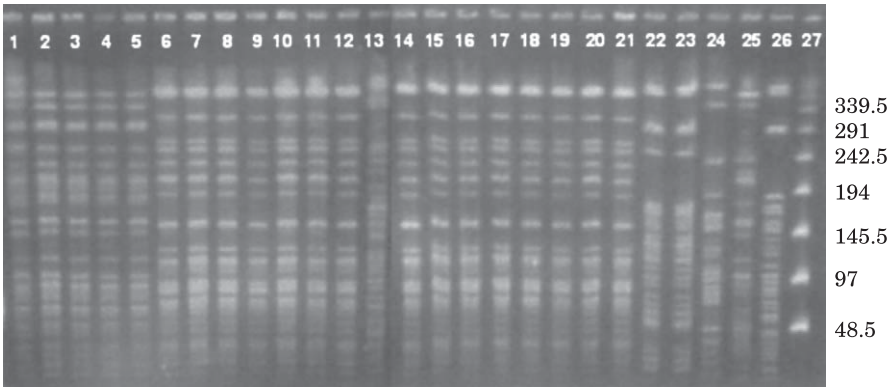

Figure 2 Pulsed-field gel electrophoresis analysis after digestion with XbaI (combined images from two gels aligned by lambda DNA marker profiles). Lanes 1-5, Isolates 1-5 (clinical); lanes 6-12, Isolates 6-12 (clinical); lane 13, Isolate 20 (environmental); lanes 14-21, Isolates 12-19; lanes 22-23, Isolates 21-22; lane 24, Isolate 23; lanes 25 and 26, Isolates 25 and 27 (unrelated); lane 27, lambda DNA marker, kilobase pairs.

Discussion

There have been previous reports in the literature of outbreaks of *S. marcescens* in NNUs. These are usually associated with significant morbidity and mortality,[6,10,11] although rates vary.[12] In the present study, there was a marked difference in the pathogenicity of the two strains that followed each other and were even cocirculating for a short period of time. The first strain was particularly invasive, giving rise to bacteraemia in three out of four infected babies, two of which also had meningitis and subsequently died. The second strain was only associated with colonization or superficial infections. There appears to be an inverse relationship between prodigiosin production and pathogenicity, but no clear explanation for this has been found to date.[13] In some bacteria, antibiotic resistance comes at a cost in terms of decreased bacterial fitness. It would be possible to hypothesize that the second, less virulent and less resistant, strain had a survival advantage, allowing it to replace the first strain as the circulating strain within the NNU. A predilection for involvement of the central nervous system in *S. marcescens* bacteraemia has been noted before.[14] When such an isolate is cultured from blood, investigations for meningitis such as a lumbar puncture are advisable.

During this study, despite extensive environmental and staff sampling, no common point source was identified. The only isolate cultured from a sink drain proved to be genotypically different, by both PFGE and RAPD-PCR, to the clinical isolates. Previous publications have reported various sources of contamination such as breast pumps,[15] plastic bottles used for umbilical irrigation,[16] scalp vein needles[17] or nail brushes,[18] but no source was identified in the majority.[9,19,20] It is possible that the original Type A isolate was introduced into the NNU by the baby transferred from another hospital. Outbreaks of multi-resistant Gram-negative organisms are often initiated by the large numbers of patients transferred between units, and some advocate screening of all interhospital transfers for resistant coliforms.[21] The authors have not adopted such a policy. There is no evidence that the Type B strain was imported from outside, but its origin remains unclear. It is likely that the same conditions that allowed the spread of the first strain favoured the emergence of the second strain. Amongst these, intrapartum antibiotic prophylaxis for group B streptococcus has been suggested as a risk factor for early-onset Gram-negative infection in neonates.[22–25] Empirical use of broad-spectrum antibiotics for treatment of suspected neonatal sepsis could also have been implicated.

It is believed that colonized and infected infants are the most frequent reservoir of *S. marcescens*.[26,27] Once affected, most of them appear to remain colonized, especially in their gut, despite antibiotic treatment. This finding seems to be particular to neonates and not to adults. Screening revealed several cases of asymptomatic colonization. The organism is probably transmitted horizontally via the hands of healthcare personnel. It is likely that the most relevant intervention, with regards to ending the outbreak, was reinforcement of hand hygiene. However, increased environmental cleaning, including the use of hypochlorite for disinfection of the sink drain from which *S. marcescens* was grown, may have contributed.

The possibility that the outbreak was due to selection by antibiotic pressure was considered. Although some authors consider *S. marcescens* to be less prone than *Enterobacter* spp. and *Citrobacter freundii* to segregate highly resistant mutants during therapy, others regard cefuroxime as a more powerful inducer of *S. marcescens* and *Morganella morganii* enzymes than those from *Enterobacter cloacae* or *C. freundii*.[28,29] Cefuroxime was used as a second-line antibiotic on the NNU and a change in policy did co-incide with the end of the outbreak. However, no causal relationship could be established.

The authors did not investigate the increased resistance of Isolate 4 obtained from a baby with meningitis who received gentamicin. It is possible that gentamicin penetrating cerebrospinal fluid in sub-bactericidal concentrations may have selected for isolates with a derepressed chromosomal aac (6′)-Ic enzyme, which is normally expressed at a very low level in *S. marcescens*. Alternatively, it may represent acquisition of an aminoglycoside-modifying enzyme containing plasmid. Other authors have noted a similar phenomenon, which makes therapy with aminoglycosides in *S. marcescens* infections susceptible to failure.[26] Cephalosporins of the first three generations should also be avoided due to the possibility of induction of the AmpC enzyme, illustrated in the Type A isolates through cefoxitin testing. Carbapenems or fourth-generation cephalosporins are stable to this enzyme and are, therefore, the antibiotics of choice for treatment of systemic sepsis.

This study employed two different molecular typing techniques to substantiate epidemiological associations. RAPD-PCR has proven to be relatively easy to perform and thus may have a role as a rapid means of screening of isolates in outbreaks, but may suffer from limited reproducibility. PFGE is both discriminatory and reproducible.

In conclusion, the investigation of an outbreak of *S. marcescens* with RAPD-PCR and PFGE revealed the presence of two outbreak clones. There was a clear difference in their pathogenicity. An inverse relationship between the amount of pigment (prodigiosin) production and the virulence of the two strains was apparent. Simple enforcement of standard measures, such as isolation and hand hygiene, were used to contain and resolve the outbreak.

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Outbreak Report

Cluster of *S. maltophilia* among patients with respiratory tract infections at an intensive care unit

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ABSTRACT

Background: Stenotrophomonas maltophilia is associated with respiratory tract infections in immunocompromised patients, and it has emerged as an important nosocomial pathogen, with admission to intensive care units (ICUs) and ventilators as recognized risk factors.

Aim: To describe the investigation of a sudden increase in patients with pneumonia caused by *S. maltophilia* at a Swedish ICU and the control measures taken.

Methods: Lower respiratory tract cultures from patients admitted to the ICU were obtained, and environmental cultures were collected from sink drains and medical equipment. Isolates identified as *S. maltophilia* were subjected to antibiotic susceptibility testing and whole genome sequencing (WGS).

Findings: A total of 17 *S. maltophilia* isolates were found (four from patients and 13 from the environment). The WGS identified two outbreak clones, sequence type (ST) 361 and ST138, and seven unique ones. Most likely, the outbreak clones originated from two sinks, and transmission was enhanced by a calorimeter. After changing the sink and calorimeter routines, no more cases were registered.

Conclusion: Acquisition of *S. maltophilia* from the hospital environment appears to be easy, especially if water is involved. To control this bacterium, better knowledge of its transmission routes in hospital environments is required.

Introduction

Stenotrophomonas maltophilia is a ubiquitous, non-fermenting Gram-negative rod with essential functions in plant ecosystems. Like *Acinetobacter* spp., it has generally been regarded as an opportunist, but in later years it has emerged as an important pathogen in hospital environments. It is mainly associated with respiratory tract infections in immunocompromised patients and bloodstream infections in neutropenic patients. It can, however, cause other serious infections, including meningitis, endocarditis and infections in bone, skin and soft tissues. Fatalities are not uncommon and admission to an intensive care unit (ICU) and ventilator use are recognized risk factors [1–3].

The productions of broad-spectrum beta-lactamases, efficient multidrug efflux pumps, low outer membrane permeability and a high ability to acquire resistance, renders

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S. maltophilia resistant to a broad array of antibiotics [2]. Trimethoprim-sulfamethoxazole is the only drug with breakpoints for *S. maltophilia*, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), but therapy failures and resistance development during therapy occur [4]. Treatment of severely ill patients can therefore be a challenge.

Apart from its multiresistance, the bacterium is characterized by its ability to form biofilms on various abiotic and biotic surfaces [2]. In the hospital environment, sinks and drains with stagnant water constitute a high risk for contamination of *S. maltophilia*. Aerosols from sinks may contaminate medical devices used in the daily care of patients, and bacteria can thereby be transmitted to vulnerable patients [1,5].

A wide range of molecular genotyping methods can be used to identify the genetic relatedness of bacterial isolates and map their transmission routes. In contrast to traditional methods, whole genome sequencing (WGS) has an ultimate resolution power by permitting discrimination between closely related isolates through comparison of single nucleotide polymorphisms (SNPs) [6]. This feature can be useful when investigating clinical isolates of *S. maltophilia* [7,8], a species with a high genetic diversity.

The objective of this study was to describe the investigation performed to elucidate the background to a sudden increase in isolation frequency of *S. maltophilia* in patients with pneumonia at an ICU at Linköping University Hospital, Sweden. Furthermore, a description of the measures taken to control the dissemination of the bacterium is given.

Methods

Setting

Linköping University Hospital is the only tertiary care hospital in southeast Sweden. The involved ICU offers a total of eight beds. All rooms have a sluice, and there is a sink in each sluice and one in each patient room. The sinks are used for hand washing, patient care, cleaning of various medical devices, etc.

Epidemiological investigation

In October 2018, three patients admitted to an ICU at Linköping's University Hospital showed growth of *S. maltophilia* in samples from their lower respiratory tracts within just a couple of days. The index patient (Patient 1) was culture positive for the bacterium prior to the admittance to the ICU. This patient shared the same room (room 1) with Patient 2, whereas the third patient (Patient 3) was located in the room next-door (room 2). For more details, see Figure 1. Due to the bacterial findings and the close proximity of the three patients in space and time, an epidemiological investigation was initiated.

The three patients had two factors in common: a calorimeter and bronchoscopy. A calorimeter is a medical device that allows clinicians to accurately assess the energy expenditure in critically ill patients (Figure 2). Calorimetry was carried out every third day on the patients admitted to the ICU. According to routines, the moist trap (Figure 2) was changed once daily, whereas the plastic tube was patientbound (Figure 2) and stored in a plastic bag in the bedside table when not used. Patients 1 and 2 had used the calorimeter the very same day. In addition, bronchoscopy had been performed on all three patients. After each bronchoscopy, the bronchoscopes were cleaned, disinfected and hung on a stand in room temperature to dry.

Sampling was obtained from different sites of the calorimeter, vaporizers and plastic buckets used in respiratory therapy, the sink drain of all sinks located at the unit (in patient rooms, sluices, preparation room and wash rooms), and bronchoscopes. In addition, all patients admitted to the ICU were screened for *S. maltophilia* in samples from the lower respiratory tract. Cases were defined as patients with carriage of, or infection with, *S. maltophilia*. An additional inclusion criterion was that there had to be a connection in time and/or space with the index patient.

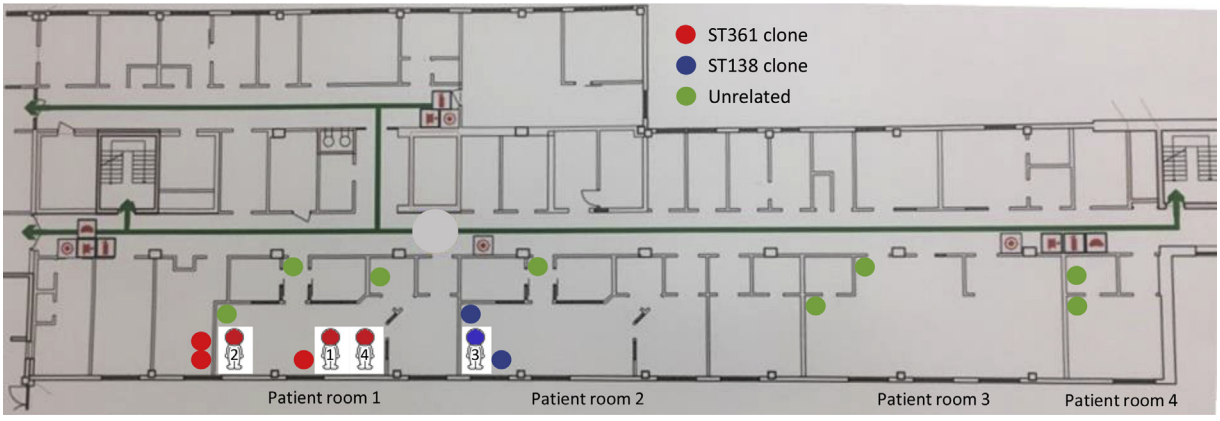

Figure 1. Drawing of the intensive care unit where the clustering of *S. maltophilia* took place. Patients and environmental sites culture-positive for the bacterium are marked with different colours depending on sequence type. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

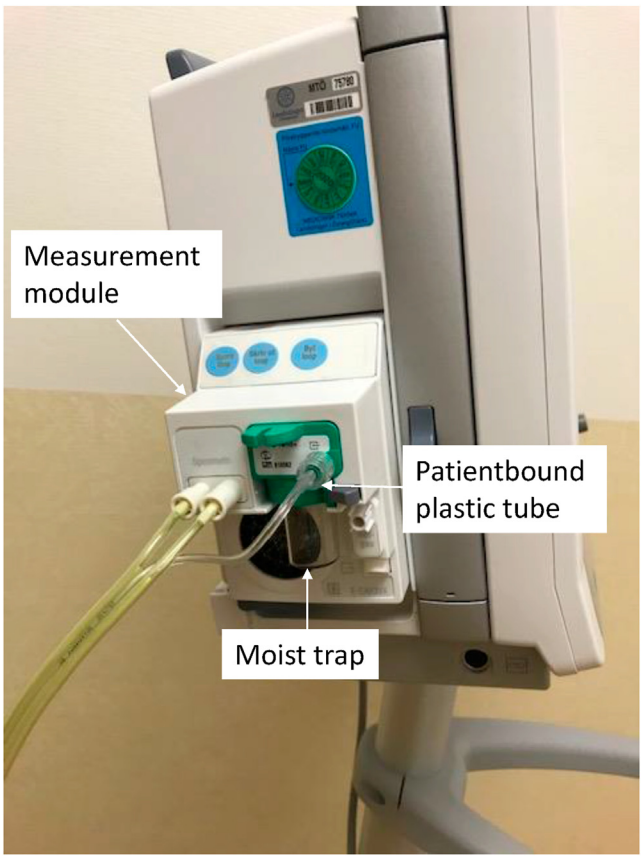

Figure 2. Photo of the involved calorimeter.

Bacterial cultures

Environmental samples were collected with ESwabs (Copan Diagnostics Inc. Murrieta, CA, USA) and inoculated onto two different types of media for Gram-negative bacteria. The plates were incubated at 35 °C for approximately 48 h. Bacteria were identified to the species level with a MALDI Biotyper 3.0 (Bruker Corporation, Karlsruhe, Germany). The antibiotic susceptibility to trimethoprim-sulfamethoxazole was tested according to the recommendations of EUCAST (www.eucast.org).

WGS

All *S. maltophilia* isolates from patients and the environment were subjected to WGS. DNA was prepared from a single colony of each isolate, using EZ1 DNA Tissue Kit (Qiagen, Germantown, MD, USA), with an included pre-heating step at 95 °C and a centrifugation step at 350 rpm for 15 min. Twenty ng of DNA was used for library preparation, using QIAseq FX DNA Library Kit (Qiagen, Germantown, MD, USA) with 8 min of fragmentation time. DNA libraries were sequenced on the MiSeq platform (Illumina, San Diego, CA, USA) with 2 x 300 bp paired-end reads, and the samples obtained an average sequencing depth of 82x.

Data analysis was performed in CLC Genomics Workbench v. 9.5.4 with the Microbial Genomics Module v. 1.6.2 (Qiagen, Germantown, MD, USA). Multilocus sequence typing (MLST) analysis was performed using the PubMLST (pubmlst.org) scheme for *S. maltophilia* [9,10]. Sequence data from previously unknown sequence types (STs) were submitted to pubMLST. Sequencing reads were mapped to the *S. maltophilia* NCBI reference genome NC_015947. Variants were called in relation to the reference genome with the following thresholds: frequency $\geq$ 90 %, sequencing depth $\geq$ 20x and quality (Phred) score $\geq$ 20 at the variant position and $\geq$ 15 in the $\pm$ 5 bp neighbourhood. Identified SNP positions were filtered based on a sequencing depth of $\geq$ 20x in all samples, a Z-value $\geq$ 1.96 and a pruning distance of 100 bp. The resulting 30 695 positions were then used to build a neighbour-joining phylogenetic tree based on the genetic distance between samples. Genomes were also assembled and searched for resistance genes using the ResFinder database [11], with thresholds of 98 % identity and 60 % length.

Results

Microbiological findings

Apart from the three patients infected with *S. maltophilia* in the lower respiratory tract, eight additional patients admitted to the ICU were screened for *S. maltophilia*. None of them were positive for *S. maltophilia*. However, one month later when the screening had been stopped, another patient (Patient 4) exhibited growth of *S. maltophilia* in a sample from the lower respiratory tract. This patient had been cared for in the same room as Patient 1 (Figure 1).

A total of 54 environmental samples were collected. Of these, 13 (24%) showed growth of *S. maltophilia*. To the culture-positive locations belonged two different sites of the calorimeter (the moist trap and the portal of the patientbound plastic tube, see Figure 2), the plastic buckets used during the respiratory therapy for Patients 1 and 3, sinks ($n = 4$) located in all four patient rooms, sinks in three of the sluices, the sink in the preparation room, and the sink in one of the washrooms (Figure 1). The bronchoscopes showed no growth of *S. maltophilia*.

All *S. maltophilia* isolates from the patients and the environment ($n = 17$) were susceptible to trimethoprim-sulfamethoxazole.

WGS results

MLST and whole genome-wide phylogenetic analysis identified a high genetic diversity among the 17 *S. maltophilia* isolates collected from the ICU, and the distribution of the isolates was the same with the two methods (Figure 3).

MLST analysis identified nine different types among the 17 isolates, with a distribution that is in agreement with the SNP analysis results (Figure 3). Three of the STs were previously unknown. Two of these received novel MLST profile numbers (361 and 362) upon submission to the *S. maltophilia* pubMLST database [10]. The third type did not receive a profile due to the presence of two different nuoD alleles.

Two outbreak clusters were recognized (Figure 3). The larger one consisted of six isolates (35%) and belonged to the ST361 clone. Among these, the isolates from Patients 1 and Patient 4 were identical. They differed by one SNP to a group of three other identical isolates: those of a calorimeter site (portal of patientbound plastic tube), the plastic bucket

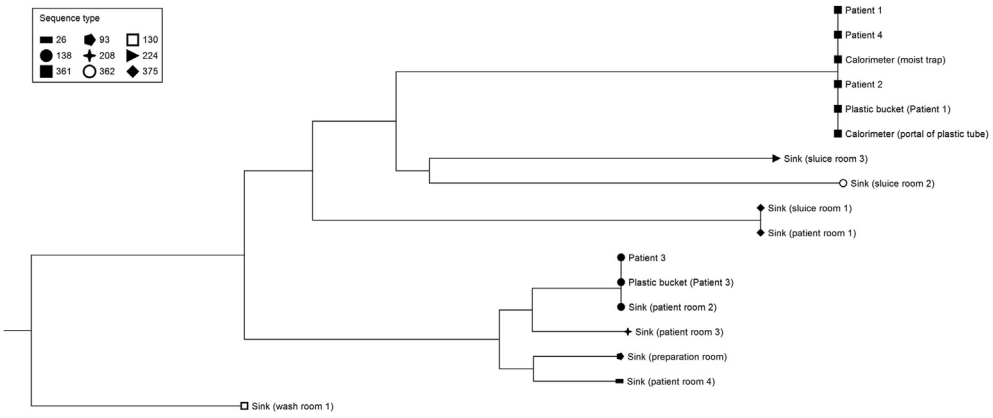

Figure 3. Phylogenetic tree based on single nucleotide polymorphism (SNP) analysis of whole genome sequencing (WGS) data from 17 *S. maltophilia* isolates. Node shapes represent sequence types (STs) based on multi-locus sequence typing (MLST). Two outbreak clones were identified: one belonging to ST361 (isolates differing by 0—2 SNPs) and one belonging to ST138 (isolates differing by 0—1 SNPs).

located next to Patient 1 and the isolate from Patient 2. The isolates of Patients 1 and 4 also differed by a single but different SNP to the second isolate from the calorimeter (moist trap). In the smaller cluster, three isolates (18%) belonging to ST138 were included. Of these, the isolate from Patient 3 and from the plastic bucket located next to this patient, had identical SNPs, whereas the isolate from the sink located next to Patient 3 (in patient room 2), differed by one SNP. None of the isolates harboured a gene encoding resistance to trimethoprim-sulfamethoxazole.

Control measures

Control measures were immediately taken, including screening of all admitted patients to the ICU and improved compliance to basic hygiene and cleaning routines. To limit bacterial growth in the water traps, every sink in the unit was rinsed once a week with boiling water. When rinsing inner cannula, a metal bowl was placed inside the original sink, and all contaminated water or other fluids were discharged in the sink located in the washrooms instead of in the patient rooms. In addition, no medical devices or items such as toothbrushes were allowed on the sinks or in their close vicinity to avoid contamination from aerosols. The plastic buckets used during respiratory therapy were replaced with single-use buckets, which were changed after each work shift. The moist trap and plastic tubes of the calorimeter were changed after each performed measurement.

Discussion

In the present study, a minor outbreak of *S. maltophilia* at an ICU at Linköping University Hospital in Sweden was described. A total of four patients were involved, and two different clones of *S. maltophilia* were identified, ST361 and ST138. The ST361 clone caused the largest cluster and originated most likely from Patient 1, whose lower respiratory tract culture yielded growth of *S. maltophilia* prior to the admittance to the ICU. The clone was thereafter transmitted by a calorimeter, which had not been properly handled by the staff. According to the manufacturer's manual, the moist trap of the calorimeter should be changed after each measurement. Instead, this was carried out only once daily. Since the calorimeter was used every third day on the patients, several patients were at risk of being infected with *S. maltophilia*. The second cluster probably originated from one of the sinks in the unit.

Environmental cultures were collected from sink drains of every sink at the ICU and growth of different strains of *S. maltophilia* was identified in almost every room. The water traps of sinks constitute a wet and relatively protected environment, which favours the growth of bacteria and production of biofilms. The exposure to liquids and the waste discarded in the sinks may serve as a breeding ground for opportunistic and multiresistant bacteria that cannot easily be eradicated [12]. It has been described that sink drains in hospitals contain 10^6—10^{10} colony forming units (cfu)/ml of bacteria of which approximately 10^3-10^5 cfu/ml are Gram-negative rods, especially waterborne bacteria [5]. These bacteria can infect patients via different transmission routes. There has been a clear increase in documented sink-associated outbreaks worldwide in recent years. However, few studies deal with the exact mechanism of transmission, i.e. from the sink to the hospitalized patients. In a study from 2017, mobilization of bacteria from biofilms in water traps of sinks to the surrounding environment was demonstrated by using green fluorescent-expressing *Escherichia coli* [12]. This was most likely the transmission route for the two last patients.

Replacement of contaminated sinks has been shown to reduce the infection rate in ICUs [13,14], but re-occurrence of growth have been described [15]. A more long-term solution would be to use sinks with a self-disinfecting function. There is already a product on the market that can disinfect the water trap [16], and similar products are needed to keep away an important source of not only carbapenemase-producing *Enterobacterales* and *Pseudomonas aeruginosa*.

Nosocomial outbreaks caused by *S. maltophilia* seem to be quite rare considering the low number of published articles. Several of them deal with airways and water in some form. In a study from 2013 [17], an outbreak of *S. maltophilia* at an ICU located in the United Kingdom was described and involved 23 patients (majority of the isolates from the respiratory tract), which were shown to belong to only two genotypes. Environmental sampling found the two outbreak strains in two sinks

and in the drinking water of the cooling unit used for providing drinking water and mouth care to ICU patients. Likewise, a Spanish study reported a bronchoscope-associated pseudo-outbreak with 39 patients, highlighting the risks with contaminated medical devices [18].

Although outbreaks are relatively rare, the genetic relatedness of isolates in suspected outbreak situations needs always to be explored. A wide range of methods have been used through the years [19]. In this study, WGS was applied. It clearly showed the high genetic diversity among *S. maltophilia* in a single unit, which is in accordance with other studies [20,21]. Furthermore, it showed a dissemination of ST361. If this clone has features that render it more epidemic than others is not yet known, but with more use of WGS, certain clones may show themselves to be more prone to dispersal than others.

Conclusions

An outbreak of *S. maltophilia* caused by two different clones and involving four patients in an ICU was confirmed by WGS. To our knowledge, this is the first study that demonstrates the involvement of a calorimeter in the transmission. The intervention was successful and no more patients were infected. To avoid transmission of *S. maltophilia*, which may cause serious infections in vulnerable patients, better attention needs to be paid to water sources and sinks located in hospital environments.

Author contributions

We have contributed to the manuscript as follows:

MG: Design, acquisition and analysis of data, and drafting and revising the manuscript.

JW: Acquisition and analysis of data, and drafting and revising the manuscript.

AM: Design, analysis of data, and drafting and revising the manuscript.

We all approve of the submitted version, and there are no conflicts of interest.

Conflict of interest statement

None.

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Hospital toilets and drainage systems as a reservoir for a long-term polyclonal outbreak of clinical infections with multidrug-resistant *Klebsiella oxytoca* species complex

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S U M M A R Y

Background: Nosocomial outbreaks with multidrug-resistant bacteria with a probable reservoir in hospital toilets and drainage systems have been increasingly reported.
Aim: To investigate an increase in bacteraemia with extended-spectrum β-lactamase (ESBL)-producing *Klebsiella oxytoca* at our hospital in 2021; the epidemiology of the outbreak suggested an environmental source.
Methods: Available clinical *K. oxytoca* isolates from patient with infection or rectal carriage from 2019 to 2022 were collected. Clinical information was gathered from included patients and sampled sinks, shower drains, and toilet water. Short- and long-read whole-genome sequencing (WGS) was performed on patient and environmental isolates to assess phylogenetic relationships, antibiotic resistance genes/mutations, and plasmid profiles.
Results: WGS revealed four clusters and a polyclonal population consisting of ESBL-producing *K. oxytoca* and *Klebsiella michiganensis*. All clusters contained both clinical and environmental isolates. The environmental sampling revealed widespread contamination of the outbreak strains in the outbreak ward, and plasmid analyses indicated possible transfer of plasmids between species and clones. Most environmental findings in the outbreak ward were from toilet water, and enhanced cleaning of bathrooms and toilets was introduced. The following year, a decrease in outbreak strains in systemic infections was observed.
Conclusion: This investigation uncovered a polyclonal outbreak of multidrug-resistant *K. oxytoca* and *K. michiganensis* and unveiled a persistent reservoir of outbreak clones

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in the drainage system and toilet water, facilitating exchange of resistance genes. The risk of toilet water as a source of clinical infections warrants further investigation.

Introduction

Adherence to standard precautions for infection control is paramount in controlling hospital outbreaks of multidrug-resistant (MDR) bacteria. Careful hand hygiene is particularly important along with surveillance cultures and contact precautions for colonized patients [1—3]. However, should new cases appear despite these measures, it is essential to investigate alternative factors supporting continued spread of infection, including persistent environmental reservoirs.

Recent studies have highlighted the importance of bacterial biofilms within hospital drainage systems as reservoirs for hospital infections. Drainage system-associated biofilms may house MDR organisms and other nosocomial pathogens and might support prolonged outbreaks [4—8]. A key challenge arises from the persistence and heterogeneity of species and strains within environmental reservoirs, which complicates outbreak detection and creates conditions supporting horizontal gene transfer encoding antimicrobial resistance [6,9,10].

K. oxytoca is commonly found in the human intestinal tract. Taxonomic studies have shown that *K. oxytoca* is a member of the *K. oxytoca* species complex (KoSC), which also includes *Klebsiella michiganensis*, *Klebsiella grimontii*, *Klebsiella huaxiensis*, *Klebsiella pasteurii*, and *Klebsiella spallanzanii* [11]. Conventional methods of identification (e.g. matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)) are often unable to identify the members of this complex at the species level. Although typically regarded as an opportunistic pathogen, there is increasing evidence of *K. oxytoca* causing nosocomial infections and outbreaks, and environmental sources include wastewater drainage systems, handwashing sinks, and contaminated detergent [11—15].

At Østfold Hospital, an unexpected rise in bacteraemia with extended-spectrum β-lactamase (ESBL; CTX-M)-producing *K. oxytoca* was identified in the late summer of 2021. In the outbreak investigation we collected retrospective and prospective data on cases with ESBL-producing *K. oxytoca*, conducted environmental screening, and performed genomic analysis of selected isolates. We hypothesized that a persistent environmental reservoir contributed to the increase in ESBL-producing *K. oxytoca*.

Methods

Study design

First, a retrospective outbreak investigation was conducted to identify risk factors for the acquisition of ESBL-producing *K. oxytoca*. In all, 21 patients were included: those with a bloodstream infection in 2021 ($N = 7$), an accessible clinical isolate (bloodstream infection or urinary tract infection) from 2019 to 2022 ($N = 12$), and a rectal carriage ($N = 2$) identified in another outbreak investigation. The corresponding hospital records were examined and the following information was collected: (i) prior admission to the Østfold Hospital within the past three months, and the ward(s) involved, (ii) prior admission to other hospitals in Norway or abroad within the past three months, and (iii) endoscopy procedures performed during the past three months. Second, aqueous environmental samples from patient rooms were collected from the ward that — based on the results from the retrospective investigations — was the likely outbreak ward, and to a lesser extent from other clinical wards. Finally, all bacterial isolates from the included patients and a selection of isolates from the environment ($N = 9$) were subjected to whole-genome sequencing (WGS). The retrospective investigation was performed towards the end of 2021, and enhanced cleaning interventions were implemented at the start of 2022. Environmental sampling was conducted during three periods of 2022.

Environmental screening

Toilet water samples were collected using a 20 mL syringe (Omnifix Luer Solo; B. Braun, Melsungen, Germany), transferred to sterile containers (Universalcontainer PS; Deltalab, Barcelona, Spain), and centrifuged precipitates were cultured on selective media (see below). Shower drain samples were collected targeting below the water trap, because of the proximity to the drainage pipes. In the outbreak ward, sink drainers and faucet in all the patient rooms were sampled. Shower drain, sink drain, and faucet samples were collected using sterile swabs (Eswab; Copan, Brescia, Italy).

Microbiological analysis

Screening samples were cultured on ESBL-selective media (CHROMagar ESBL; CHROMagar, Paris, France). MALDI-TOF (Bruker Daltonik, Bremen, Germany) was used for species identification. Antimicrobial susceptibility testing was performed using disc diffusion (Oxoid, Basingstoke, UK) according to EUCAST [16]. Phenotypic ESBL production was confirmed with clavulanate synergy using double disc synergy test (Oxoid) or combination disk test (Rosco Neo-Sensitabs, Taastrup, Denmark). Extended susceptibility testing of a selection of isolates was performed by broth microdilution using Sensititre microtitre plates (Trek Diagnostic Systems/ThermoFisher Scientific, East Grinstead, UK). Interpretation of antimicrobial susceptibility was according to EUCAST guidelines [17].

Genomic analyses

Genomic DNA was extracted using the EZ1 platform (Qiagen, Hilden, Germany). For Oxford Nanopore Technologies (ONT;

Oxford, UK) sequencing, DNA was further purified by AMPure XP beads (A63882; Beckman Coulter, Krefeld, Germany), and libraries were generated using the Rapid Barcoding kit (SQK-RBK004). Sequencing and base calling (fast mode) were performed on MinION Mk1C using FLO-MIN106 flow cells. For Illumina sequencing, 2×151 bp paired-end libraries were prepared using Nextera®XT, and sequenced on the NextSeq550 platform (Illumina, San Diego, CA, USA). Quality control of Illumina sequence and assembly data was performed using FastQC v.0.11.9 and Quast v.5.2.0 (thresholds: minimum 40× coverage; maximum 400 contigs). Species definition was confirmed using rmlst [18]. For Illumina reads, assembly was performed using Shovill v.1.1.0 (https://github.com/tseemann/shovill), and for Illumina–ONT hybrid using Unicycler v.0.4.8 in normal mode [19]. Bakta v.1.7.0 was used for annotation [20]. Sequence types (STs) were retrieved using mlst v.2.23.0 (https://github.com/tseemann/mlst) and the *K. oxytoca* database (https://pubmlst.org/) [21]. Pangenome for *K. oxytoca* were estimated using Panaroo v.1.3.2 with the sensitive mode, merging paralogs and removing invalid genes, and core genes defined using a 99% threshold for presence [22]. Maximum-likelihood phylogenies for *K. oxytoca* were separately inferred from their core genomes using RAxML v.8.2.12 with the GTR + Gamma rate model and 100 bootstraps [23]. Antimicrobial resistance genes/point mutations were identified using AMRFinderPlus v.3.11.2, database version v.2023-08-08.2 with minimum identity and coverage of 90%. Plasmid replicons were retrieved using PlasmidFinder v.2.1.6. For cluster-specific analyses, *K. oxytoca* ST323 and ST223 were separately mapped to the *K. oxytoca* reference genome ASM381292v1 (GCF_003812925.1) and *K. michiganensis* ST66 and ST376 to the *K. michiganensis* genome assembly ASM917348v1 (GCF_009173485.1). Pairwise single-nucleotide polymorphism (SNP) distances were calculated from alignments using the Nullarbor pipeline v.2.0.20191013 (https://github.com/tseemann/nullarbor). Visualizations were produced in R v.4.3.1. or EasyFig [24,25].

Statistical analysis

The proportion of ESBL-producing *K. oxytoca* was modelled by a logistic regression using location (local and national) and time (2016–2022) as covariates. An interaction term between time and location allowed for different progress over the years for the two locations. The model furthermore accounted for the intervention in 2022 at the local hospital. The national data was acquired from the Norwegian Surveillance System on Resistant Microbes (NORM) and included the local (Østfold Hospital) data within the yearly nine-month national data collection periods [26].

Results

Increasing proportion of ESBL-producing K. oxytoca

During 2021, an unusual increase in the proportion of bacteraemia cases with ESBL-producing *K. oxytoca* was observed at the Østfold Hospital in Norway (Figure 1), from 0% in 2016 to 37% in 2021. The overall number of *K. oxytoca* from blood culture remained relatively unchanged during the same period with an average of 19 cases per/year (range: 13–24). In contrast, the national proportion of ESBL among *K. oxytoca* blood culture isolates was lower and comparatively stable (0.6% in 2016 to 2.3% in 2021), indicating a local rather than a national trend. The logistic regression showed significant ($P < 0.05$) differences in the proportions of ESBL-producing *K. oxytoca* between the two sites in years 2019–2021.

Analyses of the 21 included cases with ESBL-producing *K. oxytoca* from 2019 to 2021 (Table I) showed that all had been admitted to our hospital within three months before detection. Over the preceding year, most patients also had multiple admissions across different wards. The ward with the most case-linked admissions within three months before ESBL-producing *K. oxytoca* bacteraemia was the surgical ward of urology, vascular surgery, and otorhinolaryngology (UVO ward). None of the patients had been hospitalized abroad in the 12 months preceding the identification of ESBL-producing *K. oxytoca*. Five patients had been recently admitted to another Norwegian hospital, but only two patients had stayed at the same hospital. In the three-month period before detection, five, two, and one of the included patients had undergone cystoscopy, gastroscopy, or colonoscopy, respectively. One patient had undergone endoscopic retrograde cholangiopancreatography, but at another hospital.

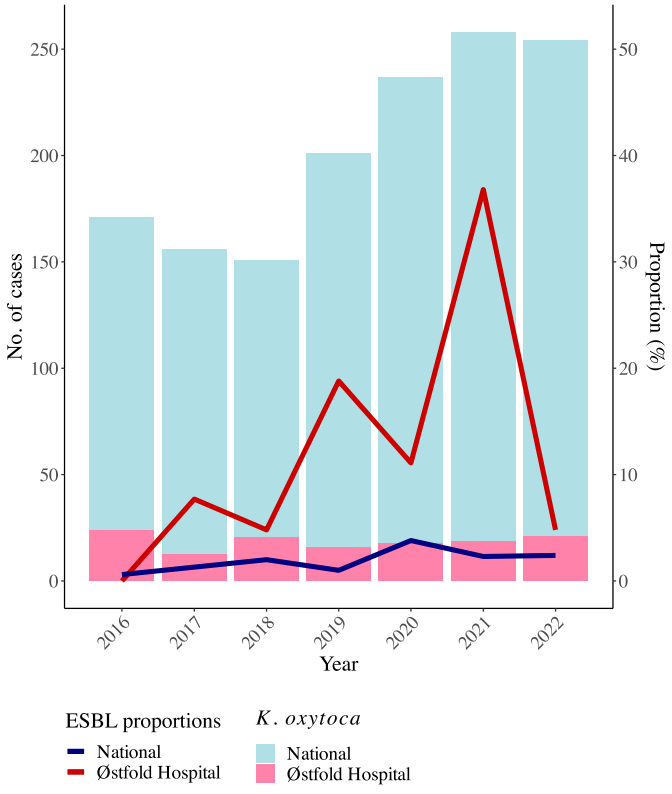

Figure 1. Number of cases of *Klebsiella oxytoca* (stacked bars) and proportion of extended-spectrum β-lactamase (ESBL)-producing *K. oxytoca* (lines) among all detected blood culture isolates of *K. oxytoca* at Østfold Hospital compared to the national level during the years 2016–2022 [26].

Table I
Relevant epidemiological and genomic characteristics of included isolates

Isolate ID	Sample material	Sample date (month/year)	*Klebsiella* species	ST	Bla_{CTX-M} variant	Bla_{CTX-M} location
Cluster 1						
kreshist0054	Blood	03/2019	*K. michiganensis*	66	$bla_{CTX-M-15}$	Plasmid, chromosome
kreshist0055	Blood	10/2019	*K. michiganensis*	66	$bla_{CTX-M-15}$	ND
kreshist0056	Toilet room A	02/2022	*K. michiganensis*	66	$bla_{CTX-M-15}$	ND
kreshist0057	Toilet water room B	06/2022	*K. michiganensis*	66	$bla_{CTX-M-15}$	ND
kreshist0058	Shower drain room C	06/2022	*K. michiganensis*	66	$bla_{CTX-M-15}$	ND
kreshist0059	Toilet water room D	06/2022	*K. michiganensis*	66	$bla_{CTX-M-15}$	ND
Cluster 2						
kreshist0063	Blood	12/2019	*K. oxytoca*	223	$bla_{CTX-M-15}$	ND
kreshist0062	Blood	02/2021	*K. oxytoca*	223	$bla_{CTX-M-15}$	Plasmid
kreshist0064	Toilet corridor	02/2022	*K. oxytoca*	223	$bla_{CTX-M-15}$	ND
Cluster 3						
kreshist0068	Blood	01/2021	*K. oxytoca*	323	$bla_{CTX-M-154}$	Plasmid, chromosome
kreshist0066	Urine	08/2021	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0065	Feces	08/2021	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0067	Urine	08/2021	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0069	Urine	11/2021	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0070	Urine	02/2022	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0071	Toilet room E	02/2022	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
kreshist0072	Toilet water room F	06/2022	*K. oxytoca*	323	$bla_{CTX-M-154}$	ND
Cluster 4						
kreshist0073	Blood	01/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	Chromosome
kreshist0076	Blood	08/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0074	Urine	09/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0075	Urine	10/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0077	Blood	11/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0078	Feces	12/2021	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0079	Toilet room A	02/2022	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
kreshist0080	Shower drain room E	06/2022	*K. michiganensis*	376	$bla_{CTX-M-15}$	ND
Non-clustered isolates						
kreshist0053	Blood	02/2020	*K. michiganensis*	52	$bla_{CTX-M-15}$	Plasmid
kreshist0081	Blood	07/2021	*K. michiganensis*	384	$bla_{CTX-M-15}$	Plasmid
kreshist0060	Urine	10/2021	*K. michiganensis*	183	$bla_{CTX-M-154}$	Chromosome
kreshist0061	Blood	11/2021	*K. oxytoca*	199	$bla_{CTX-M-15}$	Plasmid
kreshist0052	Blood	03/2022	*K. michiganensis*	11	$bla_{CTX-M-15}$	ND

ST, sequence type; ND, not determined.

Environmental screening

The strong association with admissions to a specific ward and previous *K. oxytoca* outbreak characteristics indicated a potential environmental reservoir. Consequently, during three periods in 2022 (February, June, and August), 251 aqueous environmental samples were collected from the affected ward and from adjacent and distantly located wards for comparison. Contact points were also sampled in designated rooms. The environmental screening unveiled growth of ESBL-producing *K. oxytoca* in multiple wards in a total of 36/235 (15.3%) samples (Table II).

The UVO ward was predominantly affected, with ESBL-producing *K. oxytoca* detected in 58% (15/26) of the rooms. No environmental contamination was observed in the respiratory disease and maternity wards, though the sample number was small. In the UVO ward, defined as the outbreak ward, toilet bowl water reservoirs emerged as the predominant environmental source with 81% (21/26) of samples supporting growth of

Table II
Findings of ESBL-producing *Klebsiella oxytoca* in the drainage system of different hospital wards including all aqueous environmental samples collected during 2022

Ward	ESBL-producing *K. oxytoca* positive rooms/rooms sampled (%)
Gastro surgery	4/27 (14.8%)
Urology, vascular surgery, otorhinolaryngology	15/26 (57.7%)
Nephrology, geriatrics, gastrointestinal diseases	1/9 (11.1%)
Oncology	1/26 (3.8%)
Respiratory diseases	0/9 (0%)
Children's ward	1/6 (16.7%)
Maternity ward	0/8 (0%)
Infectious diseases	1/24 (4.2%)

ESBL, extended-spectrum β-lactamase.

ESBL-producing *K. oxytoca*, in contrast to 19% (5/26) and 8% (2/26) of the shower and sink drain samples, respectively.

Interventions

Based on the results from the environmental samples, enhanced cleaning of the bathrooms was implemented in the most affected hospital wards. Disinfection was performed twice a day during most of 2022, including disinfecting the toilet water trap. Different disinfectants were applied initially, finally selecting disinfection of toilets, shower drains and sinks with Perasafe (Brage Medical AS, Drammen, Norway) and deposition of 50 mg (10 tablets) Rely+On Virkon (Lanxess, Köln, Germany) in the toilet water trap at the end of cleaning as the routine. The introduction of targeted measures specifically addressing the cleaning and disinfection of toilets and bathrooms had an abrupt effect on the occurrence of ESBL-producing *K. oxytoca* blood culture isolates (Figure 1).

Genomic and microbiological characterization

To explore the increased incidence of ESBL-producing *K. oxytoca*, short-read WGS was performed for all clinical isolates and a subset of environmental isolates ($N = 9$), 30 in total (Table I and Supplementary Table S1).

The sequencing revealed two species, *K. oxytoca* and *K. michiganensis*, each displaying two clusters: *K. oxytoca* ST323 and ST223, and *K. michiganensis* ST376 and ST66 (Table I, Figure 2). All clusters contained both clinical and environmental isolates, confirming the overlap between the human and environmental niche(s). The SNP distance within each cluster varied; ST323 (15–39 SNP), ST223 (11–64 SNP), ST376 (18–76 SNP), and ST66 (36–68 SNP). In addition, single isolates of *K. michiganensis* ST11, ST52, ST183, ST384, and *K. oxytoca* ST199 were identified, all associated with infections, but from patients who had not been admitted to the outbreak ward in the period before detection.

The isolates within both *K. michiganensis* clusters and *K. oxytoca* ST223 harboured $bla_{\text{CTX-M-15}}$. In contrast, *K. oxytoca* ST323 carried $bla_{\text{CTX-M-154}}$, a single nucleotide variant of $bla_{\text{CTX-M-15}}$. Notably, $bla_{\text{CTX-M-154}}$ was also identified in the *K. michiganensis* ST183 isolate, whereas $bla_{\text{CTX-M-15}}$ was present in the other single ST isolates. This dissemination pattern suggests the genetic sharing of one common resistance determinant but with a single mutation in the CTX-M-encoding gene. Resistance profiles, including additional resistance genes and mutations, generally followed the cluster and ST profile, albeit with some variability (Figure 2 and Supplementary Table S1). Each ST featured a distinct variant of the intrinsic OXY β-lactamase. Except for one isolate, all were classified as MDR.

The plasmid replicon profile varied both between clusters and ST, as well as within clusters. Nonetheless, the consistent presence of the IncFII(K) replicon in all isolates (Figure 2) suggested a potential role of this plasmid type in the dissemination of the ESBL-encoding genes. To explore this hypothesis and investigate the ESBL determinant's genetic surroundings, additional long-read sequencing was conducted for eight isolates representing each cluster and the individual STs.

Sequence comparisons revealed that six isolates contained IncFII(K) plasmids carrying $bla_{\text{CTX-M-15/-154}}$ (Figure 3A). The plasmids identified in *K. michiganensis* (ST52, ST66, and ST384) and *K. oxytoca* (ST323) exhibited high sequence identity (99.7–99.9%) with coverage ranging from 94% to 100%, suggesting potential plasmid transmission. Despite minor observed inversions and insertions, these plasmids shared gene content and synteny, including a second replicon repB(R1701), resistance encoding genes, and a complete transfer module, supporting their capability for conjugative transfer. In two isolates, *K. oxytoca* ST199 and ST223, $bla_{\text{CTX-M-15}}$ was located on similar IncFII(K)-IncFIB hybrid plasmids (~240 kb), sharing the set of resistance genes and a putative transfer module, but otherwise distinct from the first plasmid group.

However, all six plasmids contained $bla_{\text{CTX-M-15/-154}}$ along with additional resistance genes (*sul2*, *aph(3″)-Ib*, and $bla_{\text{TEM-1}}$) and IS elements on a common ~16 kb region (Figure 3B). This genetic element was bounded by direct repeats of IS*26*, as described for highly transferable class I transposons [27]. This element (100% coverage and 100% identity) appeared in diverse plasmids ($N = 73$) within the NCBI completed plasmid database of Klebsiella (taxid:570). None was closely related to those in our study, supporting its mobility. Among the plasmids in the current study, two variants of this $bla_{\text{CTX-M}}$ element were observed: a short truncation in *K. oxytoca* ST323 and a duplication in *K. michiganensis* ST52. This duplicated region corresponds precisely to an ~3 kb segment containing IS*Ecp1* and $bla_{\text{CTX-M-15}}$, also found as a second $bla_{\text{CTX-M-15}}$ copy in the *K. oxytoca* ST199 plasmid (Figure 3A) and as chromosomal insertions (Figure 3B).

In ST376 ($bla_{\text{CTX-M-15}}$) and ST183 ($bla_{\text{CTX-M-154}}$) the ESBL gene was located on the chromosome rather than within their IncFII(K) plasmids; for ST376 in three copies (I, II, and III). In contrast to the plasmid-encoded elements, these chromosomal insertions were delineated by IS*5075* or IS*Ecp1* together with IS*26* due to 5′-end truncations (Figure 3B). Additionally, we detected insertions of the ~3 kb IS*Ecp1*-$bla_{\text{CTX-M}}$ element into various chromosomal positions in *K. michiganensis* ST66 and *K. oxytoca* ST323, where the CTX-M-encoding element was also present within the plasmid. The finding of $bla_{\text{CTX-M}}$ elements inserted into chromosomal positions and in different genetic backgrounds indicates independent transposition events.

These findings show that the IncFII(K) replicon type plasmid facilitates the horizontal spread of the ESBL determinant across distinct species and STs. Additionally, this study highlights IS-mediated mobilization of flexible-sized CTX-M-encoding modules between and within plasmids and chromosomes.

Discussion

Contaminated toilet and water drainage systems have increasingly been recognized as reservoirs for nosocomial outbreaks with MDR bacteria [4–8]. In our outbreak investigation, we hypothesized that biofilm formation within the hospital drainage system created a diverse and persistent reservoir of bacteria that could infect patients. Phylogenetic analysis identified genetically and timely related isolates from clinical and environmental samples, supporting our hypothesis of a shared origin. The detection of highly conserved genetic elements responsible for the dissemination of the ESBL phenotype between species and strains, along with the discovery of outbreak clusters dated back to 2019, strongly indicates the presence of a persistent, mixed outbreak originating from a common reservoir.

The identified high-risk hospital ward also had the highest prevalence of positive environmental samples, including ESBL-producing *K. oxytoca* in 21 out of 26 toilets. Transmission of

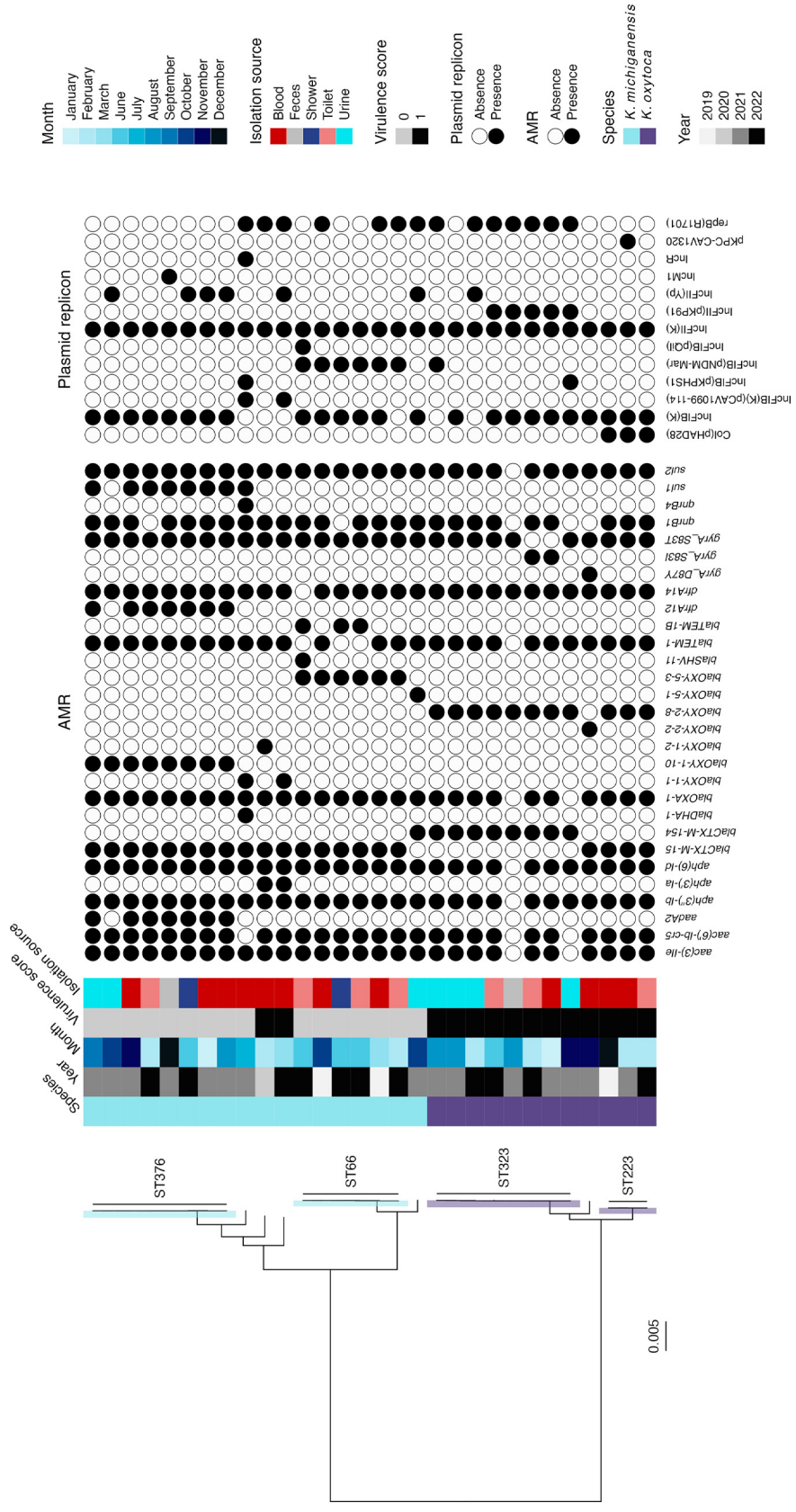

Figure 2. Maximum-likelihood phylogeny on core genome alignment, aligned with species information, temporal data, virulence scores, isolation sources, resistance profiles, and plasmid replicons of the environmental and clinical *Klebsiella michiganensis* and *Klebsiella oxytoca* isolates selected for whole-genome sequencing.

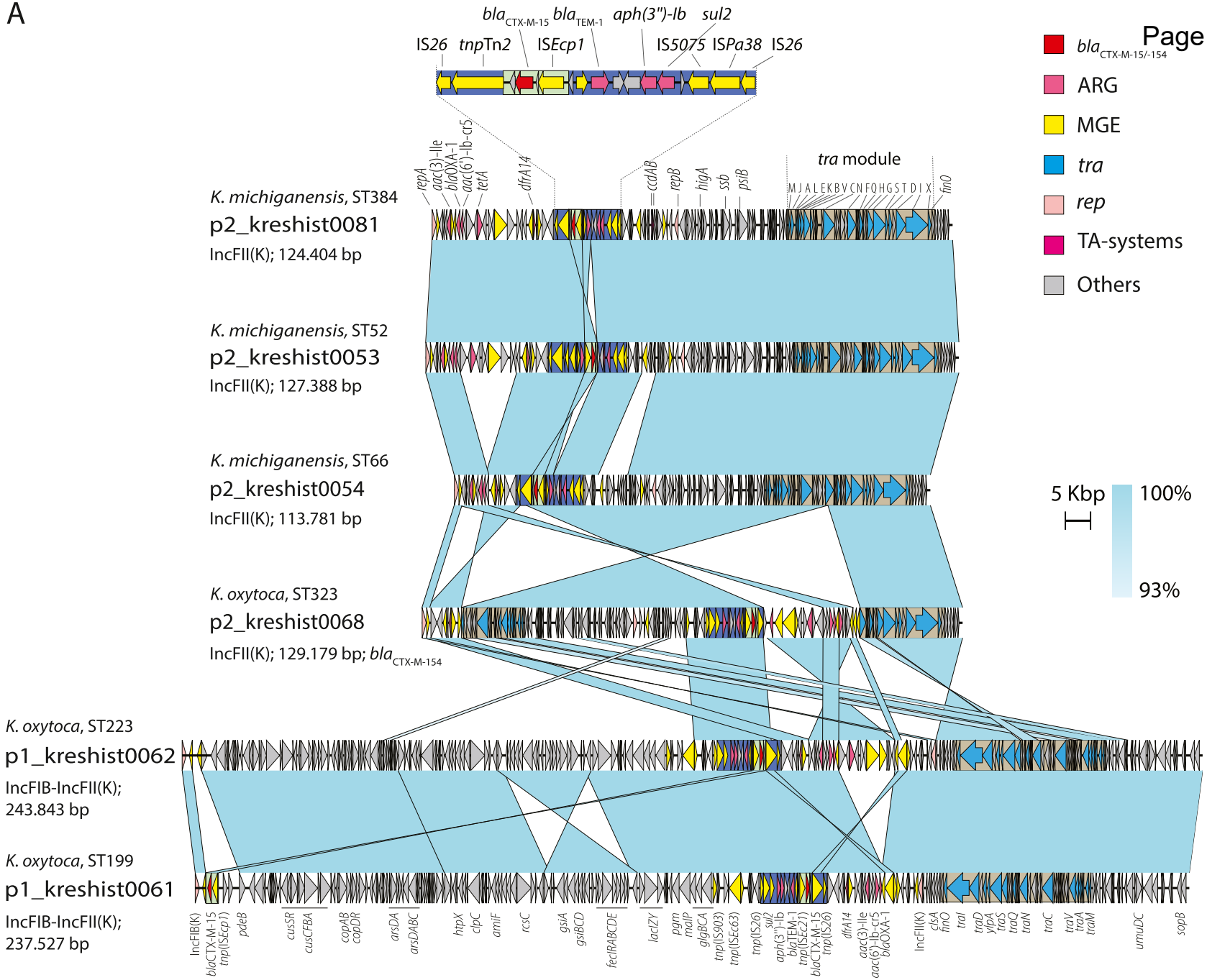

Figure 3. Genetic context of the extended-spectrum β-lactamase (ESBL)-encoding gene in *Klebsiella oxytoca* and *Klebsiella michiganensis* from the outbreak investigation. Comparisons of plasmids (p) in (A) and chromosomal (ch) regions (B) containing $bla_{\text{CTX-M-15/-154}}$ for the indicated isolates. Turquoise shading between pairs of sequences indicates identity (93–100% or 98–100%). Annotated CDS representing $bla_{\text{CTX-M-}}$ and other antibiotic resistance genes (ARG), mobile genetic elements (MGE), genes involved in conjugative transfer (*tra*), replication initiation (*rep*), toxin–antitoxin (TA) systems, and others are represented by arrows with the given colour codes. Regions included in the ~16 kb (blue) and ~3 kb (light green) $bla_{\text{CTX-M}}$ elements and the transfer module (dark grey) are boxed. The specific positions are provided for the chromosomal integrations.

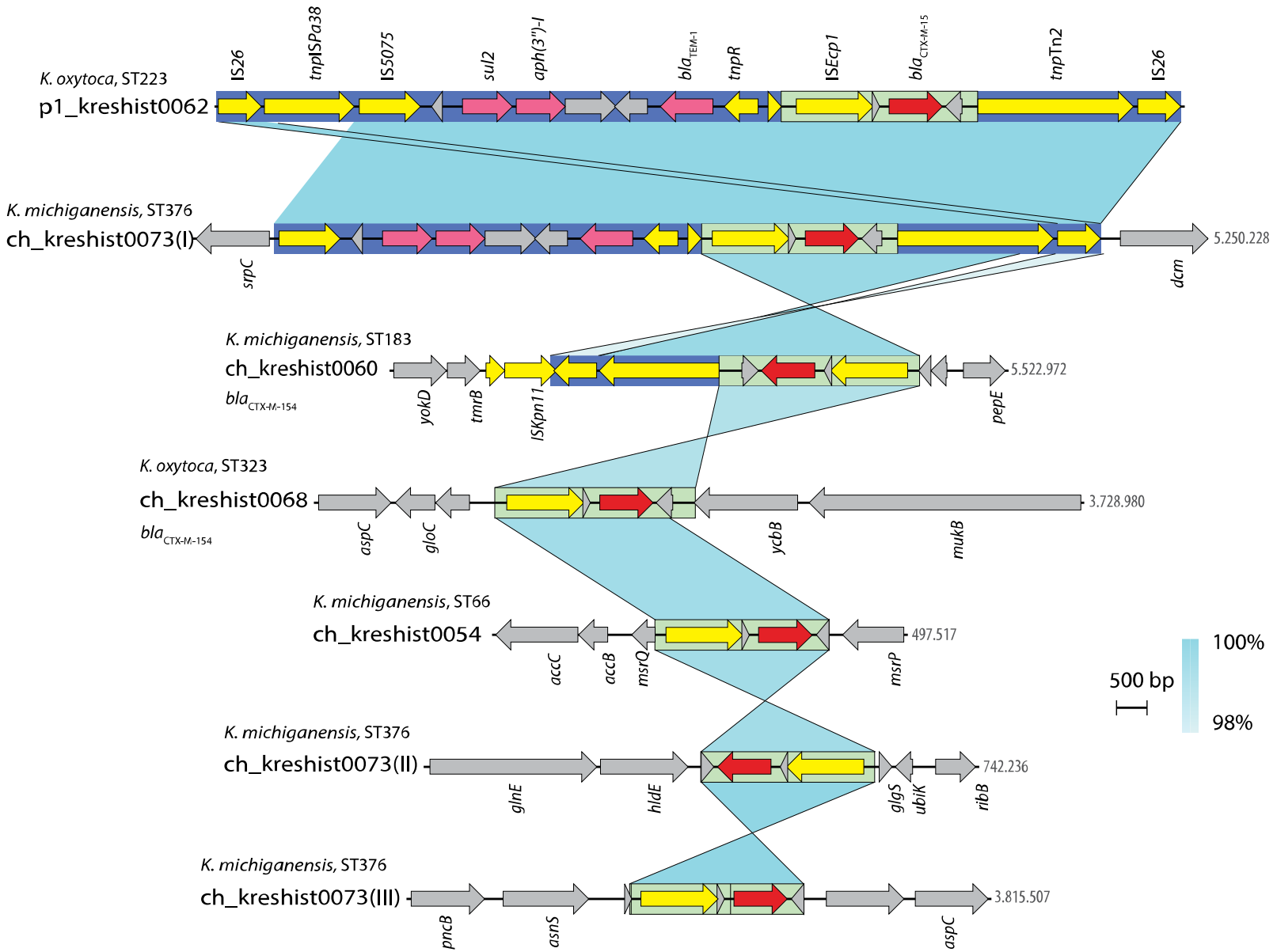

Figure 3. (*continued*).

bacteria from toilet water to patients may occur through aerosolization during flushing and surface contamination of contact points [28—31]. Toilet flushes can produce a strong, chaotic jet capable of transporting aerosols, which may contain micro-organisms from faecal waste [30,32]. Indeed, toilets have been identified as a source of hospital contamination and associated outbreaks [33—35].

In an outbreak of OXA-48-producing *K. pneumoniae*, where toilet drain water was suspected to be the source of room-to-room transmission, several drainpipe obstructions occurred leading to the retrograde flow of wastewater, and disinfection efforts were only temporarily successful [33]. The likelihood of room-to-room transmission via the drainage system is diminished in our hospital due to few horizontal drainpipes. However, factors such as high patient turnover, frequent readmissions and switching of rooms may have contributed to the spread of the outbreak strains. Nevertheless, the enhanced disinfection, targeting toilets, shower drains, and sinks led to a notable reduction in the number of bloodstream infections caused by ESBL-producing *K. oxytoca/michiganensis* in 2022 compared to 2021. This outcome further emphasizes the importance of the environment as the source for transmission.

Only a small number of studies on hospital outbreaks due to *K. oxytoca* have determined the specific species/STs involved (as reviewed by [11]), leaving the differential outbreak potential among members of the *K. oxytoca* species complex unclear. Our study identified two species within the *K. oxytoca* complex (*K. oxytoca* and *K. michiganensis*) implicated in the outbreak. It underscores the importance of accurate species identification and demonstrates that other species within the *K. oxytoca* species complex may contribute to outbreaks. Among the STs identified, *K. oxytoca* ST223 and ST323 have been identified from clinical samples in multiple countries, including the USA, Australia, Switzerland, and Denmark, indicating a global distribution of these lineages [36,37].

Long-read sequencing enabled a comprehensive characterization of the genomic architecture of the ESBL-encoding elements shared among the outbreak bacteria, providing valuable insights into transmission mechanisms. Comparative analyses of the bla_{CTX-M}-containing IncFII(K) plasmids revealed several cases of horizontal transfer within and among the two *Klebsiella* species, consistent with observations in previous outbreaks [6,9,10]. The location of bla_{CTX-M} on both plasmids and chromosomes illustrates the dynamic nature of resistance genes linked to IS elements, where involvement of IS*26* is known to mediate efficient transposition [27]. These findings underscore the complexity of outbreaks originating from environmental sources challenging outbreak investigations.

Our study has several limitations. The selection of a subset of isolates for genomic investigation posed a limitation in fully elucidating the extent of the outbreak and obtaining a comprehensive understanding of it. Moreover, we did not systematically screen patients in the affected wards, and the environmental screening was not initiated before 2022. Consequently, we lack data on asymptomatic colonization and cannot determine when the strains were initially established in the environment. Screening patients in and out of the hospital ward could have strengthened the assumption that patients are infected during admission. However, the identification of genetically closely related clinical isolates more than three years before the environmental screening underlines long-term environmental contamination.

Conclusion

This study has investigated a polyclonal hospital outbreak of MDR *K. oxytoca* species complex causing urinary tract infections and invasive disease. It delineates the dissemination of the outbreak bacteria in the wastewater system of the implicated hospital wards, investigating the relatedness between isolates from patients and the environment, along with the genetic context of the ESBL determinant. Our findings underscore the persistence of resistant micro-organisms within sewage pipes, the capability of the bacteria to migrate from the pipes to toilet bowls, and their role as an environmental source of serious nosocomial infections. Consequently, toilets should be recognized as a reservoir for nosocomial transmission of MDR bacteria, affecting hospital hygiene protocols and cleaning procedures.

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Conflict of interest statement

None declared.

Ethics approval

Ethical approval was not required after evaluation by the Regional Ethical Committee (REK South-East A, 553976). The project was approved and defined as a quality assurance project by the Data Protection Officer at Østfold Hospital Trust (22/07500-3).

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Data availability

Whole-genome sequences are available in the European Nucleotide Archive under BioProject PRJEB76256.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.infpip.2024.100430.

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Polyclonal outbreak of *Burkholderia cepacia* complex bacteraemia in haemodialysis patients

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KEYWORDS
Burkholderia cepacia complex; *Burkholderia vietnamiensis*; Haemodialysis; Outbreak

Summary We report a polyclonal outbreak of bacteraemia involving 24 patients at a haemodialysis facility in Recife (Brazil). During the outbreak period (4 June to 11 July, 2001), three *Burkholderia cepacia* complex strains were isolated from human blood and from various water samples collected at different sites in the haemodialysis unit and from dialysate fluids. Out of 14 patients with positive blood cultures, six were infected by *Burkholderia cepacia* complex bacteria: three with *Burkholderia cepacia* genomovar III, two with a first strain of *Burkholderia vietnamiensis*, and one with the *Burkholderia cepacia* genomovar III strain and a second *B. vietnamiensis* strain.

Introduction

Burkholderia cepacia complex bacteria are primarily known as serious pathogens in cystic fibrosis and chronic granulomatous disease patients, but are not a major concern in immune-competent patients.[1] However, *B. cepacia* complex species are notoriously resistant to disinfectants and antiseptic solutions, and are therefore regularly seen as hospital-acquired pathogens.[2–10] In haemodialysis clinics, where this organism successfully colonizes water supplies, filter membranes, and antiseptic solutions, *B. cepacia* complex bacteraemia is an increasing concern.[3–5]

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Materials and methods

Between 4 June and 11 July, 2001, two to three blood cultures were taken at intervals of 30 min from 24 febrile patients under treatment at a private haemodialysis clinic in Recife, Brazil. Blood cultures were carried out using the Bactec system (Becton Dickinson, Maryland, USA) as recommended by the manufacturer. Positive broth cultures were plated on tryptic soy agar supplemented with 5% of defibrinated sheep blood and onto eosin methylene blue medium. During the outbreak period a total of 60 samples of water and dialysate fluids were cultured quantitatively. Water samples included reservoir water, tap water from haemodialysis rooms, de-ionized water, (water before and after filtering) and post-osmosis water. At this haemodialysis unit, water treatment and bacterial colony counting are done in accordance with the standards of the Association for the Advancement

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of Medical Instrumentation.[11] Briefly, municipal water passes through one sand filter, two softeners, two activated charcoal filters, and a set of reverse-osmosis membranes. Bacteriological control of water, collected from different places in the unit, is carried out monthly. Dialysers are reprocessed after use with 2% formaldehyde or 1% peracetic acid solutions. For bacterial counting, plates containing sheep blood agar, eosin methylene blue medium, and *B. cepacia* selective agent (BCSA)[12] (included in the routine analyses after the first recognition of *B. cepacia* complex in blood cultures), were inoculated with 1 mL undiluted and 1:10 diluted samples, dried at 37°C, and incubated at 35°C for 48 h. BCSA plates were maintained for additional 48 h at room temperature. Cultures were identified using standard procedures[13] and those resembling *B. cepacia* complex were submitted to a panel of conventional phenotypic tests,[14] whole-cell protein electrophoresis,[15] *recA* restriction fragment length polymorphism (RFLP) analysis,[16] and pulsed-field gel electrophoresis (PFGE) of SpeI digested genomic DNA.[17]

Results

Blood cultures of 14 patients were positive. Of these, six were identified as *B. cepacia* complex, four as *Staphylococcus aureus*, and one each as *Streptococcus agalactiae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii*. In 10 out of 24 patients with febrile reactions blood cultures were negative suggesting the involvement of endotoxin, other bacterial products, or undetected organisms in these patients. Although the colonies of *B. cepacia* genomovar III were indistinguishable from those of *Burkholderia vietnamiensis* on BCSA plates, the β-haemolytic *B. vietnamiensis* strains were easily recognized on sheep blood agar. This β-haemolytic capacity of the *B. vietnamiensis* strain linked to this outbreak permitted the recognition of a case of double infection in one of the bacteraemic patients. All patients, including those without positive blood cultures were successfully treated with 1 g ceftazidime intravenously every 12 h for seven days. There were no relapses. The outbreak stopped after rigorous cleaning of water reservoir and ducts, and replacing filters and membranes of the haemodialysis system.

During the outbreak, seven *B. cepacia* complex isolates were recovered from blood samples of six patients and 37 *B. cepacia* complex isolates were recovered from fluids analysed, mainly post-osmosis water and dialysates. Altogether these *B. cepacia* complex isolates represented three different whole-cell protein profiles, referred to as profile a, b, and c (Figure 1(a)). Isolates characterized by whole-cell protein profile a were identified as *B. cepacia* complex but further genomovar identification was equivocal. Subsequent *recA* RFLP analysis identified these isolates as *B. cepacia* genomovar III RFLP type H (data not shown).[16] Isolates characterized by whole-cell protein profiles b and c were identified as *B. vietnamiensis*. This was confirmed by *recA* RFLP analysis which demonstrated that these isolates represented RFLP types B and A, respectively[16] (data not shown).

All *B. cepacia* complex isolates were biochemically similar. They were all delayed oxidase positive, grew at 42°C, oxidized lactose and saccharose, produced β-galactosidase and decarboxylated lysine (within 48 h). They did not oxidize adonitol, hydrolyze esculin or gelatin, or decarboxilize ornithine. However, *B. vietnamiensis* caused

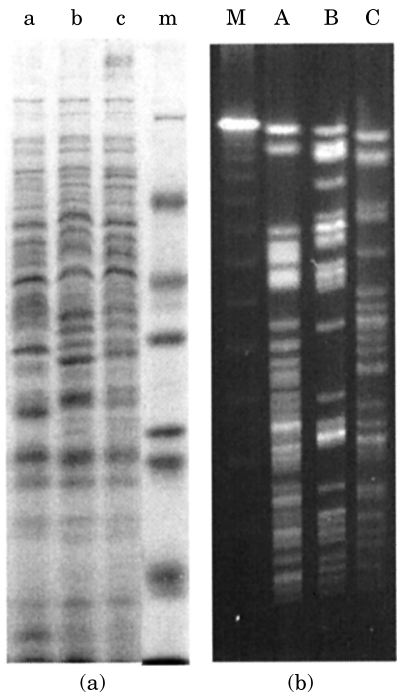

Figure 1 Whole-cell protein (1a) and pulsed-field gel electrophoresis patterns (1b) of *B. cepacia* complex bacteria. Profiles a and A correspond to the *B. cepacia* genomovar III clone; profiles b, B, c and C correspond to the two *B. vietnamiensis* clones as described in the text. Molecular weight markers are in lane m. The molecular weight markers used were (from bottom to top) lysozyme (molecular weight, 14 500), trypsin inhibitor (20 100), trypsinogen (24 000), carbonic anhydrase (29 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), egg albumin (45 000), bovine albumin (66 000) and β-galactosidase (116 000). Lane M is a 48.5-1000 kb lambda concatemer ladder.

haemolysis on sheep blood agar while *B. cepacia* genomovar III did not.

Of the human isolates, four belonged to *B. cepacia* genomovar III, two belonged to *B. vietnamiensis recA* RFLP type B (protein profile b), and one belonged to *B. vietnamiensis recA* RFLP type A (protein profile c). The latter isolate and one of the *B. cepacia* genomovar III isolates were obtained from blood cultures of a single patient. All seven human isolates and seven water isolates (three isolates representing the protein profile types a and b each, and one isolate representing the protein profile type c) were subjected to PFGE of SpeI-digested genomic DNA. All isolates with identical whole-cell protein profiles generated identical PFGE macrorestriction profiles. The macrorestriction profiles of strains with different protein profiles were clearly distinct (Figure 1(b)) and were different from that of a non-outbreak *B. vietnamiensis* strain, isolated from a blood culture of a patient at the same haemodialysis unit six months before onset of the outbreak (data not shown). The latter isolate had a protein profile that differed from that of the other *B. vietnamiensis* isolates and belonged to *recA* RFLP type B (data not shown).

Discussion

B. cepacia complex bacteria are widely distributed in nature and can produce opportunistic infections in several groups of individuals, especially those compromised by underlying diseases including cystic fibrosis and chronic granulomatous disease.[1,18] Numerous cases of nosocomial infections and pseudo-epidemics of bacteraemia have been reported.[2,4,6–10] In Thailand, Kaitwatcharachai *et al.*[5] reported '*B. cepacia*' (the genomovar status was not specified) bacteraemia among chronic renal patients who were using subclavian catheters for haemodialysis. Blood samples and a diluted chlorhexidine–centrimide solution contained isolates with identical genotypes and '*B. cepacia*' grew as biofilms on the inner wall of the catheters. Camargo *et al.*[3] reported another outbreak of '*B. cepacia*' bacteraemia among haemodialysis patients in São Paulo (Brazil). The patients had arterial–venous fistulas for blood vessel access and contaminated water was assumed to be the source of infection. In the present study, the outbreak was associated with three different strains of *B. cepacia* complex species, and all three strains were detected in water samples from the haemodialysis equipment or rooms. As in the São Paulo situation, patients had arterial–venous fistulas and the source of infection was contaminated water. Two samples of post-osmosis water received from the haemodialysis unit during the outbreak period had a bacterial load above 8000 cfu/mL. Conversely, most samples collected before the passage of the water through the reverse-osmosis membranes yielded less than 100 cfu/mL and only a few specimens showed counts above 200 cfu/mL. These findings suggested a probable bacterial colonization of reverse-osmosis membranes. In most water samples the high bacterial counts were due to the presence of *B. cepacia* complex organisms. However, *P. aeruginosa* and *A. baumanii* which were isolated from blood cultures as well, were also present in water samples. Interestingly, *Achromobacter* species, though present in most water samples examined (sometimes in high numbers), were not recovered from patients. During haemodialysis sessions bacteria could move across contaminated dialysate compartments directly reaching the patient's bloodstream.[3]

Measures to reduce costs in dialysis units may include re-use of dialysis filters[19–20] or multiple use of single-use drug vials.[21] Due to such practices a range of environmental bacteria (often Gram-negative non-fermenters) without known virulence factors become the main source of infection at haemodialysis clinics.[3] The high bacterial numbers detected mainly in post-osmosis water and dialysates suggest that, in the present study, bacterial colonization of the reverse osmosis membranes was the most plausible cause of the outbreak, and not dialyser reuse.[3] This was corroborated by the strong decrease in bacterial counts in post-osmosis water samples when the reverse-osmosis membranes were replaced.

A salient characteristic of the present outbreak was the involvement of three different *B. cepacia* complex strains: one *B. cepacia* genomovar III and two *B. vietnamiensis*. The outbreak of septicaemia on a cardiology ward reported by Van Laer *et al.*[10] involved two types of '*B. cepacia*' that were both, in retrospect, identified as *B. stabilis* (P. Vandamme, unpublished data). In addition, an outbreak of subclinical mastitis in dairy sheep in Spain and a double infection in a cystic fibrosis child in Recife were caused by both *B. cepacia* genomovar III and *B. vietnamiensis*.[22,23] *B. cepacia* complex bacteria are environmental organisms that thrive in nutrient-poor environments and are highly resistant to antiseptics. They present an obvious risk to vulnerable patient groups such as haemodialysis patients. The polyclonal nature of such outbreaks might be an under-reported phenomenon due to the variable colonial morphology of *B. cepacia* complex bacteria.

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Major article

Consecutive *Serratia marcescens* multiclone outbreaks in a neonatal intensive care unit

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Key Words:
Bacteremia
Carriage
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Total parenteral nutrition
Infection control

Background: This report describes 3 consecutive outbreaks caused by genetically unrelated *Serratia marcescens* clones that occurred in a neonatal intensive care unit (NICU) over a 35-month period.
Methods: Carriage testing in neonates and health care workers and environmental investigation were performed. An unmatched case-control study was conducted to identify risk factors for *S marcescens* isolation.
Results: During the 35-month period, there were 57 neonates with *S marcescens* isolation in the NICU, including 37 carriers and 20 infected neonates. The prevalence rate of *S marcescens* isolation was 12.3% in outbreak 1, 47.4% in outbreak 2, and 42% in outbreak 3. Nine of the 20 infected neonates died (45% case fatality rate). A total of 10 pulsed field gel electrophoresis types were introduced in the NICU in various times; 4 of these types accounted for the 9 fatal cases. During outbreak 3, a type VIII *S marcescens* strain, the prevalent clinical clone during this period, was detected in the milk kitchen sink drain. Multiple logistic regression revealed that the only statistically significant factor for *S marcencens* isolation was the administration of total parenteral nutrition.
Conclusions: Total parenteral nutrition solution might constitute a possible route for the introduction of microorganisms in the NICU. Gaps in infection control should be identified and strict measures implemented to ensure patient safety.

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] The microorganism is able to survive in a moist nosocomial environment and colonize the gastrointestinal tract of neonates and the hands of health care workers (HCWs).[2,3] Outbreaks in NICUs of up to 16 months duration,[4] a 55% attack rate,[5] and up to 120 cases[6] have been described, and the sources of infection have been traced to HCWs,[6] contaminated milk,[5] medical equipment,[6] soaps,[7] and disruption of infection control measures,[6] whereas no point source was identified in others.[4,8-10] We describe 3 consecutive outbreaks caused by genetically unrelated *S marcescens* clones that occurred in a NICU of a tertiary care hospital over a 35-month period and the investigations and interventions that ensued.

METHODS

Setting

The outbreaks occurred between December 2007 and September 2010 in the NICU of Alexandra General Hospital, a 463-bed gynecology and maternity hospital in Athens. The hospital also serves as a reference hospital for southern Greece, receiving

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approximately 1,700 admissions annually. Approximately 300 neonates are admitted to the NICU annually, including 100 weighing <1,500 g. The NICU consists of 57 cots distributed in 4 rooms, including 1 room with 11 cots for critical care (level 3), 1 room with 11 cots for intermediate care (level 2), and 2 rooms with 35 cots for standard care. The distance between cots is up to 50 cm. Access to rooms is accomplished through a common corridor, with no direct connection between them. There are 2 non–hand-operated sinks per room. Hand hygiene is performed using a 70% alcohol-based disinfecting solution provided next to each cot. An isolation room is not available. A total of 51 HCWs work in 3 shifts in the NICU, including 16 neonatologists, 30 midwives, 1 nurse, 2 nursing assistants, and 2 cleaners. HCWs work in all rooms. Milk formulas and total parenteral nutrition (TPN) solutions are prepared in a specifically dedicated kitchen outside the NICU but within the same building. TPN solutions are prepared exclusively by 2 nursing assistants. First-line empiric treatment for infants with suspected sepsis consists of imipenem plus gentamicin effective for *S marcescens* bacteremia.

Temporal evolution of the outbreaks

Outbreaks of *S marcescens* bacteremias occurred in the NICU between December 2007 and August 2008 (outbreak 1), involving 5 neonates, and between September 2009 and February 2010 (outbreak 2), involving 10 neonates. Both outbreaks were investigated by the local infection control team, and infection control measures were applied. On July 8, 2010, when an additional cluster of 4 cases of *S marcescens* bacteremia was identified (outbreak 3), the Hellenic Center for Disease Control and Prevention in Athens was notified, and a complete microbiological and epidemiologic investigation was initiated. At that time, 56 neonates were hospitalized in the NICU, including 11 neonates in critical care and 10 in intermediate care.

Microbiological investigation

Carriage testing

The NICU performs carriage testing (pharyngeal and rectal swabs, but not endotracheal swabs) from neonates when a cluster of 2 or more cases of infection is detected. After the onset of outbreak 3, pharyngeal and rectal swabs were also collected from all HCWs in the NICU.

Environmental investigation

Extensive environmental investigation was conducted during the course of all 3 outbreaks. In particular, multiple samples were collected, including samples from milk formulas, milk powder, sterilized milk bottles, milk bottle brushes, TPN solutions, sterilizers, surfaces and floors, sink tabs, sink drains, cleaning sponges, water samples, hand gel alcohol antiseptic, alcohol antiseptic pump, soap, incubators (internal and external surfaces), suction tubes, oxygen masks, oxygen tubes, water collector champers, refrigerators, tables, laryngoscopes, and ventilation system.

Microbiological methods

The blood samples were inoculated in BACTEC Peds Plus medium and incubated in the automated BACTEC 9240 blood culturing system (BD, Franklin Lakes, NJ). Identification of the clinical isolates to the species level was performed by standard laboratory tests using API 20E (BioMerieux, Lyon, Marcy-l'Etoile, France) and automated MicroScan system (Siemens Healthcare diagnostics Inc. Microscan Systems, Renton, WA). Antibiotic susceptibility testing was performed by the disk diffusion technique, and the MICs were determined using the MicroScan system and Etest strips (AB Biodisk, Solna, Sweden) according to Clinical and Laboratory Standards Institute guidelines.[11] Environmental samples were processed as clinical samples (the swabs) on routine media or with standard (International Standards Organization) protocols in use from the Central Public Health Laboratory of the Hellenic Center for Disease Control and Prevention for solid and liquid materials. Molecular typing of all available isolates was performed by pulsed-field gel electrophoresis (PFGE) as described previously.[12] The pattern of restriction fragment bands was interpreted visually and using GelCompar II software (Applied Maths, Austin, TX), which resulted in the construction of a dendrogram. Cluster analysis using the Dice coefficient gave cutoff values of 83% similarity for related isolates.

Epidemiologic investigation

An unmatched case-control study among neonates hospitalized in the NICU from May 15 through July 15, 2010 was conducted to identify risk factors associated with the onset of *S marcescens* carriage or infection. The medical records of all neonates hospitalized during this period were reviewed, and demographic, clinical, and microbiologic data were collected using a single standardized form. Cases were defined as neonates with infection or colonization with *S marcescens*. Only the first positive culture was used to define a case. Colonization was defined as a positive rectal and/or pharyngeal swab culture for *S marcescens* in the absence of clinical signs and/or symptoms of infection. *S marcescens* infection was defined as the presence of signs and/or symptoms compatible with infection and *S marcescens* isolated from a normally sterile site. Controls were defined as neonates hospitalized in the NICU between May 15 and July 15 2010, in whom *S marcescens* was not isolated in any clinical specimen. In addition, the clinical and microbiologic records of all neonates with a positive *S marcescens* culture hospitalized in the NICU between December 2007 and September 2010 were reviewed, and data were collected.

Statistical analysis

Statistical analysis was performed using SPSS version 13 (SPSS Inc, Chicago, IL). Categorical variables were compared using the χ^2 test, and continuous variables were compared using the Student *t* test. Multiple logistic regression analysis (forward selection) was applied to identify factors significantly associated with the onset of *S marcescens* carriage or infection. A *P* value $\leq$.05 was considered statistically significant.

RESULTS

Description of outbreaks

Figure 1 shows the temporal distribution of *S marcescens* clinical isolates (infection and colonization) detected in the NICU between December 2007 and September 2010 clustered in 3 well-defined outbreaks each lasting between 5 and 9 months. The 9 neonates with bacteremia out of the 20 neonates with *S marcescens* invasive infection died; thus, the overall *S marcescens*–associated case fatality rate was 45%. All 20 neonates had late-onset invasive infection, at a mean age at onset of 21 days (range, 1-75 days).

Microbiological investigation

Carriage testing

A total of 37 neonates had at least one positive rectal or pharyngeal culture for *S marcescens*; all were born in the hospital.

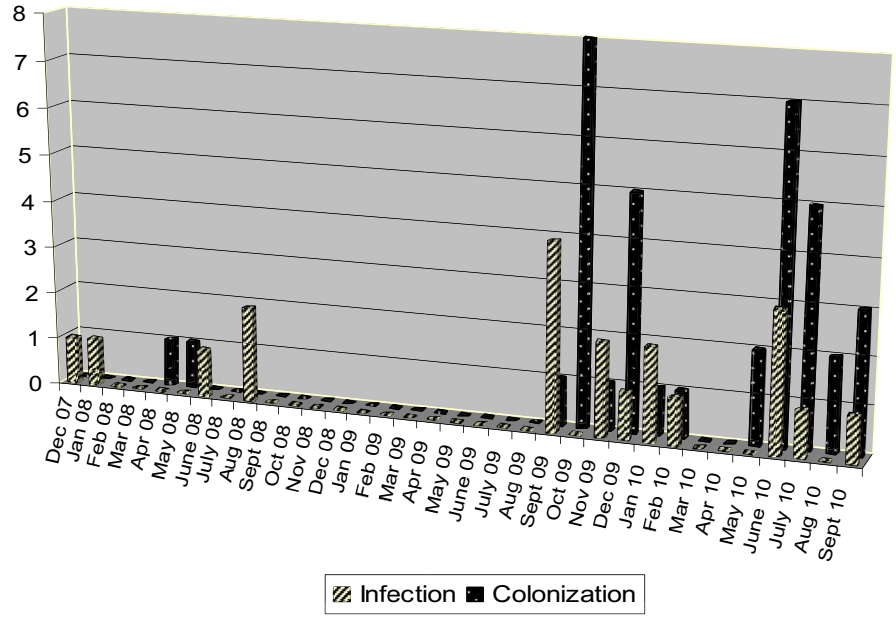

Fig 1. Temporal distribution of *S marcescens* clinical isolates.

In addition, 1 HCW was found to be colonized, with the remaining 50 HCWs testing negative. The colonized HCW was a pediatric resident who subsequently rotated to another hospital. The prevalence rate of *S marcescens* isolation (carriage and infection) was 12.3% in outbreak 1, 47.4% in outbreak 2, and 42% in outbreak 3. No mother was infected with *S marcescens*, thus excluding the possibility of vertical transmission as opposed to horizontal transmission.

Environmental investigation

An environmental investigation during outbreak 1 found 6 *S marcescens* isolates from sink drains. No environmental isolates were detected during outbreak 2. Three environmental isolates were found during outbreak 3 from sink drains in standard care rooms and the milk kitchen. All other samples collected from nursing assistants, materials, objects, and surfaces implicated in the preparation of TPN solutions and milk formulas in the milk kitchen tested negative.

Molecular typing

All *S marcescens* isolates from the 3 outbreaks were found to be sensitive, revealing only the intrinsic resistance of the genus. PFGE allocated 57 clinical and 9 environmental isolates into 10 PFGE types, of which 3 types were found to prevail during the 3 outbreaks. In outbreak 1, all 7 *S marcescens* isolates from infections and colonizations were genetically indistinguishable, belonging to PFGE type I, whereas 5 of the 6 environmental isolates were allocated into PFGE type II, not found in clinical isolates. Interestingly, 1 environmental isolate detected in a sink drain from standard care room belonged to type I, most prevalent in clinical samples.

In outbreak 2, 2 PFGE types prevailed: type III in the first part of the outbreak, isolated from 14 patients, and type VII in the last part of the outbreak, isolated from 10 patients. Three other PFGE types (IV, V, and VI) were recovered from 1 patient each.

In outbreak 3, a new PFGE type VIII was the prevalent clone, isolated from 21 cases. Two additional PFGE types, IX and X, were detected in 1 neonate and 1 HCW, respectively. Interestingly, type VII (from the previous outbreak) was also present in 1 case in this outbreak, 2 months after the last type VII carrier had been discharged. The environmental isolate from the milk kitchen sink drain was allocated to PFGE type VIII, the prevalent clinical clone during this period, whereas the other 2 environmental strains isolated from sink drains of both standard care rooms were found to belong to type II, unrelated to clinical samples. Figure 2 shows the number of *S marcescens* infected or colonized individuals (neonates and 1 HCW) in time relation to the different PFGE types.

Overall, PFGE types I, III, VII, and VIII accounted for all invasive infections and the vast majority of colonizations (Fig 2). PFGE type IV was detected in 1 case of sporadic bacteremia, and types V, VI, IX, and X were detected once each in carriage cultures exclusively. PFGE type X from the HCW was not found in any neonate; PFGE type II was detected only in environmental samples. Finally, PFGE types I, III, IV, and VIII accounted for the 9 fatal cases (3, 3, 1, and 2 cases, respectively).

Case-control study

A total of 94 neonates were included in the case-control study, including 17 cases (18.1%; 13 with isolated carriage and 4 with infection) and 77 controls (81.9%). Table 1 shows the characteristics significantly associated with the development of *S marcencens* carriage or clinical illness. No association between a positive *S marcescens* culture and any of the following factors was demonstrated: sex, birth weight, gestational age, cesarean delivery, underlying diseases, peripheral venous catheter use, respirator use, incubator use, room where care was provided, previous use of antibiotics, total number of antibiotics used, and total duration of antibiotic therapy. Multiple logistic regression analysis revealed that the only factor statistically significantly associated with the development of *S marcencens* carriage or infection (Nagelkerke $R^2 = 0.474$) was the administration of TPN solution (odds ratio, 49,871.7).

Hygiene inspection

Direct observation of HCWs involved in the preparation and handling of milk formulas and TPN solutions revealed gaps in hand

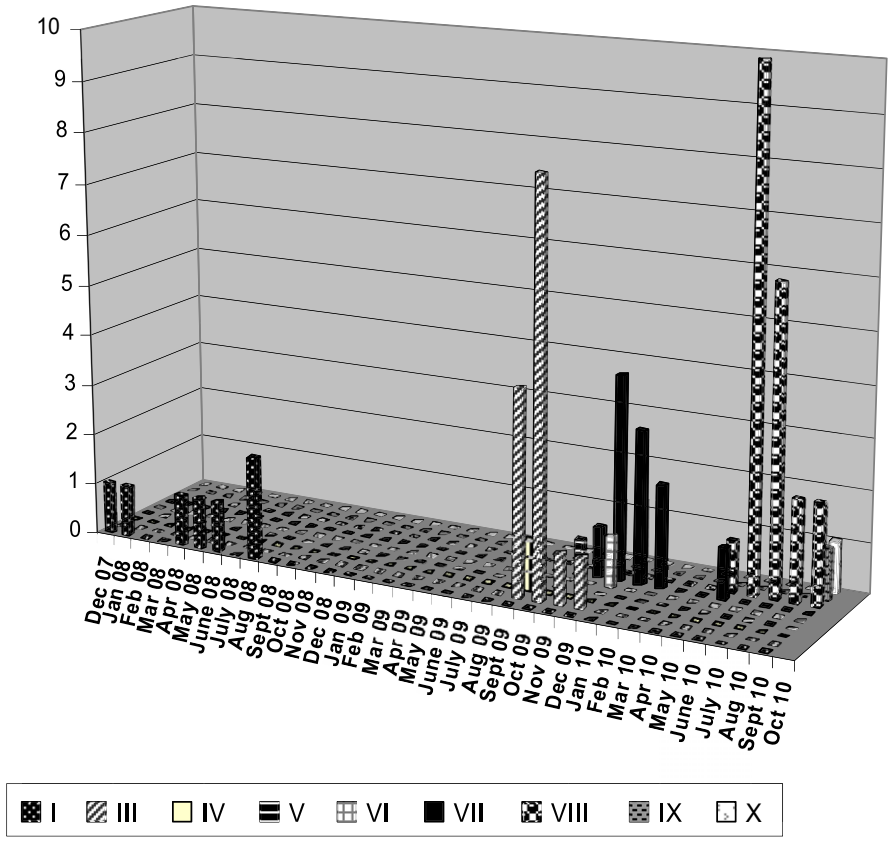

Fig 2. Number of *S marcescens* infected or colonized neonates in time relation to different PFGE types.

hygiene and environmental cleaning practices. In particular, there was no strict spatial separation between the clean area for TPN and milk preparation and storage and the dirty area used for bottle cleaning and sterilization, indicating a possible route for spread of *S marcescens*. Overall, hand hygiene was not practiced consistently among HCWs in the NICU, including deficient use of alcohol-based antiseptics and gloves.

Interventions

During outbreak 1, colonized or infected neonates were cohorted within the critical care or the intermediate care room, depending on their nursing needs. During outbreaks 2 and 3, one room was used solely for cohorting neonates with *S marcescens* carriage or infection, whereas trained personnel from other departments were allocated in the NICU. Given the rapid spread of *S marcescens* among neonates, the NICU was closed for admissions from other hospitals for 4-8 weeks during the evolution of all 3 outbreaks, but remained open for neonates born within the hospital. In addition, the following measures were implemented during outbreak 3. Decontamination with 5% hydrogen peroxide fumigation was applied in the critical care room, where most colonized or infected neonates were confined. Clean areas dedicated to the preparation and storage of TPN solutions and milk formulas were strictly separated from the dirty area used for bottle cleaning. All sink drains in the NICU, including the kitchen, were replaced. A 10% hypochlorite solution was poured down the sink drains twice per day and left there for at least 15 minutes; repeat samples were negative. Strict infection control practices were enforced, and educational sessions were provided on hand hygiene, cleaning of bottles and environmental surfaces, sterilization practices, and proper preparation, handling, and storage of TPN solutions and milk. Written algorithms and guidelines were posted onsite. One month later, inspection of hygiene practices revealed high rates of compliance with guidelines regarding implementation of infection control measures in the NICU.

DISCUSSION

We describe 3 consecutive, rapidly spreading, prolonged outbreaks in a NICU during a 3-year period each caused by a genetically distinct *S marcescens* clone, consistent with the repeated introduction and spread of new clones in the NICU. *S marcescens* (like *Klebsiella pneumoniae*) may cause carriage and survive on hands more efficiently compared wirh *Escherichia coli* and *Pseudomonas aeruginosa*, and thus can transfer readily from patient to patient through the hands of HCWs.[13] To the best of our knowledge, consecutive outbreaks caused by genetically distinct *S marcescens* clones have been reported only rarely to date.[5] In the present study, the fact that no *S marcescens* PFGE type was found to persist during the entire period under investigation indicates good decontamination and disinfection policies in the NICU. Nonetheless, the entrance and explosive spread of new clones in the NICU together with the observed high proportion of colonized or infected neonates with *S marcescens* in the NICU (up to 50%), highlights the gaps in existing infection control practices. It is possible that the prevalence rates of *S marcescens* infections would be higher were respiratory samples collected for carriage testing in addition to pharyngeal and rectal swabs.[14] The 45% case fatality rate in the present series is also noteworthy. Case fatality rates ranging from 14% to as high as 100% among neonates with *S marcescens* bacteremia have been reported,[5,15-17] possibly attributed to the

Table 1
Statistically significant characteristics for *S marcescens* carriage or clinical illness in 94 neonates, univariate statistical analysis

Characteristic	*S marcescens* isolated*			
	No (n = 77), n (%)	Yes (n = 17), n (%)	Total (n = 94), n (%)	*P* value
Prematurity				
No	27 (96.4)	1 (3.6)	28 (29.8)	.017
Yes	50 (75.8)	16 (24.2)	66 (70.2)	
Apgar score at 1 minute				
≤5	4 (66.7)	2 (33.3)	6 (6.4)	.026
>5	37 (74.0)	13 (26.0)	50 (53.2)	
Unknown	36 (94.7)	2 (5.3)	38 (40.4)	
Apgar score at 5 minutes				
≤5	1 (100.0)	0 (0.0)	1 (1.1)	.018
>5	39 (72.2)	15 (27.8)	54 (57.4)	
Unknown	37 (94.9)	2 (5.1)	39 (41.5)	
Central venous catheter				
No	77 (82.8)	16 (17.2)	93 (98.9)	.032
Yes	0 (0.0)	1 (100.0)	1 (1.1)	
Levine tube				
No	46 (100.0)	0 (0.0)	46 (48.9)	<.001
Yes	31 (64.6)	17 (35.4)	48 (51.1)	
Umbilical catheter				
No	76 (83.5)	15 (16.5)	91 (96.8)	.026
Yes	1 (33.3)	2 (66.7)	3 (3.2)	
TPN				
No	52 (100.0)	0 (0.0)	52 (55.3)	<.001
Yes	25 (59.5)	17 (40.5)	42 (44.7)	
Powdered infant formula				
No	74 (84.1)	14 (15.9)	88 (93.6)	.036
Yes	3 (50.0)	3 (50.0)	6 (6.4)	
Critical care room				
No	28 (96.6)	1 (3.4)	29 (30.9)	.014
Yes	49 (75.4)	16 (24.6)	65 (69.1)	

*Carriage or clinical illness.

invasiveness of specific epidemic strains, as well as to the several coexisting risk factors associated with high mortality in our patients.

Identified risk factors for *S marcescens* infections in neonates include low birth weight, prematurity, prolonged intubation, prolonged administration of antibiotics, and use of a central venous catheter and arterial line.[6,8,17-19] In the present study, although we looked for these factors, TPN administration emerged as the sole statistically significant risk factor for *S marcescens* carriage or infection. This finding identifies a likely route for the introduction of the microorganism in the NICU, given the fact that the responsible *S marcescens* clinical clone VIII was detected in the kitchen sink drain where TPN solutions were prepared. However, no such strain was isolated from TPN solution samples or from the flora of the nursing assistants who prepared the solution. Nosocomial infections and outbreaks in NICUs have been linked to TPN solutions[20-22]; however, to the best of our knowledge, to date only one reported outbreak has been associated with the use of *S marcescens*—contaminated TPN solutions in a NICU.[16] Although the possibility of reciprocal introduction of *S marcescens* from the NICU to the kitchen via TPN solutions and milk bottles used in the NICU, with subsequent contamination of the kitchen sink drain, cannot be excluded,[1] hygiene gaps in the kitchen (mainly in separation of the clean area from the dirty area) appeared to play a crucial role in the evolution of at least the last outbreak. Similar to our results, in a study investigating 3 consecutive *S marcscens* NICU outbreaks caused by genetically unrelated strains,[5] the authors concluded that although strain C was detected in milk during the third outbreak, milk might have played an important role in the propagation of the microorganism in the first outbreak as well, given the fact that strain A was identified in the sink of the milk kitchen.[5] Nevertheless, in the present study extensive microbiological investigations were undertaken during all 3 outbreaks, and isolates with identical PFGE patterns to clinical isolates were detected only in sink drains. Overall, overcrowding and understaffing were recorded within the NICU, which, in association with low compliance with hand hygiene and appropriate glove use, most likely provided the conditions conducive to propagation of the outbreaks, with neonates serving as reservoirs of *S marcescens* in each outbreak. *S marcescens* has been shown to survive on HCWs' hands for up to 3 months,[2,3] emphasizing the significance of appropriate glove use.

We faced difficulties in containing the 3 outbreaks, and closure of the NICU was inevitable to prevent further transmission of the epidemic clones. *S marcescens* outbreaks tend to be prolonged, an environmental source almost always remains unidentified, closure of NICUs is frequent, and estimated costs are high.[1] Containment of such outbreaks requires a multi-interventional approach to quickly identify gaps in infection control practices and implement strict measures to ensure patient safety. Moreover, the value of molecular typing in elucidating the epidemiology of these outbreaks must be emphasized.

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Serratia marcescens outbreak in a neonatal intensive care unit and the potential of whole-genome sequencing

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SUMMARY

Background: *Serratia marcescens* is notorious for its increasing antimicrobial resistance and potential to cause outbreaks in neonatal intensive care units (NICUs). A promising tool in outbreak investigations is whole-genome sequencing (WGS).

Objectives: To describe a *S. marcescens* outbreak (2018–2019) in an NICU and discuss which infection control measures contributed to containment, addressing the potential of WGS.

Methods: *S. marcescens* isolates from patients and the environment isolated during the 2018–2019 NICU outbreak were analysed. In comparison, isolates from previous presumed NICU outbreaks and adult blood cultures were included. WGS and whole-genome multi-locus sequence typing analysis were performed.

Results: Sixty-three *S. marcescens* isolates were analysed. The 2018–2019 outbreak was divided into three clusters, including four environmental strains (drains, *N*=3; baby scale, *N*=1). The strains differed significantly from those of an NICU outbreak in 2014 and adult blood cultures. Besides standard infection control measures, the siphons were replaced and weekly decontamination was performed with acetic acid 10%. Seven acquired-resistance genes and 29 virulence-associated genes were detected.

Conclusions: It was assumed that both neonates and drains were reservoirs of *S. marcescens* cross-contamination via the hands of healthcare workers and parents. Initially, standard measures, including hand hygiene, were reinforced. However, definitive containment was achieved only after replacement of the siphons and weekly decontamination with acetic acid. WGS enables faster recognition of an outbreak with accurate mapping of the spread, facilitating the implementation of infection control measures. WGS also provides interesting information about the spread of antibiotic resistance and virulence genes.

Introduction

Serratia marcescens is a Gram-negative bacterium belonging to the Enterobacteriaceae, one of the seven families of the order Enterobacterales. The micro-organism is notorious for its

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increasing antimicrobial resistance and potential to cause outbreaks in critical care units, predominantly neonatal intensive care units (NICUs) [1–7].

S. marcescens strains involved in outbreaks are often multidrug resistant, with intrinsic as well as acquired resistance [3]. As the genetic determinants can be transmitted easily from one species to another, rigorous implementation of infection control measures is of utmost importance in the hospital setting [4,6].

In a European multi-centre study of paediatric nosocomial infections, *S. marcescens* was responsible for 15% of culture-positive nosocomial infections on NICUs [8]. Neonates admitted to an NICU are at high risk for acquisition of nosocomial infections because of their immature immune system and the intensity of the medical interventions [1,9]. Risk factors significantly associated with *S. marcescens* infection are low birth weight (<1500 g), prematurity (<37 weeks), length of stay, mechanical ventilation and antibiotics [2,3]. Neonates may remain colonized for a long period despite adequate antimicrobial therapy, particularly in the gastrointestinal and respiratory tracts [10].

Early identification of colonized and infected patients and prompt implementation of infection control measures are crucial to stop the spread of *S. marcescens* [3,4]. A new and promising technique in outbreak investigation is whole-genome sequencing (WGS), which enables detailed typing of micro-organisms with higher resolution than older techniques such as pulsed-field gel electrophoresis, the current standard method in outbreak investigation for most bacterial species including *S. marcescens* [1,6,11]. WGS could be used to map the spread and identify potential sources of an outbreak. Furthermore, WGS enables rapid and accurate identification of antibiotic resistance and virulence genes [1,11,12].

The aim of this study was to describe an outbreak of *S. marcescens* (2018–2019) at the neonatal unit in the authors' hospital and discuss which infection control measures contributed to the containment. Isolates from previous presumed NICU outbreaks and adult blood cultures were included for comparison. Phylogenomic links, antibiotic resistance genes and virulence genes were examined using WGS, addressing the potential of this tool in outbreak investigations.

Methods

Setting

The University Hospital of Brussels is a tertiary teaching hospital with >700 beds. The neonatal unit has 16 intensive care cots and 11 medium care cots, with approximately 350 admissions annually from both in-born and referred neonates. The NICU consists of two open areas with seven incubators each, and two isolation boxes (Appendix A, see online supplementary material). The medium care cots are placed in a distinct area; however, the same team of healthcare workers (HCWs) cares for both intensive care and medium care patients.

Patients and samples

Under normal circumstances, surveillance cultures for *S. marcescens* are not performed at the NICU. Colonization screening (oral and rectal) and environmental screening are initiated in response to a suspected outbreak. Screening specimens are taken with an ESwab (Copan, Brescia, Italy). This study consisted of different subgroups:

- Patient isolates from NICU outbreak in 2018–2019 (N=36):
  - screening specimens (October 2019–March 2020); and
  - clinical specimens (August 2018–March 2020).
- Environmental isolates from NICU outbreak in 2018–2019 (N=4).
- Patient isolates conserved in response to two previous presumed NICU outbreaks:
  - screening and clinical specimens from May 2014 to August 2015 (N=12); and
  - clinical specimens from January to December 2017 (no screening initiated) (N=3).
- All *S. marcescens* isolates from adult blood cultures taken in 2019 (N=8) were included to investigate if there was a hospital-wide problem.

One isolate per patient was analysed, except for one adult with two episodes of bacteraemia (cultures taken >7 months apart; Isolate Nos Patient 21_19/0284 and Patient 21_19/1798).

Ethical approval was obtained from the Medical Ethics Committee of Universitair Ziekenhuis Brussel (B.U.N 1432020000001).

Strain isolation and identification

The strains were isolated on MacConkey (bioMérieux, Brussels, Belgium) and sheep blood agar (Thermo Fisher Scientific, Merelbeke, Belgium) with 24–48 h of incubation (35°C, 5% CO_2 for blood agar). Isolates were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry using a Microflex LT mass spectrometer with MBT Compass Software Version 4.1.90 and Bruker Database Taxonomy Version 8 (Bruker Daltonik GmbH, Bremen, Germany), and stored at -80°C prior to WGS. Antimicrobial susceptibility testing was performed using disk diffusion and SIRscan (i2a, Montpellier, France) in accordance with the European Committee on Antimicrobial Susceptibility Testing guidelines.

Whole-genome sequencing

Genomic DNA was extracted from *S. marcescens* isolates using the Maxwell RSC Cell DNA purification kit on the Maxwell RSC instrument (Promega Corporation, Madison, WI, USA). Fragmentation of genomic DNA was performed using the NEBNext Ultra II FS module. Sequencing libraries, with an insert size of 550 bp on average, were prepared using the KAPA Hyper Plus kit (Kapa Biosystems, Wilmington, MA USA) and a Pippin Prep size selection. In order to avoid polymerase chain reaction (PCR) bias, the PCR amplification step was excluded and a 500-ng input of genomic DNA was used. After equimolar pooling, libraries were sequenced on a Novaseq 6000 instrument (Illumina, San Diego, CA, USA) using an SP-type flow cell with 500 cycli. For this, the library was denatured and diluted according to the manufacturer's instructions. A 1% PhiX control library was included in each sequencing run. Sequence quality was assessed using FastQC Version 0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). De-novo

assembly was performed using the SPAdes genome assembler (http://bioinf.spbau.ru/spades).

In-silico identification of antibiotic resistance and virulence genes

Identification of acquired antibiotic resistance genes was performed using a tool available from the Centre for Genomic Epidemiology (https://cge.cbs.dtu.dk/) (ResFinder 3.2). The presence of resistance genes was determined with an identity threshold of 90% over the length of the reference sequence.

Virulence factor-related genes were selected according to Abreo *et al.* and used to search in the Prokka annotated gene list produced by the PGAdb-builder [12,13]. The datasets generated by PGAdb-builder for the analysis of presence/absence of virulence genes are available via the following links: http://wgmlstdb.imst.nsysu.edu.tw/disProfileDB.php?folder=1397548617 and http://wgmlstdb.imst.nsysu.edu.tw/disProfileDB.php?folder=1397548617.

wgMLST analysis

The sequencing data were analysed using the whole-genome multi-locus sequence typing (wgMLST) scheme for *S. marcescens* available in BioNumerics Version 7.6.3 (Applied Maths, bioMérieux). This scheme consists of 9377 loci [6]. Both assembly algorithms were used for allele calling: the assembly-free k-mer-based approach using the raw reads, and the assembly-based BLAST approach. The default settings were used for both the assembly-free and assembly-based algorithms. The quality of the sequence read sets, the de-novo assemblies, and the assembly-free and assembly-based allele calls were verified using the quality statistics window in BioNumerics. Minimal spanning trees were generated using the wgMLST allelic profiles as input data in BioNumerics. Branch lengths reflect the number of allelic differences between the isolates in the connected nodes. For clustering, the maximum distance between nodules in the same partition was set at 19 [6].

Results

Epidemiological outbreak investigation

In April 2019, the Department of Infection Control and Prevention became concerned following the isolation of *S. marcescens* from the specimens of three NICU patients (two blood cultures, one bronchial aspirate) in one week. Based on the prevalence of *S. marcescens* in NICU patients, there seemed to have been a problem since August 2018 with a slight decrease at the beginning of 2019, followed by a second peak in Spring 2019.

An outbreak investigation was started. The case definition was described as all neonates admitted to the NICU from August 2018 who were infected or colonized with *S. marcescens* (Figure 1).

From August 2018 to March 2020, 36 patients admitted to the NICU became infected or colonized with *S. marcescens*. They were in different cots and even in different areas of the department at the time of the first positive culture (Figure 1). This led to the hypothesis of a common source in the environment and/or transmission between the neonates via the hands of HCWs and parents.

The following environmental surfaces were sampled: ultrasound gel (N=1); bath tap filters (N=4); showerhead filters (N=4); showerhead (N=1); bath drains (N=4); sink drains (N=6); thermometer (N=1); hand soap (N=2); oil cleansing

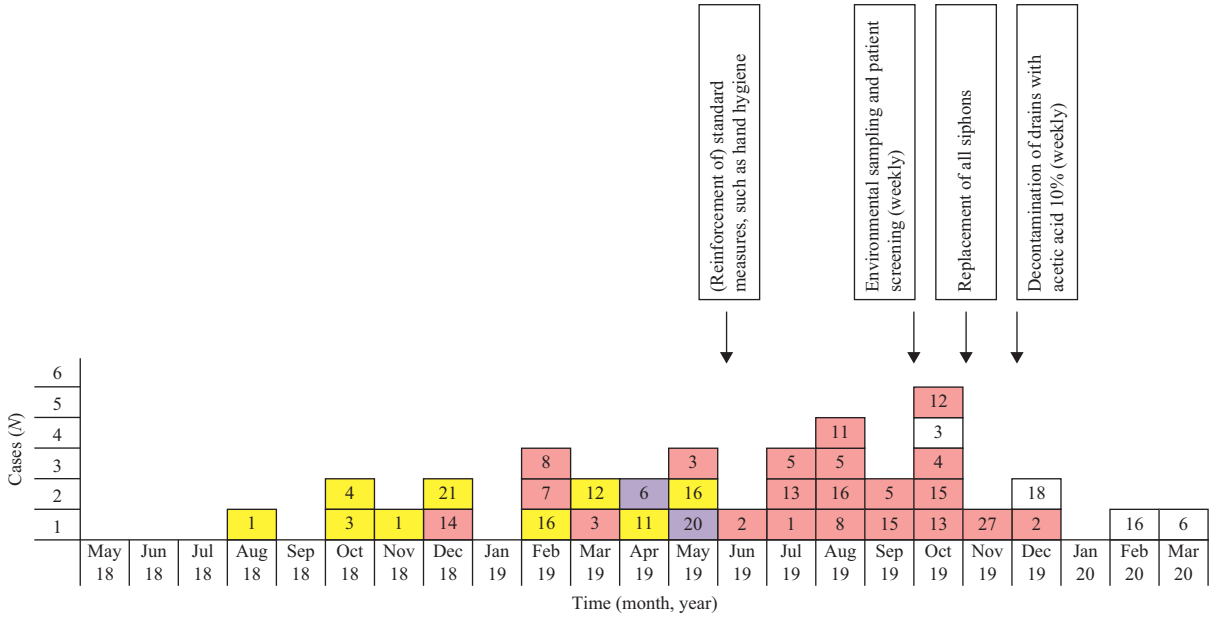

Figure 1. Outbreak curve (monthly incidence) of *Serratia marcescens* infection/colonization in the *neonatal intensive care unit* at the University Hospital of Brussels, Belgium (May 2018–March 2020; N=36). The implementation of infection control and prevention measures is indicated by arrows. The colours of the boxes correspond to the colours of the distinct outbreak clusters represented in Figure 2; the white boxes are cases that did not belong to any of the outbreak clusters. The numbers in the boxes represent the cots where the neonates were admitted at the time of sampling.

(*N*=2); incubators (*N*=4); breast pumps (*N*=6); seats (*N*=12); changing pad (*N*=1); baby scale (*N*=1); box of disinfectant wipes (*N*=2); surfaces in isolation boxes (*N*=20); high-touch surfaces of milk fridge (*N*=3) and freezer (*N*=2); baby bottles from patients colonized with *S. marcescens* (*N*=2); human milk fortifier powder (*N*=1); and soy oil (*N*=1).

Four environmental screening sites tested positive for *S. marcescens* (Appendix A): drains (*N*=3) and baby scale (*N*=1).

In total, 63 *S. marcescens* isolates (patients, *N*=59; environment, *N*=4) were included for WGS and wgMLST analysis, revealing five clusters (Figure 2; Appendixes B and C, see online supplementary material).

The presumed 2018–2019 outbreak turned out to be divided into three distinct clusters. A difference of 7438 (Clusters 2 and 3) to 26,281 (Clusters 1 and 3) alleles was observed between the different clusters (Figure 2). Cluster 1 consisted of 24 isolates (patients, *N*=21; environment, *N*=3), differing by zero to 13 alleles. The environmental isolates were taken from the sink drain in Area A, and the baby scale and bath drain in Area B. Cluster 2 contained isolates from two patients and one environmental surface (sink drain in Isolation Box 1), differing by zero to one allele. Noteworthy, the patients from this cluster stayed in the NICU simultaneously but were not admitted to the isolation box. Cluster 3 contained nine patient isolates, differing by zero to eight alleles. No environmental surfaces were linked to this cluster.

One neonatal screening specimen taken on 28th October 2019 showed a *S. marcescens* strain that differed significantly from the outbreak strains. Furthermore, the strains isolated from 25th December 2019 (patients, *N*=3) were significantly different from the outbreak strains and from each other, suggesting that the outbreak had stopped.

The *S. marcescens* strains isolated from adult blood cultures (*N*=8) were not linked to each other nor to any of the NICU outbreak strains. The adult patients were admitted to six different units.

A slightly elevated prevalence of *S. marcescens* at the NICU at the end of 2016 and beginning of 2017 suggested an outbreak; however, the three included isolates from 2017 were not linked to each other nor to any of the outbreak strains.

In 2014, two clusters could be distinguished: Cluster 4 with seven patient isolates (allelic difference of zero to two) and Cluster 5 with two indistinguishable patient isolates. The allelic difference between these two clusters was 8023. The outbreak clusters from 2014 differed from the 2018–2019 clusters by 11,369 (Clusters 2 and 4) to 26,830 (Clusters 3 and 5) alleles (Figure 2).

The presence of *S. marcescens* during these outbreaks predominantly represented colonization; infection was observed

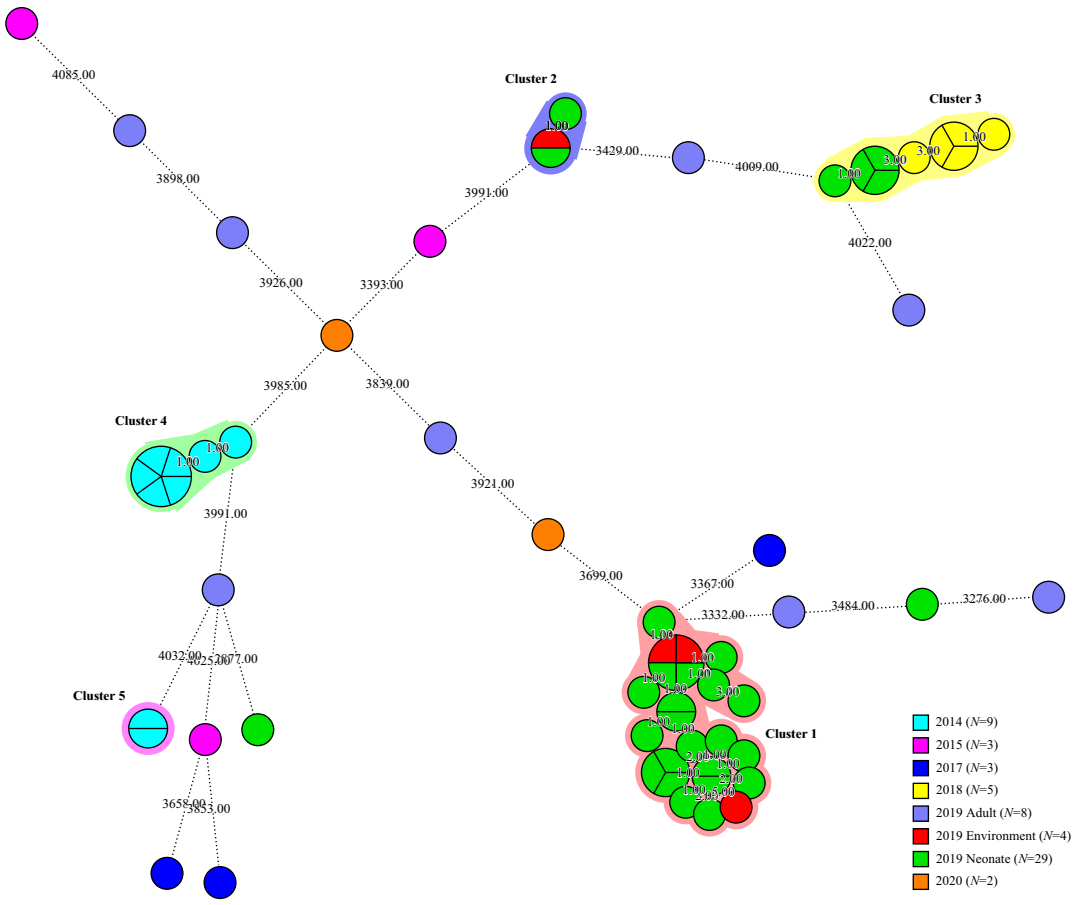

Figure 2. Minimum spanning tree for all *Serratia marcescens* isolates (*N*=63), with indication of the year of isolation. Colours around the circles identify the epidemiological clusters. Circle sizes and divisions are associated with the number of isolates per type. The numbers on the branches denote the numbers of allelic differences between the connected isolates.

in seven of 21 (33%) patients from Cluster 1; one of two (50%) patients from Cluster 2; two of nine (22%) patients from Cluster 3; two of seven (29%) patients from Cluster 4; and zero of two (0%) patients from Cluster 5. The respiratory tract was mainly affected (N=7), followed by bloodstream (N=4) and central nervous system (N=1) infections. In total, three patients of the outbreak clusters died as a consequence of *S. marcescens* infection (one each from Clusters 1, 3 and 4).

In comparison, the presence of *S. marcescens* in neonates who did not belong to any of the outbreak clusters (N=10) represented colonization and infection equally. These neonates mainly suffered from respiratory tract infections (N=3), followed by bloodstream (N=1) and urinary tract (N=1) infections. There were no deaths in this population.

Infection control and prevention measures

The infection control team critically observed the processes of internal and external care providers (e.g. physiotherapists, sonographers).

The NICU staff and external care providers were not always compliant with good hand hygiene practice; insufficient attention was paid, especially before touching patients. Not all parents were well educated. Gloves were not always used during patient care involving contact with mucous membranes. As such, good hand hygiene practice was reinforced, including glove use (from June 2019 onwards; Figure 1). The use of gowns by physiotherapists during procedures where contact with secretions is anticipated (e.g. percussion) and during care of infected patients was implemented from June 2019 onwards.

Disinfection of common medical devices and environmental cleaning was intensified from June 2019 onwards.

From October 2019 onwards, all neonates on the NICU were screened weekly with oral and rectal swabs. Although the best screening method combines a respiratory and gastrointestinal sample [10], oral swabs were used in this study as respiratory samples are more difficult to obtain. In addition, clinical specimens were obtained when applicable. Dedicated staff were assigned to take care of either the infected/colonized neonates or the unaffected neonates.

Environmental sampling (27th September–16th October 2019) led to isolation of three *S. marcescens* strains from drains linked to two outbreak clusters (Clusters 1 and 2). Upon this, all the siphons in the NICU were replaced at the beginning of November 2019 (baths, N=4; sinks, N=6) and weekly decontamination with acetic acid 10% was introduced from December 2019 onwards. The drains are left to soak for 30 min before flushing with water.

Following the implementation of these infection control measures, the outbreak was contained and surveillance was stopped at the end of March 2020 (Figure 1).

Antibiotic resistance and virulence genes

Aac(6′)-Ic, associated with aminoglycoside resistance, was the only resistance gene present in all included isolates (N=63) (Appendix D, see online supplementary material). Five beta-lactamase genes were observed: SST-1 (N=14), SRT-1 (N=3), SRT-2 (N=45), CTX-M-15 (N=1) and TEM-1B (N=1). The presence of an extended-spectrum beta-lactamase (ESBL) was also detected phenotypically for the isolate containing CTX-M-15 and TEM-1B. Tet(41), a tetracycline resistance determinant, was observed in one isolate from an adult blood culture.

All isolates belonging to an outbreak cluster contained two acquired resistance genes: aac(6′)-Ic and an AmpC beta-lactamase (SST-1 or SRT-2). Isolates belonging to the same cluster contained the same antibiotic resistance genes.

Twenty-nine virulence-associated genes were observed, divided into six groups according to the function of the encoded proteins: virulence regulation; haemolysin; peptidoglycan hydrolase; biofilm related; chaperone precursor; and proline/betaine transporter (Appendix E, see online supplementary material). The highest number of virulence-related genes in one isolate was 21. Ten genes were observed in all isolates: bvgS; bvgA; shlB_4; shlA; flgJ; bdlA; tabA_1; proP_2; proP_4; and proP_5.

The isolates belonging to the same cluster contained the same genes, except for one: Patient 42_19/1428 had one extra gene (virB9) compared with the other patients in Cluster 1.

Discussion

This article describes an outbreak of *S. marcescens* in the NICU at the study hospital analysed by WGS and wgMLST, and comparison of the strains with those of presumed previous outbreaks.

The 2018–2019 outbreak appeared to consist of three distinct clusters that were cocirculating over several months. The simultaneous presence of multiple clones has been described previously, suggesting the involvement of multiple sources [1,4,14–16].

Environmental sampling revealed the presence of *S. marcescens* strains in drains (Cluster 1, N=2; Cluster 2, N=1). The positive drain from Cluster 2 was located in the isolation box, but neither of the two patients in this cluster had been admitted to this box. Thus, the drains may have been a source of contamination, as was the case in previously published outbreak reports, but there should have been a vehicle of transmission from the sink drain to the infants in Cluster 2, and there should have been another source of infection as none of the drains could be linked to Cluster 3 [1,16–18]. Based on previous outbreaks, it is believed that the most important reservoir is the gastrointestinal tract of infected/colonized newborns, which remain colonized for a long period [2,4,14]. From this reservoir, cross-contamination can occur through the hands of HCWs [3,4]. However, parents can become contaminated transiently as well, subsequently contaminating the environment and vice versa. It is not possible to know whether the drain microbiota colonized the infants or the infant microbiota led to contamination of the drains, but it is hypothesized that both act as a reservoir for contamination through the hands of HCWs and parents.

Furthermore, two concurrent outbreaks at the NICU in 2014 were observed, indicating that *S. marcescens* outbreaks are a recurring problem. However, those strains differed significantly from the 2018–2019 outbreak cluster strains. Therefore, it can be assumed that there was no environmental surface acting as a continuous reservoir. This confirms the hypothesis that neonates, and not the environment, are the most important reservoir and the starting point of outbreaks.

The strains of adult blood cultures were not related to each other nor to the NICU outbreak strains. This confirms the assumption that it was not a hospital-wide problem, justifying that infection control measures were only taken in the NICU.

Furthermore, this indicates that neonates are more prone to *S. marcescens* outbreaks than adults due to the immaturity of their immune system and the intensity of medical interventions [1,9].

In response to the outbreak, several interventions were implemented in different steps (Figure 1). First, standard measures such as hand hygiene were reinforced. As the bacterium is transmitted by hands, it is evident that this is the most important procedure to prevent transmission [2–4,14,19]. Re-education of staff was undertaken, as this has proven to be effective in previous outbreaks [2,4,14,15,19,20]. In addition, parents were educated at the NICU as it is likely that they play a role in transmission. Weekly colonization screening, assignment of dedicated staff, and environmental screening were also implemented [15,19]. As no new cases were detected from June 2019 onwards, it appears that the spread of *S. marcescens* strains belonging to Clusters 2 and 3 was terminated by this first set of standard measures. Nevertheless, these measures seemed to be insufficient to terminate the spread of Cluster 1 and resolve the outbreak.

Subsequently, all the siphons in the NICU were replaced. This was based on previous research in the adult ICU at the study hospital indicating the sink drains as a potential source of (multi-resistant) *Enterobacteriaceae*. As biofilm formation can occur, the bacterial burden in the siphons is very high and aerosols containing bacteria can be spread when water is running [21]. In several NICU outbreaks, drains have been replaced to control the spread [16,20]. However, the replacement of siphons alone seems to be insufficient to stop transmission definitively [16,21]. David *et al.* and Maltezou *et al.* contained NICU outbreaks with *S. marcescens*, at least in part, by disinfection of the drains with hypochlorite 10% [14,16]. Smolders *et al.* reported that decontamination of sink drains with acetic acid 25% seemed to be a cheap and effective alternative [22]. However, concentrations >10% can cause irritation on contact with skin and eyes, and similar effects are observed in the airways after inhalation [23]. Due to the frailty of the NICU population, weekly decontamination of the drains with acetic acid 10% was introduced in the study NICU. After replacement of the siphons (November 2019) and introduction of weekly decontamination with acetic acid 10% (December 2019), the outbreak seemed to be under control as the last new patient infected/colonized by an outbreak strain was observed on 2nd December 2019.

The WGS results from this study highlight the potential of this tool to improve outbreak investigations. Initially, *S. marcescens* cases observed in NICU patients from August 2018 to April 2019 were thought to be sporadic cases. If WGS had been easily accessible in the study hospital, faster recognition of the outbreak would have been possible. Subsequently, environmental sampling was performed, leading to clues regarding the possible sources. These sources could be linked to the different outbreak clusters by WGS, elucidating the transmission pathways and facilitating the implementation of targeted measures. The effectiveness of the infection control measures was proved by WGS as the *S. marcescens* strains isolated after December 2019 were not linked to the outbreak strains. Furthermore, as *S. marcescens* outbreaks are a recurrent problem at the NICU, it was important to know that previous NICU outbreaks and adult infections were caused by other strains, indicating that there was no continuous source and no hospital-wide problem.

Furthermore, information about antibiotic resistance genes was provided by WGS. All included strains contained the aminoglycoside-resistance-associated gene aac(6′)-I-C, as was the case in previous reported outbreaks [1,6]. Three AmpC beta-lactamases (Ambler class C) were observed: most commonly SRT-2, followed by SST-1 and SRT-1. In a report by Saralegui *et al.* concerning an *S. marcescens* outbreak in a paediatric unit, SRT-2 was most commonly detected [24]. Two beta-lactamases belonging to Ambler class A were present in a single adult isolate in this study: CTX-M-15 and TEM-1B. Although CTX-M-type enzymes are the ESBLs most commonly carried by *S. marcescens* [17].

Virulence factors produced by opportunistic *Serratia* spp. are not understood well in general, and only limited data have been published based on WGS in particular [12,17]. Three virulence factors were found in all isolates included in a study by Abreo *et al.* (N=45), as was the case in the present study (N=63): bvgS, shlA and tabA_1 [12]. On the contrary, seven genes detected by Abreo *et al.* in a limited number of isolates could not be found in the isolates in the present study: bvgS_2 (N=2), evgS (N=1), hpmA_2 (N=1), flgJ_2 (N=5), bigR_2 (N=2), fimC_1 (N=3) and fimC_2 (N=3). These data indicate that some common virulence-associated genes can be found in almost all *S. marcescens* isolates, and other genes are only found in sporadic cases.

In conclusion, both the infected/colonized neonates and the affected drains were considered to be reservoirs of *S. marcescens*, with cross-contamination occurring via the hands of HCWs and parents. Based on this assumption, it is likely that the reinforcement of hand hygiene measures was the most important standard intervention to stop the spread. However, definitive containment was only achieved after replacement of the siphons and weekly decontamination with acetic acid 10%.

WGS has proven to be of great value in outbreak investigations. It enables faster recognition of an outbreak with accurate mapping of the spread and identification of potential sources, facilitating the implementation of infection control measures. Furthermore, WGS can provide interesting information about the spread of antibiotic resistance and virulence genes.

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Appendix A. Supplementary data

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Brief communication

Polyclonal outbreak of bacteremia caused by *Burkholderia cepacia* complex and the presumptive role of ultrasound gel

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ABSTRACT

A nosocomial polyclonal outbreak associated to bacteremia caused by different *Burkholderia cepacia* complex (BCC) species and clones is reported. Molecular characterization identified *Burkholderia stabilis*, *Burkholderia contaminans*, and *Burkholderia ambifaria* among BCC isolates obtained from patients in neonatal and adult intensive care units. BCC was also isolated from an intrinsically contaminated ultrasound gel, which constituted the presumptive BCC source. Prior BCC outbreak related to contaminated ultrasound gels have been described in the setting of transrectal prostate biopsy. Outbreak caused strains and/or clones of BCC have been reported, probably because BCC are commonly found in the natural environment; most BCC species are biofilm producers, and different species may contaminate an environmental source. The finding of multiple species or clones during the analysis of nosocomial BCC cases might not be enough to reject an outbreak from a common source.

The *Burkholderia cepacia* complex (BCC) encompasses at least 17 related Gram-Negative bacilli species as judged by different phenotypic and genotypic analyses.[1] BCC members can cause infections in cystic fibrosis, chronic granulomatous disease, and hospitalized patients.[2] BCC members are among the most frequent sources of nosocomial outbreaks due to intrinsically contaminated substances other than blood products.[2] Here, we describe an outbreak of bacteremia caused by BCC strains between April and July 2013. Subject's clinical charts were reviewed and microbiological testing of substances and solutions representing potential sources of the outbreak was performed. Eighty samples from different wards

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Table 1 – Description of the 12 *Burkholderia cepacia* complex strains isolated from blood cultures and from ultrasound lubricant gel.

Date of positive culture	Origin	Patient (P) or gel sample (G) number	BCC species by *recA* sequencing	Clone by DO-PCR
04/05/2013	NU	P1	*B. ambifaria*	A
04/09/2013	NU	P2	*B. ambifaria*	A
04/13/2013	ICU	P3	*B. stabilis*	B
04/14/2013	ICU	P3	*B. stabilis*	B
04/27/2013	ICU	P4	*B. ambifaria*	C
04/30/2013	ICU	P4	*B. ambifaria*	C
05/04/2013	ICU	P5	*B. stabilis*	B
05/04/2013	ICU	P4	*B. ambifaria*	C
05/08/2013	NU	P6	*B. contaminans*	D
06/05/2013	UO	G1	*B. contaminans*	E
06/05/2013	OW	G2	*B. contaminans*	E
06/12/2013	Gel container	G3	*B. contaminans*	E

NU, Neonatal Unit; ICU, Intensive Care Unit; UO, Ultrasound Office; OW, Obstetric Ward, DO-PCR, degenerate oligonucleotide-PCR.

and commercial products commonly used in the Neonatal Unit (NU) and Intensive Care Unit (ICU) were tested for BCC presence using selective culture media (Burkholderia agar, BioMerieux Inc, Mercy Létoil, France). All suspected isolates were phenotypically identified as belonging to the BCC by oxidase, OF-glucose, sculin hydrolysis, lysine decarboxylation, and DNA hydrolysis tests, as well as by the semi-automatized API-20NE (BioMerieux Inc, Mercy Létoil, France) method. Regrettably, only nine BCC isolates were available for molecular analysis (six from blood cultures and three from ultrasound gels). These strains were identified to the species level by *recA* gene sequence comparisons[3] and analyzed for genomic relatedness by both degenerate oligonucleotide-primed PCR[4] and repetitive extragenic palindromic-PCR.[5]

The outbreak involved 11 patients with 17 BCC isolates recovered from blood cultures; seven of these 11 subjects were hospitalized in the NU, all of them were preterm neonates with respiratory distress, three other patients were in the ICU, two of which had recent cardiovascular surgery, and one patient was in the General Ward. The mean (range) time of hospitalization of these patients until the development the bacteremia was 5.55 (0–15) days; one neonate developed BCC bacteremia the date of birth, probably reflecting horizontal transmission. In seven patients, BCC strains were recovered only from baseline blood cultures; two more patients had positive surveillance blood cultures on day 2, and two other patients had bacteremia also in a 3rd set of blood cultures. Three of the 11 patients died (two adults and one neonate) during the hospitalization, although none of these deaths were attributed to the BCC bacteremic episode. It was noted that the seven neonates and the four adult patients underwent a mean (range) of 5 (1–10) ultrasounds, including transthoracic and transfontanellar ones, and 2 (1–3), respectively.

Eighty environmental samples were taken for culture, including several solutions of antiseptics (iodopovidone, hydrogen peroxide, cholorhexidine, and alcohol-gel), drugs (fentanyl citrate, morphine, tobramycin drops), multiple surfaces in the surgical room, ICU and NU, and other commonly used materials such as gels for ultrasound, liquid soap, and vaseline; BCC strains were isolated only from ultrasound scanning gels (Table 1). A quantitative culture done from an unopened 5-L container ultrasound gel displayed growth of 4.66 $\log_{10}$ CFU/mL (mean of two samples) of BCC cells. Molecular analyses based on *recA* gene sequence of nine isolates obtained from six patients in NU and ICU wards indicated the presence of three different species of the BCC complex (Table 1): *Burkholderia ambifaria* was identified in patients 1 and 2 (NU) and patient 4 (ICU), *Burkholderia stabilis* in patient 3 and 5 (ICU), and *Burkholderia contaminans* in patient 6 (NU). All BCC isolates were susceptible to ceftazidime, meropenem, minocycline, and trimethoprim-sulfamethoxazole by disk diffusion methods.

Among the clinical isolates, genotypic characterization revealed two different clones of *B. ambifaria* and a single clone of both, *B. stabilis* and *B. contaminans* (Table 1). Two different BCC species coexisted in the NU (*B. ambifaria* and *B. contaminans*) and the ICU (*B. stabilis* and *B. ambifaria*). In addition, two different *B. ambifaria* clones were detected, both from different wards (Table 1). *B. contaminans* was isolated in different samples of ultrasound gel as it was from patient 6 although this isolate was a different clone (Table 1). Repetitive extragenic palindromic-PCR confirmed the clonal distinctness of the BCC isolates analyzed above (data not shown). These results support the polyclonal outbreak of BCC strains caused by multiple species (*B. ambifaria*, *B. stabilis*, and *B. contaminans*) and clones (e.g. *B. ambifaria*, *B. contaminans*).

Interesting, BCC members can hydrolyze parabens, which are p-hydroxybenzoic acid esters with antimicrobial properties commonly added as stabilizers to ultrasound gels[6]; BCC strains can therefore survive and proliferate in these gels.[6] Even though the sterility of substances in contact with intact skin such as ultrasound gels is generally not required, the US Food and Drug Administration had to recall commercial ultrasound gels contaminated with *Pseudomonas aeruginosa* and *Klebsiella oxytoca*, recommending the use of sterile ultrasound gel for invasive procedures, leaving the use of non-sterile

(open) containers for procedures performed on intact skin and for low risk patients.[7] Of note, BCC invasive infections related to contaminated ultrasound gels have been only described in the setting of transrectal prostate biopsy.[6,8] Current report represents the third outbreak of BCC presumably associated to ultrasound gel. We speculate that the invasive procedures done in neonate hosts and patients undergoing cardiovascular surgery might have predisposed them to develop bacteremia after significant BCC skin colonization from contaminated gel.

The striking feature of this outbreak is the presence of multiple BCC species and clones since most BCC outbreaks have been associated to a single clone. However, outbreaks caused by different strains and/or clones have been reported,[9–11] including BCC contamination of hospital water,[9] intravenous bromopride vials,[10] and non-identified environmental sources.[11] Since BCC bacteria are commonly found in the natural environment and most BCC species are biofilm producers, different species may contaminate an environmental source (as it has been described in cystic fibrosis patients[12]), eventually leading to a polyclonal nosocomial outbreak. Unfortunately, we could not confirm this hypothesis as we only recovered one *B. contaminans* clone from the ultrasound gel samples.

In summary, the sudden appearance of BCC invasive cases, the isolation of BCC from ultrasound gels including an unopened container, and the abrupt interruption of new cases after removal of ultrasound gel stocks led us to speculate that this substance might have been the source of the nosocomial BCC outbreak. The finding of multiple species or clones during the analysis of nosocomial BCC cases might not be enough to reject an outbreak from a common source.

Conflicts of interest

The authors declare no conflicts of interest.

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Recurrent *Sphingomonas paucimobilis*-bacteraemia associated with a multi-bacterial water-borne epidemic among neutropenic patients

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Summary: A cluster of septicaemias due to several water-related species occurred in a haematological unit of a university hospital. In recurrent septicaemias of a leukaemic patient caused by *Sphingomonas paucimobilis*, genotyping of the blood isolates by use of random amplified polymorphic DNA-analysis verified the presence of two distinct *S. paucimobilis* strains during two of the separate episodes. A strain of *S. paucimobilis* identical to one of the patient's was isolated from tap water collected in the haematological unit. Thus *S. paucimobilis* present in blood cultures was directly linked to bacterial colonization of the hospital water system. Heterogeneous finger-printing patterns among the clinical and environmental isolates indicated the distribution of a variety of *S. paucimobilis* clones in the hospital environment. This link also explained the multi-microbial nature of the outbreak.

Keywords: *Sphingomonas*; bacteraemia; nosocomial infection; random amplified polymorphic DNA technique.

Introduction

Non-enteric Gram-negative bacilli and non-tuberculous mycobacteria which may induce severe nosocomial infections in patients with underlying debilitating conditions and/or preceding medical interventions are known to colonize water distribution systems in hospitals.[1,2] An unusual cluster of bacteraemic infections was observed among adult haematological patients in Kuopio University Hospital during a several months' period in 1994. Among the 195 bacterial isolates recovered from their blood cultures during the outbreak, 25% ($N = 48$) were glucose non-fermenting Gram-negative bacilli, e.g., *Pseudomonas aeruginosa, Acinetobacter* sp., *Achromobacter xylosoxidans, Sphingomonas paucimobilis* and *Stenotrophomonas maltophilia*. These species caused septicaemia in 10 patients.

The recognition of a cluster of infections associated with bacteria potentially derived from environmental reservoirs prompted a survey of the microbiological quality of the hospital water supply. Septicaemia in a leukaemia patient, caused by *Mycobacterium fortuitum*, had been linked to the hospital water distribution systems, as described

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earlier.[2] Further investigations suggested the same water source as an apparent reservoir of bacteraemic infections caused by a variety of species growing in similar conditions.

We describe here results of molecular epidemiological analyses of *S. paucimobilis* isolated from blood cultures of a neutropenic patient with recurrent bacteraemic episodes, and her hospital environment. As far as we are aware, only two previous epidemiological reports on *S. paucimobilis* have been based on molecular typing of clinical and environmental isolates in a hospital setting,[3,4] and this is the first time hospital water has been linked to nosocomial bacteraemias.

Materials and methods

Case report

A 57-year-old female presented with acute lymphoblastic leukaemia in October 1993. Induction chemotherapy was carried out through a central tunnelled catheter (Chemo-Cath®, HDC) and was followed by morphological remission. During the neutropenic periods following the induction chemotherapy and the first consolidation chemotherapy cycle, the patient suffered from three septic episodes. The first of the episodes was caused by *Streptococcus mitis* and *Enterococcus faecium*, the second by *Bacteroides fragilis* and *Lactobacillus* sp. and the third by *Streptococcus sanguis*. All episodes were successfully treated with appropriate antibiotics. There was a purulent infection on the exit site of the tunnelled catheter during the third septic episode caused by *S. sanguis*, but no cultures were performed from the exit site.

In April 1994 the patient received her fifth course of chemotherapy, which resulted in severe neutropenia (neutrophils $< 0.5 \times 10^9$/L) lasting 7 days. She presented with fever and oral mucositis, with *Candida albicans* and Herpes simplex virus I detected in the oral lesions. No signs indicating catheter infection were present. Blood cultures collected from a cubital vein on the second day after admission revealed a slowly growing non-enterobacterial Gram-negative rod. Treatment with piperacillin and netilmycin was followed by rapid clinical response without the removal of the central catheter. The blood culture isolate was initially incorrectly identified as *Sphingobacterium multivorum* and later as *S. paucimobilis*. Because of susceptibility results, piperacillin was replaced by ceftazidime while the patient was already afebrile. Prior to the first blood culture positive for *S. paucimobilis*, the patient had been hospitalized for 142 days in the preceding 6 months.

The patient returned to the hospital in the end of May 1994 to receive the final planned chemotherapy cycle. On admission, she had no signs or symptoms of infection. Laboratory tests revealed normal white blood cell and neutrophil counts. Following flushing of the Chemo-Cath she developed a fever of 40.1°C with chills. Blood cultures obtained from a cubital vein and through Chemo-Cath were again positive for *S. paucimobilis*. Treatment with ceftazidime and netilmycin was followed by resolution of fever. After a 10-day course of antibiotic treatment she received the chemotherapy cycle, and returned home with Chemo-Cath in place for removal after recovery of the bone marrow hypoplasia. She was re-admitted to hospital 29 days later with fever and sore throat. Laboratory tests showed neutropenia (below 0.5×10^9) lasting for further 19 days. Blood cultures were again positive for *S. paucimobilis*, and the infection again responded to treatment with ceftazidime and netilmycin. After this episode, the Chemo-Cath was removed and oral maintenance treatment for acute leukaemia was started. Bacterial culture of the removed Chemo-Cath was negative.

Bacterial strains

Clinical isolates

Blood cultures were performed in a semi-automated system (BACTEC 730, Becton Dickinson, Sparks, MD, USA). Non-fermentative Gram-negative rods were biotyped with API20 NE (bioMérieux, Marcy l'Etoile, France), and isolates were stored in skimmed milk at −70°C.

Environmental isolates

After recognition of a cluster of bacteraemias in April 1994, 22 environmental samples were collected from taps, showers and detergent dilutions used by the patients in the haematology ward. Water samples were collected in sterile containers after removal of the aerator or showerhead and flushing for 30 seconds. The aerators and showerheads were also sampled with sterile cotton swabs. Water samples were diluted 1:10 in sterile water and aseptically filtered through a 0.45 μm membrane (10 mL/membrane) (Schleicher and Schuell, Dassel,

Germany). The membranes were placed on CLED (cystine-lactose-electrolyte-deficient) agar plates (Becton Dickinson, Cockeysville, MD, USA) and incubated at 36°C for 2 days, and on Sabouraud medium (Becton Dickinson, Cockeysville, MD, USA) at 30°C for 4 weeks. Swab samples were streaked on to CLED agar and incubated at 37°C for 2 days. Detergent dilutions were analysed according to the method of Kelsey–Maurer.[5] The following autumn, a similar surveillance, comprising 30 environmental cultures, was performed in the haematology ward.

A total of 71 randomly selected colonies, each representing different colony types detectable on separate plates, were subcultured for further studies. Non-fermentative Gram-negative bacillary isolates, initially identified using API20 NE, were stored in skimmed milk at −70°C for later molecular analyses.

Genotyping

Two to six parallel subcultures of each isolate of *S. paucimobilis* were grown in 5 mL tryptone soy broth at 36°C for 48 h. The bacteria were pelleted by centrifugation and washed once with phosphate-buffered saline (PBS). DNA was extracted by using a commercial nucleic acid isolation kit (High Pure PCR Template kit, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Twenty commercial 10-mer primers (Operon Technologies, Inc., Alameda, California) were screened in a pilot study using two to four strains. The primers with the best discriminatory power, OPB10 (5′ CTGCTGGGAC) and OPB17 (5′ AGGGAACGAG) (Operon Technologies, Inc., Alameda, CA, USA), were selected for the final random amplified polymorphic DNA (RAPD) analyses. PCR amplification was carried out as described earlier,[6] in a final volume of 27 μL. Amplified DNA was electrophoresed on 2.0% agarose gels containing 0.5 μg/mL ethidium bromide and 1x Tris-acetic acid-EDTA running buffer, and photographed under UV light. Different RAPD types were designated by a two-letter code showing the pattern for each of the primers OPB10 (letters A–D) and OPB17 (letters a–e).

16S rRNA analysis

Selected isolates were analysed for their partial 16S rRNA sequences to verify their identification. The partial 16S rRNA gene was amplified by using primers pA and pE′.[7] PCR was performed in a 50 μL reaction mixture containing 25 pmol of each primer, 25 ng bacterial DNA, 200 μM dNTP, 1 × DynaZyme buffer, and 1 U DynaZymell polymerase (Finnzymes, Espoo, Finland). The thermal cycler was programmed as described previously.[7] The amplification products were purified for sequencing with MicroSpin S-400 HR columns (Pharmacia, Piscataway, NJ, USA) and sequenced with primers pD′ and pE′.[8] Sequencing was done by using a Dye Terminator Cycle Sequencing kit and an automated ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Clinical isolates

Eight isolates were recovered from six blood culture samples of the index patient. They were yellow-pigmented, glucose non-fermenting Gram-negative rods. All gave an API20 NE code 0463304 indicating either *Sphingobacterium multivorum* (64.8%), *S. paucimobilis* (21.1%) or *Sphingobacterium spiritivorum* (14.0%) at a low discrimination level.

Environmental isolates

Twelve yellow-pigmented isolates from eight water samples produced an API20 NE code similar to the patient's. These were selected for further comparative studies. The other species isolated from the same water systems included *S. multivorum, Stenotrophomonas maltophilia, Pseudomonas* sp., *Acinetobacter* sp. and *Mycobacterium fortuitum*. The detergents examined did not contain non-enteric Gram-negative bacilli.

16S rRNA analysis

Twelve isolates, two clinical and 10 environmental isolates, were selected for partial 16S rRNA sequencing as representatives of the different RAPD-patterns detected (see below). Nine, including the clinical isolates initially identified as *Sphingobacterium multivorum* by phenotypic characteristics, were identified as *S. paucimobilis* by gene sequencing. They all showed 100% similarity in partial 16S rDNA sequences of the first variable region. This sequence was identical to that of the *S. paucimobilis* type strain (Genebank accession number U37337). The remaining three isolates represented other distinct environmental species.

Genotyping

The results of the RAPD fingerprinting demonstrated that the index patient harboured two *S. paucimobilis* strains in her bacteraemic episodes. In the first two episodes, she had both strains simultaneously, whereas in the last one she only had one of the strains. As shown in Figure 1, the

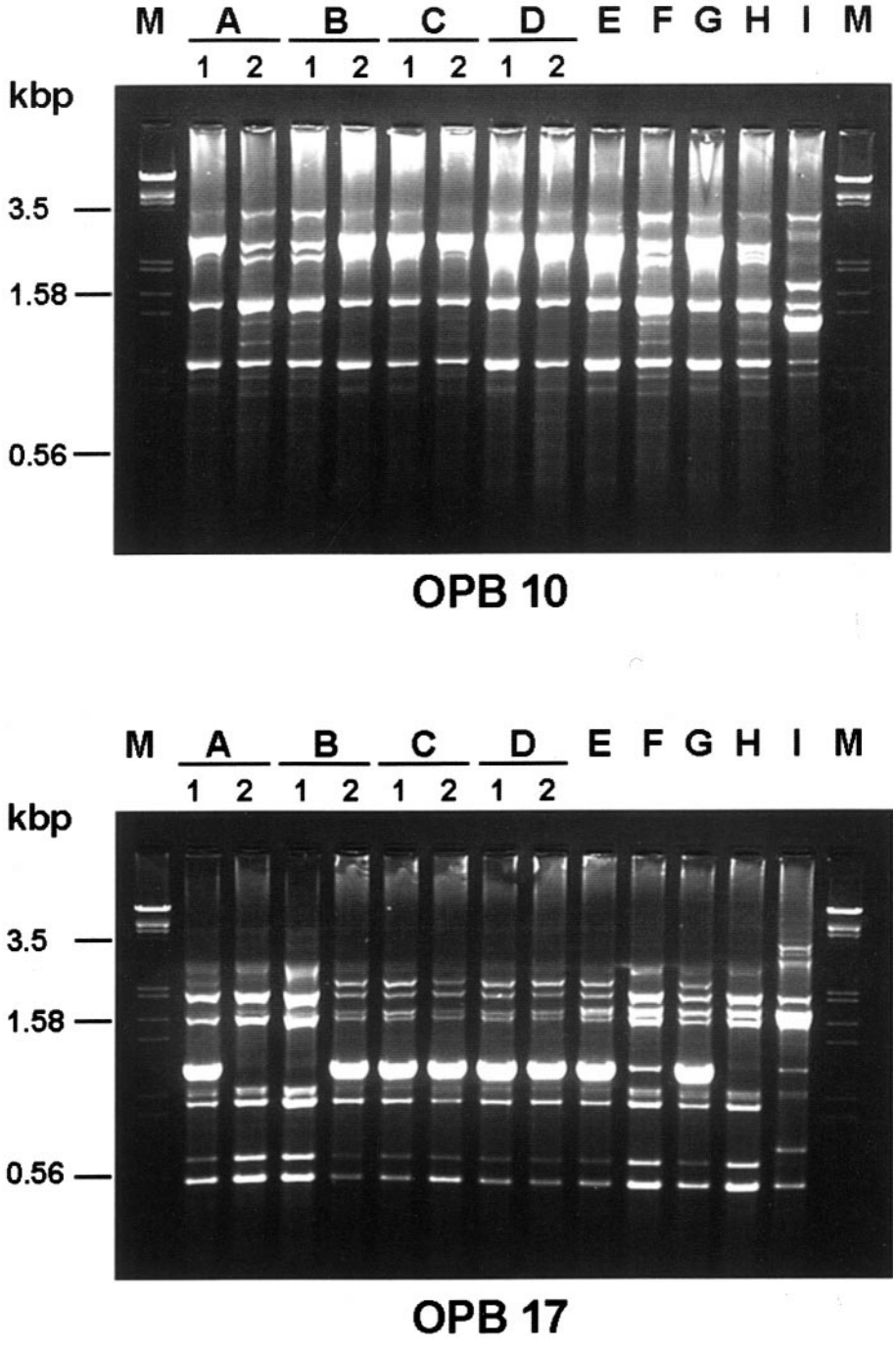

Figure 1 RAPD fingerprint patterns using primers OPB10 and OPB17 for *Sphingomonas paucimobilis* isolates from the blood cultures of the index patient obtained on four distinct dates during recurrent episodes (A–D) and from the tap water at the haematological department (E–I). For pattern and isolate descriptions refer to Table I. Lane M; molecular weight marker (Lambda DNA/*Eco*R I + *Hind* III).

first two patient isolates (lanes A–B) had distinct RAPD-patterns ('Aa' and 'Bb'), whereas the latter patient isolates (Figure 1, lanes C–D) shared the pattern 'Aa'.

In all, five different finger-printing patterns were generated by the 13 isolates using the two primers applied (OBP10 and OBP17) (Figure 1, Table 1). Two water isolates (Figure 1, lanes E and G) had a pattern identical to the patient's isolates of type 'Aa'. These water isolates were recovered at four-week intervals from a single tap of a patient bathroom at the haematological ward. Another water isolate from the same tap (Figure 1, lane F) had a pattern closely similar to pattern 'Bb' of the index patient (Figure 1, lanes A2 and B1). The patient had a direct access to this bathroom, located next to the patient room she occupied. The other two tap water isolates recovered from different rooms at the same ward had unique patterns (Figure 1, lanes H and I).

Discussion

To our knowledge, this is the first time that a nosocomial *S. paucimobilis* bacteraemia has been epidemiologically linked to the hospital water system using molecular typing methods. Although the culture of the removed central venous catheter of the patient was negative on culture, clinical findings indicated that a contaminated and colonized catheter was the likely portal of entry of the organism into the bloodstream. We demonstrated two clones of *S. paucimobilis* in the bacteraemic episodes of our patient. A similar observation of several clones in a catheter-related infection of a single patient has recently been published by Hsueh *et al.*[4] The heterogeneity of RAPD patterns of *S. paucimobilis* isolates most likely reflect the diversity of *S. paucimobilis* clones in the hospital water system, regarded as the potential source of colonization of the patient.

Contamination of faucet aerators has recently been linked to colonization or infection in patients by using molecular epidemiological methods.[9] In our study, faucet aerators and showerheads were colonized with several bacterial species, mainly non-enteric Gram-negative rods. To diminish colonization, the faucet aerators and showerheads were mechanically washed and disinfected in chlorine periodically. After initiation of these procedures, clinical isolation rates of environment related bacteria returned to the level before the outbreak described.

We have found RAPD-method a useful basic tool for epidemiological studies in clinical settings. Due to its adaptability to different species, it can easily be applied to a variety of epidemiological situations.[2,6,10] It has high discriminatory power and good reproducibility if done carefully.[6,10] Due to interlaboratory variability in banding patterns, the results of different laboratories can only be compared to the level 'identical vs. non-identical'.

Increasing clinical evidence implicates water as a source of nosocomial infections. To decrease the risk of water-derived infections in tertiary care hospitals, rational limits for acceptable quality of hospital water need to be defined, and simultaneously, reasonable and sound sanitation procedures developed.

Table 1 *Recovery of different* S. paucimobilis *strains from blood cultures of the index patient and environmental waters at the adult haematological ward in 1994*

Date of isolate	Source	Specimen type	Isolate	RAPD-pattern
19 April	Patient	Blood	A1	Aa
19 April	Patient	Blood	A2	Bb
19 April	Hall	Tap water	I	De
31 May	Patient	Blood	B1	Bb
31 May	Patient	Blood	B2	Aa
1 June	Patient	Blood	C1	Aa
1 June	Patient	Blood	C2	Aa
4 July	Patient	Blood	D1	Aa
4 July	Patient	Blood	D2	Aa
14 November	Bathroom	Tap water	E	Aa
29 November	Bathroom	Tap water	F	Bc
14 December	Bathroom	Tap water	G	Aa
14 December	Room	Tap water	H	Cd

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Polyclonal Spread and Outbreaks with ESBL Positive Gentamicin Resistant *Klebsiella* spp. in the Region Kennemerland, The Netherlands

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Abstract

Objective: The objective of this study was to analyze the transmission dynamics of ESBL positive *Klebsiella* spp. with an additional resistance towards gentamicin (ESBL-G) in a Dutch region of 650,000 inhabitants in 2012.

Methods: All patient related ESBL-G isolates isolated in 2012 were genotyped using both Amplification Fragment Length Polymorphism (AFLP) and High-throughput MultiLocus Sequence Typing (HiMLST). HiMLST was used to analyze the presence of (unidentified) clusters of ESBL-G positive patients. Furthermore, all consecutive ESBL-G isolates within patients were studied in order to evaluate the intra-patient variation of antibiotic phenotypes.

Results: There were 38 ESBL-G isolates, which were classified into 18 different sequence types (STs) and into 21 different AFLP types. Within the STs, four clusters were detected from which two were unknown resulting in a transmission index of 0.27. An analysis of consecutive ESBL-G isolates (with similar STs) within patients showed that for 68.8% of the patients at least one isolate had a different consecutive antibiotic phenotype.

Conclusion: The transmission of ESBL-G in the region Kennemerland in 2012 was polyclonal with several outbreaks (with a high level of epidemiological linkage). Furthermore, clustering by antibiotic phenotype characterization seems to be an inadequate approach in this setting. The routine practice of molecular typing of collected ESBL-G isolates may help to detect transmission in an early stage, which opens the possibility of a rapid response.

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Introduction

In Europe, there is an alarming increase in the prevalence of Multi Drug Resistant Organisms (MDROs) seen in recent years [1]. The increase of resistance in Gram-negative bacteria appears to be largely based on the production of Extended Spectrum Beta Lactamases (ESBLs) [2]. ESBL producing bacteria are able to deactivate the antibacterial properties of beta lactam antibiotics by hydrolysis [3]. In the Netherlands, the prevalence of ESBL producing *Klebsiella pneumoniae* increased from 3.3% in 2008, to 6.0% in 2012 [4]. The prevalence of patients cultured positive for ESBL positive *Klebsiella* spp. with an additional resistance towards gentamicin (ESBL-G) among all *Klebsiella* spp. culture positive patients in the region Kennemerland (650.000 inhabitants), was 2.6% in 2012 (unpublished data). Infections caused by ESBL-G isolates are not covered by the Dutch working party on antibiotic policy (SWAB), in which treatment with cephalosporins in combination with aminoglycosides (such as gentamicin) are advised as empirical therapy for sepsis [5]. Therefore, patients infected with ESBL-G are at risk for treatment failures, and as a consequence this infection is associated with higher morbidity, mortality and treatment costs compared to infections without ESBL-G [6–8]. For patients infected with an ESBL-G, 'last-line antibiotics' like intravenously applied carbapenems, are the preferred choice of treatment [6].

Between 1999 and 2011 several (small) outbreaks with Multidrug Resistant Klebsiella (MRK) were described in Dutch health care settings which comprised more than 100 patients [9–13]. Because patients-exchange regularly occurs between hospitals and nursing homes in the region Kennemerland, and the presence of ESBL-G positive patients can cause treatment failures, this finding has raised the following question: what are the transmission dynamics of ESBL-G and have there been (yet unidentified) clusters of ESBL-G in the region Kennemerland in 2012?

Methods

Ethics statement

According to the Dutch regulation for research with human subjects, neither medical or ethical approval was required to

conduct the study since the data were retrospectively recorded. Additionally we received approval to conduct the study from the institutional review board of the Kennemer Gasthuis which waived the need for participant consent. The data were anonymized and analyzed under code.

Study design and bacterial isolates

To answer the main question, all patient related ESBL-G isolates (from January 2012 to December 2012), which were routinely collected by the Regional Public Health Laboratory Kennemerland (RPHLK), were retrospectively included in our study. We genotyped all ESBL-G isolates by using both Amplification Fragment Length Polymorphism (AFLP) and High-throughput Multilocus Sequence Typing (HiMLST). Subsequently, we analyzed the spread and possible presence of (yet unidentified) clusters of ESBL-G positive patients that could be detected by applying these two different genotyping techniques. The data were analyzed with regard to admission dates and in house location of the ESBL-G positive patients. Furthermore, we compared the results of the HiMLST, AFLP and a phenotypical method based on species type and antibiogram to determine their discriminatory capacity. Additionally, we analyzed all consecutive ESBL-G isolates (with similar sequence types (STs)) within patients to evaluate the intra-patient variation of phenotypes based on the antibiogram. The RPHLK stored all first isolated ESBL positive isolates (phenotypic distinctive, per sampling date) per patient in the freezer for future analysis, allowing the possibility to conduct this study. In total 177 isolates were found to be ESBL-G positive. Of these 177 isolates 75 isolates were excluded for various reasons: 20 isolates were not patient related, 40 isolates were marked as a double isolate (identical phenotype from the same patient) and therefore not stored in the freezer. For five isolates the AFLP and/or HiMLST was not reliable due to mixed reads. This resulted in 102 isolates with complete antibiogram and genotyping results. Among these 102 isolates, only the first positive isolate per patient was included resulting in 38 patient related ESBL positive *Klebsiella* spp. isolates which were analyzed. Additionally, we considered 76 consecutive ESBL-G isolates (with similar STs) within patients to analyze the intra-patient variation of phenotypes. The RPHLK performs microbiology for all (three) hospitals, most general practitioners and most nursing homes in the region Kennemerland which comprise over 650.000 inhabitants. Hospital one is a 260 bed regional hospital, hospital two and three are 500 bed teaching hospitals.

Microbiological methods

All isolates were tested for resistance patterns using the Vitek2 System (BioMérieux). Isolates suspected for ESBL production (lowered susceptibility for ceftazidime and/or cefotaxime) were further determined using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics, Germany). Phenotypic confirmation of ESBL was performed using the combination disk method using cefotaxim and ceftazidime with and without clavulanic acid (Becton Dickinson, Netherlands). All tests were performed and interpreted according to the National Guideline for Laboratory Detection of ESBL [14].

Molecular typing by Amplified Fragment Length Polymorphism

Dna-lysates of ESBL positive *Klebsiella* spp. isolates were genotyped by Amplified Fragment Length Polymorphism (AFLP) using the restriction enzymes *Eco*RI and *Mse*I according to previously described methods [15]. Digested-ligated products were amplified with adaptor-specific primers with selective extensions, *Mse*+C and *Eco*RI+A. Primer *Eco*RI+A was labeled with D3 for fragment separation with the CEQ8000 Genetic Analysis System (Beckman-Coulter). The collected raw data were analyzed using the Bionumerics v6.6 software (Applied Math). To analyze and group AFLP patterns a Pearson correlation UPGMA with a curve smoothing of 0.5%, and an optimization of 2% was used. Isolates were considered clonally related when *Klebsiella* strains were identical, including strains with a concordance higher than >90%.

Molecular typing by High-throughput MultiLocus Sequence Typing

All the available *Klebsiella* spp. isolates were subjected to MLST. For this, partial DNA sequences of the seven housekeeping genes *gap*A, *inf*B, *mdh*, *pgi*, *pho*E, *rpo*B and *ton*B were generated using the High-Throughput-MultiLocus Sequence Typing (HiMLST) strategy as described by Boers et al [16]. The oligonucleotides used for PCR amplification reported in the standardized MLST scheme by Diancourt et al were modified to reduce amplicon sizes and to contain universal tails for the employment of HiMLST (Table S1) [17]. Allele variant numbers and corresponding sequence types (STs) were obtained by performing queries in the *Klebsiella* MLST database, which is available online [18].

Antibiotic phenotype

The antibiotic phenotype characterization of the isolates was based on species type (*Klebsiella pneumoniae* or *Klebsiella oxytoca*) combined with a number of selected antibiotics: ciprofloxacin-co trimoxazol-tobramycin-carbapenem-nitrofurantoin. Resistance towards carbapenem was defined as resistance towards either (or both) meropenem and imipenem.

Definition of ESBL-G clusters, epidemiological linkage and transmission index

A cluster of ESBL-G was defined as two or more patients with identical Sequence Types (STs) and epidemiological linkage, which was defined as patients who had stayed on the same ward in the same hospital or primary care institution within a maximum time window of four weeks [19]. The transmission index was calculated as the number of secondary cases (number of patients with epidemiological linkage, without the index patient) divided by the number of index patients plus the number of primary cases (single patients without clustering).

Intra-patient (antibiotic phenotypic) comparison of consecutive ESBL-G isolates

All consecutive isolates (with equal ST as the first isolate) of ESBL-G positive patients were compared with the first isolate and included in these analyses. A consecutive isolate was marked as different when resistance towards one of the included antibiotics changed (as an example: from resistant to susceptible or the other way around).

Results

Demographic and clinical characteristics of ESBL-G carriers

In 2012, 38 patients were diagnosed with an ESBL positive gentamicin resistant *Klebsiella* spp. isolate in the region Kennemerland, the Netherlands (Table 1). The mean (SD) age of these patients was 66.5 (18.8) years, and 19 (50%) patients were male. Sixteen (42.1%) patients were diagnosed in the primary care

setting, ten (26.3%) patients in hospital 2, and six (15.8%) patients in both hospital 1 and 3. The isolates were derived from a broad range of non-sterile body sites (see Table 1). The majority of isolates were determined as *Klebsiella pneumoniae* (34 isolates (89.5%)) followed by *Klebsiella oxytoca* (four isolates (10.5%)). All isolated *Klebsiella oxytoca* isolates were diagnosed in hospital 1.

HiMLST, AFLP and phenotypical method

The results of the molecular and phenotypical analyses are shown in Table 2. The isolates were classified into 18 different STs and 21 different AFLP types. The phenotypical analyses consisting of species type and sensitivity patterns for a selection of antibiotics (ciprofloxacin-co trimoxazol-tobramycin-carbapenem-nitrofurantoin) classified the isolates into 17 different antibiotic phenotypes. As displayed in Table 2, the AFLP types showed similar results in comparison with HiMLST, except for ST 405 (two different AFLP types, type M and type J), ST 37 (two different AFLP types, type I3 and K) and ST 17 (three different AFLP types, type L1, L2 and L3). These discrepancies resulted in a concordance between typing techniques of 84.6% (number of different AFLP types divided by the number of isolates of which two of more STs were available).

The phenotypical characterization showed a high variation between isolates with the same sequence type and AFLP type. All STs (with more than one strain) showed two or more different antibiotic phenotypes.

Clustering and transmission

Based on sequence type (obtained from the HiMLST analysis) and clustering definitions, we could differentiate four clusters (Figure 1). Three clusters were detected in the hospitals, and one cluster was detected in a nursing home (Table 2). No transmission was detected between hospitals and none of the patients was transferred to another hospital. The largest cluster comprised four patients colonized/infected with ST 405 located in hospital 2 (cluster B). Furthermore, we identified three other clusters: ST 193 (cluster A, hospital 2), KO_01 (cluster C, hospital 3) and ST 1207 (cluster D, primary care). Based on AFLP typing, we could differentiate the same four clusters that were detected with HiMLST (Cluster A–D). Instead of the four patients in cluster B who were identified by HiMLST, AFLP typing identified only three of these patients.

Three patients of cluster B (patient B1, B2 and B3, hospital 2) were part of a known cluster. We additionally linked one more patient (patient B4) to this cluster based on the HiMLST analyses performed in this study. All patients in cluster B were diagnosed between November 2012 and December 2012. After patients B1, B2, and B3 were identified and an ESBL-G isolate was isolated from a siphon (located in the room of the colonized patients) infection prevention procedures consisting of contact isolation on a single room (following the national directive for MDRO) and replacement of the siphon successfully stopped transmission.

The patients of cluster C (hospital 1) were also known before the start of this study. All patients were diagnosed between January 2012 and May 2012. After infection prevention procedures, consisting of contact isolation on a single room were installed (following the national directive for MDRO), transmission was stopped successfully.

Additionally, two new clusters (A and D) were identified after the molecular analysis performed in this study. Cluster A was detected in hospital 2 and consisted of two patients (diagnosed between May 2012 and July 2012). Cluster D comprised three patients living in a nursing home which were linked based on strain typing results (diagnosed between June 2012 and August 2012). Without this study these clusters were not identified.

Table 1. Demographic and clinical characteristics of patients and isolates.

Patient characteristics	Total	Hospital 1	Hospital 2	Hospital 3	Primary care
Number of isolates and patients	38 (100)	6 (15.8)	10 (26.3)	6 (15.8)	16 (42.1)
Gender					
Male	19 (50)	3 (50)	6 (60)	5 (83.3)	5 (31.3)
Mean age, yrs (SD)	66.5 (18.8)	69.3 (9.9)	66.2 (21.1)	67.7 (10.0)	65.3 (23.2)
Sample sites					
Non sterile					
Gastro-intestinal tract †	11 (28.9)	1 (16.7)	4 (40)	1 (16.7)	5 (31.2)
Catheter	1 (2.6)	0	0	1 (16.7)	0
Throat	1 (2.6)	0	1 (10)	0	0
Sputum	3 (7.9)	2 (33.3)	1 (10)	0	0
Urine	9 (23.8)	0	1 (10)	2 (33.3)	6 (37.5)
Urine catheter	7 (18.5)	1 (16.7)	1 (10)	1 (16.7)	4 (25)
Wound	4 (10.5)	2 (33.3)	1 (10)	0	1 (6.3)
Other	1 (2.6)	0	1 (10)	0	0
Sterile					
Blood	1 (2.6)	0	0	1 (16.6)	0
Species					
***Klebsiella* pneumoniae**	34 (89.5)	2 (33.3)	10 (100)	6 (100)	16 (100)
***Klebsiella* oxytoca**	4 (10.5)	4 (66.7)	0	0	0

†including faeces, perineum, rectum and peri-anal samples.
Data are presented as numbers (%) unless indicated otherwise.
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Table 2. All first isolated patient related strains with corresponding sequence type, AFLP type and phenotype.

Strain number †	HiMLST	AFLP	Phenotype *	Location of diagnosis	Cluster (HiMLST) ‡	Cluster (AFLP) ¥
1	147	N	KP-RRISI	Primary care	–	–
2	161	I2	KP-RRISR	Primary care	–	–
3	17	L1	KP-IRRSI	Primary care	–	–
4	17	L1	KP-IRRSI	Hospital 3	–	–
5	17	L1	KP-RRRSR	Primary care/Hospital 3	–	–
6	17	L2	KP-IRRSS	Hospital 3	–	–
7	17	L3	KP-IRRSI	Hospital 1	–	–
8	17	L3	KP-RRRSR	Primary care	–	–
9	193	R	KP-RRRSI	Hospital 2	A	A
10	193	R	KP-RRRSI	Primary care	–	–
11	193	R	KP-RRRSR	Hospital 2	A	A
12	37	I3	KP-SRISR	Hospital 3	–	–
13	37	I3	KP-SRISR	Primary care	–	–
14	37	K	KP-RRRSR	Hospital 1	–	–
15	392	P	KP-RRRRS	Hospital 3	–	–
16	405	J	KP-IRRSR	Hospital 2	B	–
17	405	M	KP-IRISI	Hospital 2	–	–
18	405	M	KP-IRISI	Primary care/Hospital 2	–	–
19	405	M	KP-IRRSR	Hospital 2	–	–
20	405	M	KP-IRRSR	Hospital 2	B	B
21	405	M	KP-RRISR	Primary care/Hospital 2	–	–
22	405	M	KP-RRRSR	Hospital 2	B	B
23	405	M	KP-RRRSR	Hospital 2	B	B
24	414	H	KP-SRSSI	Hospital 2	–	–
25	45	I4	KP-SRISI	Primary care	–	–
26	641	W	KP-SSISI	Hospital 3	–	–
27	946	I5	KP-IRRSR	Primary care	–	–
28	KO_01	A	KO-RRRSI	Hospital 1	C	C
29	KO_01	A	KO-RRRSS	Hospital 1	C	C
30	KO_01	A	KO-RRRSS	Hospital 1	C	C
31	KO_02	B	KO-SRRSI	Hospital 1	–	–
32	1418	I1	KP-SRSRR	Primary care	–	–
33	1420	U	KP-RRISR	Hospital 3	–	–
34	1421	E1	KP-IRRSS	Hospital 2	–	–
35	1423	X	KP-IRRSS	Primary care	–	–
36	1207	I4	KP-RRRSI	Primary care (nursing home A)	D	D
37	1207	I4	KP-RRRSR	Primary care (nursing home A)	D	D
38	1207	I4	KP-RRRSR	Primary care (nursing home A)	D	D

HiMLST = High-troughput multilocus sequence typing.
AFLP = Amplification Fragment Length Polymorphism.
*Phenotype = Species type and resistance patterns for ciprofloxacin-co trimoxazol-tobramycin-carbapenem-nitrofurantoin. S = susceptible I = intermediate R = resistant.
‡A cluster of ESBL-G was defined as two or more patients with epidemiological linkage and the same ST-type.
†Only the first positive isolate per patient is included.
¥A cluster of ESBL-G was defined as two or more patients with epidemiological linkage and the same AFLP-type.
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However all patients were isolated following the national directive for MDROs. In total, eight patients acquired ESBL-G as a result of transmission (following the criteria of epidemiological linkage and clustering), while four patients were classified as index patients. 26 patients were classified as primary cases. The overall transmission index of ESBL-G in the region Kennemerland in 2012 was 0.27.

Intra-patient comparison of phenotypes

Sixteen of the 38 ESBL-G positive patients had more than one consecutive ESBL-G isolate (42.1%) with an identical sequence type as the first isolate (all first isolates shown in Table 2) available. After analyzing all 76 consecutive ESBL-G isolates of these sixteen

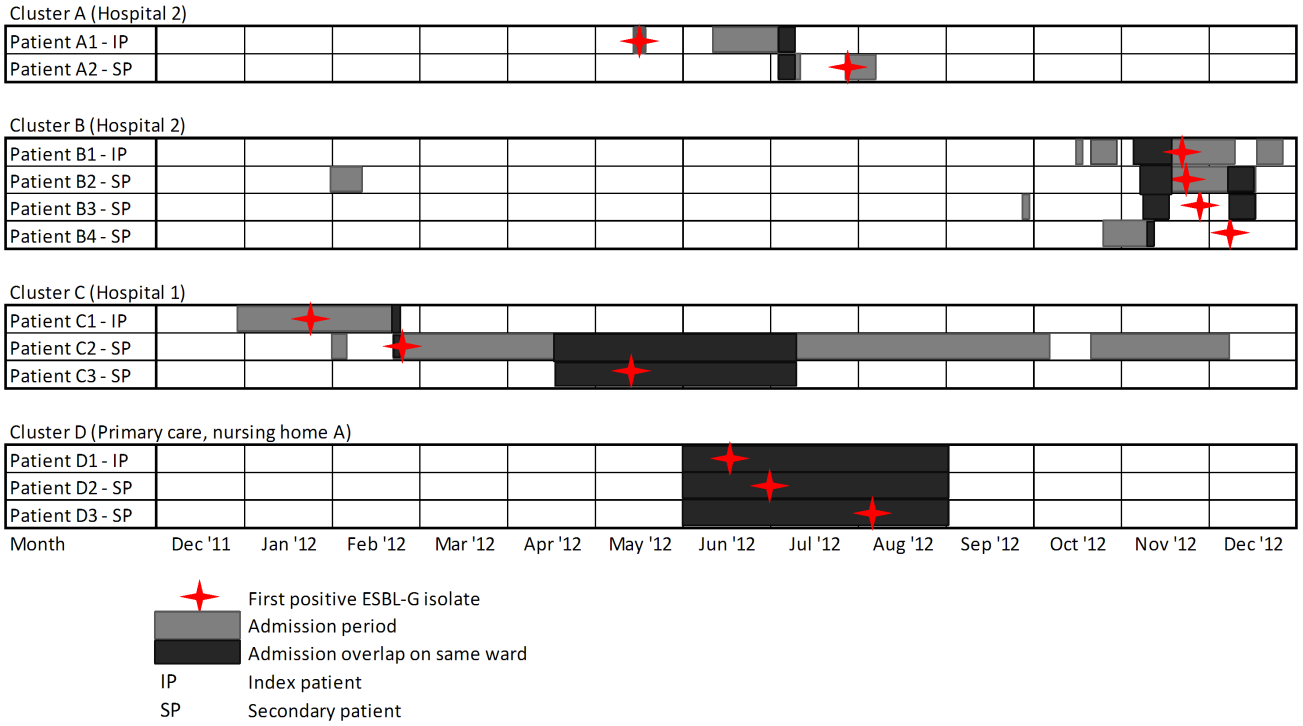

Figure 1. Transmission dynamics of ESBL positive *Klebsiella* strains with an additional resistance towards gentamicin (ESBL-G) in 2012. Grey boxes correspond with admission periods for each patient. The star symbol marks the date of first ESBL-G positive culture. Black boxes represent the periods of overlap in admission time and ward between patients. IP represents index patients and SP represents secondary patients according to the definitions.
doi:10.1371/journal.pone.0101212.g001

patients, the data showed that eleven patients (68.8%) had at least one different consecutive antibiotic phenotype.

Discussion

For this present study, we genotyped all isolated ESBL positive *Klebsiella* spp. with an additional resistance towards gentamicin (ESBL-G) derived from patients in the region Kennemerland, the Netherlands in 2012, by using AFLP, HiMLST and an antibiotic phenotypical method. Although the prevalence of ESBL-G positive patients among all *Klebsiella* spp. positive patients was relatively low (2.6% in 2012), several clusters were identified. By genotyping (using AFLP and HiMLST) all ESBL-G strains, we detected four clusters, of which two were previously unknown. No transmission between institutions was detected. A possible explanation for this finding is that none of the positive patients was transferred between hospitals. The genotyping data showed comparable results for AFLP and HiMLST: both techniques identified four clusters. Only in cluster B one patient was missed when only AFLP was used. Seen from an epidemiological point of view this patient is correctly classified as part of cluster B since there was an epidemiological link present (admission on same ward in a time window of 4 weeks). Although we found some discrepancies between AFLP and HiMLST (concordance of 84.6%) it is difficult to explain the cause of these discrepancies as most of these patients had no epidemiological link.

The phenotypical method, consisting of the identification of species type and resistance towards several selected antibiotics, could not adequately detect these clusters. These data suggest that, in this setting, the phenotypical method (using an antibiogram) is not suitable for the identification of clusters among ESBL-G isolates. However, the antibiotic susceptibility testing does provide relevant information with respect to the treatment of patients, as the different ESBL-G strains isolated within patients showed high variation in the antibiogram.

Although several studies have described outbreaks of multiresistant *Klebsiella* spp. (MRK) including ESBL-G, this study is (to our knowledge) the first Dutch study showing regional transmission dynamics of ESBL-G in both hospitals and primary care patients [9–13]. In 2011, the TRIANGLE study described the transmission of highly resistant gram-negative microorganisms including MRK in 18 Dutch hospitals by analyzing routine clinical samples during a six month period [13]. The same study showed low horizontal transmission rates (ranging from 0.0 to 0.2) and detected 22 clusters (in 18 hospitals) by using AFLP. Most of the isolated enterobacteriaceae (54.3%) were ESBL producers. In the present study we found a transmission rate of 0.27. Although this rate is not directly comparable with the results of the previous mentioned study (because different bacterial species were studied) it does indicate a considerable transmission capacity of ESBL-G *Klebsiella* spp. A comparison between other studies is difficult because different definitions, other bacterial species and/or single centers were studied.

When analyzing the consecutive ESBL-G *Klebsiella* isolates (with identical STs) within patients, we found a high percentage of variation in antibiogram of these intra-patient consecutive isolates. A possible explanation for the variation in antibiograms is that the genes encoding for resistance of these antibiotics (especially aminoglycosides and quinolones) are frequently found on plasmids, and could be selected out by the use of these antibiotics in the treatment protocol of the patients. Several previous studies have reported this plasmid mediated co-resistance in ESBL positive bacteria [20,21].

Genotyping MRK can be performed using different techniques. These techniques contain fragment based methods such as: AFLP, PFGE, Rep-PCR and MLVA but also DNA sequence techniques such as MLST [22,23]. In this study, we used AFLP and HiMLST to genotype ESBL-G isolates. One of the advantages of using AFLP is the faster procedure time, which may be essential for genotyping in local epidemiological and outbreak investigations. Furthermore, with AFLP almost the whole genome is covered, resulting in a higher discriminatory capacity [24]. A possible drawback of the AFLP technique is the absence of an inter-laboratory database and the low inter-laboratory reproducibility of this technique, caused by the different platforms that are used worldwide. As a result, no comparison in global epidemiology is possible with the results of the AFLP typing data. On the other hand MLST uses an internationally accepted nomenclature, targeting seven housekeeping genes regardless of the platform used to generate them, showing its comparability [24]. In term of costs and labor, MLST is much more labor intensive and expensive than the AFLP technique. However, the introducing of HiMLST has resulted in a sharp reduction of costs since this method allows the simultaneous analysis of a large number of isolates. By using HiMLST, the price per analyzed isolate is comparable to that of AFLP [16]. Considering these arguments, HiMLST seems to be the most suitable technique for regional monitoring procedures including genotyping.

Clustering patients on the basis of equal STs (without an epidemiological link) can be interpreted in several ways. (1) The appearance of the strain could simply reflect polyclonal spread rather than transmission [25]. (2) On the other hand transmission could be present, but not identified since carriership is generally asymptomatic [26]. As a result, intermediate patients are missed and no epidemiological link can be made. However, when patients with ESBL-G isolates with identical STs in addition show epidemiological linkage, transmission is probably the case. One must be careful to conclude there is no transmission, taking into account the possibility of asymptomatic carriership. Nevertheless, in our study the difference between a time window of one day or four weeks in the epidemiological link definition did not increase the number of secondary cases.

The present study has several limitations. First of all, no plasmid typing was performed on the collected ESBL-G isolates. Since we described regional transmission of ESBL-G, it would be interesting to assess ESBL producing genes, as plasmid transmission is possible between bacteria of the same and other species [27]. This could possibly clarify transmission routes, or help to identify yet unknown reservoirs. Second, we have retrospectively described the regional transmission on the basis of clinical samples, collected from symptomatic patients which is a major drawback of this study. As it is well known that colonization of ESBL positive bacteria is not uncommon among the hospitalized population, the extent of the transmission could be much larger than described, since colonization could be established without infection [28]. For future studies we would advice using a prospective study design, including for example screening of all contact patients when an ESBL-G positive patient is detected (in clinical samples). This prospective study design requires a lot of cooperation and effort to perform regionally, especially in all participating nursing homes.

In conclusion, our results show that the transmission of ESBL-G *Klebsiella* in the region Kennemerland is polyclonal (without transmission between institutions) with several outbreaks (with the majority of patients being part of clusters with a high level of epidemiological linkage) that could be identified. Furthermore, clustering by antibiogram phenotype characterization seems to be an inadequate method in this setting. The routine practice of molecular typing of collected ESBL-G isolates may help to detect nosocomial spread in an early stage, which opens the possibility of a rapid response.

Supporting Information

Table S1 MLST target gene-specific primers used in this study. Nucleotides in black represent the gene-specific part and universal tails are shown in red or blue.
(DOC)

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Author Contributions

Conceived and designed the experiments: DS DV SME JK JWB. Performed the experiments: DV SAB. Analyzed the data: DS SME. Contributed reagents/materials/analysis tools: DV SAB. Wrote the paper: DS SAB DV SME JK JWB.

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ORIGINAL

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Patterns of colonization by *Pseudomonas aeruginosa* in intubated patients: a 3-year prospective study of 1,607 isolates using pulsed-field gel electrophoresis with implications for prevention of ventilator-associated pneumonia

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Abstract *Objective:* To identify routes and patterns of colonization with *Pseudomonas aeruginosa* in intubated patients to design strategies of prevention for respiratory infection. *Design and setting:* Prospective and observational study in the 16-bed intensive care unit of a teaching hospital. *Patients and participants:* Ninety-eight intubated patients were investigated over a 3-year period. Those ventilated less than 72 h were excluded. *Measurements and results:* Samples from the tap water from each patient's room, stomach, oropharynx, subglottic secretions, trachea, and rectum were collected when the patient was intubated, and then three times per week. Pulsed-field gel electrophoresis was performed to type the strains. We identified 1,607 isolates pertaining to 35 different pulsotypes. Overall 54.2% of patients presented colonization, and tracheal colonization was present in 30.5%. Ten patients had colonization at intubation, and four of these developed ventilator-associated pneumonia (VAP) after a mean of 4±2 days. ICU-acquired colonization occurred in 31 patients, and 4 of these developed VAP after a median of 10±5 days. *P. aeruginosa* was isolated from the room's tap water in 62.4% of samples. More than 90% of tap water samples had pulsotypes 1 and 2, which were frequently isolated in the stomach (59%) but were only rarely associated with VAP. *Conclusions:* Although colonization/infection with *P. aeruginosa* in intubated patients tends to be endogenous, exogenous sources should not be ruled out. A combination of early identification (and eradication) of airways colonization by *P. aeruginosa* plus infection control measures targeted to reduce cross-contamination should be the basis to prevent pulmonary infection.

Keywords *Pseudomonas aeruginosa* · Colonization · Pneumonia · Mechanical ventilation · Intensive care

Introduction

Pseudomonas aeruginosa (PA) is highly endemic in intensive care units (ICUs), where it causes urinary tract infections, surgical wound infections, bacteremia, and pneumonia [1, 2]. According to the report of the National Nosocomial Infections Surveillance System [3], PA is the second most frequent pathogen in ICUs. Although colonization by PA frequently precedes overt infection, the origin of the organism and the precise mode of transmission are often unclear [4, 5]. The origin may be exogenous (the environment, other patients, or respiratory equipment) or endogenous (the oropharynx, stomach, or gut). Establishing the source of strains causing colonization is crucial for the development of effective preventive measures.

The main goal of this study was to identify the routes of colonization by PA in intubated patients and the sources of the strains causing colonization or respiratory infection over a long period and without an epidemic outbreak. The findings may have important implications for the design of evidence-based preventive measures for ventilator-associated pneumonia (VAP). A secondary goal was to determine whether patients were colonized or infected with a single or with multiple genotypes of PA, an issue that may have implications for diagnosis.

Material and methods

This prospective study was conducted over a 35-month period (1 April 1996 to 28 February 1999) in a medical-surgical ICU of a teaching hospital. The Human Subjects Review Committee of the Hospital approved the study protocol (96-027).

Study population

The study originally enrolled 98 patients, 26 of whom were later excluded because the duration of MV was less than 72 h; thus 72 patients were finally evaluated (Table 1). All patients intubated with Hi-Lo Evac (Mallinckrodt Laboratories, Athlone, Ireland) were eligible; patients with tracheotomies and different tubes were not included. A maximum of three patients were evaluated simultaneously. The nurse-patient ratio was 1:2. The Acute Physiology and Chronic Health Evaluation II score [6] was used to assess the severity of illness at admission. The infection control measures used in the ICU during the study period consisted of hand hygiene with clorhexidine, use of gloves, and cleaning once daily with tap water of the sink and environment surfaces using glutaraldehid solutions. Eight patients died during the study.

Table 1 Characteristics of the 72 patients included in the colonization study

Age (years)	65±17
Sex: M/F	51/21
APACHE II at admission	20±8
Cause of ICU admission	
Respiratory failure	40.8%
Cardiovascular failure	22.5%
Neurological diseases	15.5%
Renal failure	8.5%
Metabolic disturbances	5.6%
Gastrointestinal diseases	4.2%
Hematological disturbances	2.8%
Days in the study (range)	7.6±5.5 (3–21)
Days of ICU stay (range)	18.5±17.1 (3–91)

Surveillance cultures

Samples from gastric aspirate, oropharyngeal swab, subglottic secretions, internal surface of endotracheal tube, tracheal secretions, and rectal swab were taken in each patient after intubation and then three times per week. Tap water from the individual ICU room was also cultured at admission and every 72 h. Follow-up was considered complete at 21 days of mechanical ventilation (MV) or when the patient was extubated, a tracheotomy was carried out, the patient died, or an episode of VAP by PA was diagnosed. Surveillance cultures from environmental surfaces of the ICU and from the hands of health care workers (HCW) were taken on five separate days during the study period, without prior warning.

Definitions

Colonization was defined as the isolation of PA from specimens taken from any body site studied in the absence of infection. Colonization at intubation was defined as that occurring within 24 h of entry into the study. When colonization occurred more than 48 h after entry in the study, it was defined as ICU acquired. The trachea was considered as the initial site of colonization when no prior colonization was documented at other sites. The routes of PA colonization leading to the trachea were determined by analyzing the chronological comparison of the pulsotypes (PTs) obtained from the different samples.

Primary or endogenous colonization was defined as colonization by a strain of PA that had not been previously isolated from another studied patient, the hands of HCW, or another site analyzed in the ICU environment. Exogenous colonization was defined as colonization by a strain of PA with a PT previously isolated from another studied patient, hands of HCW, or another environmental surface in the ICU. Diagnosis of VAP has been reported elsewhere and confirmed by a positive protected specimen brush culture containing at least 10^3 colony-forming units (CFU)/ml, a positive bronchoalveolar lavage culture with at least 10^4 CFU/ml or by quantitative endotracheal aspirate with 10^6 CFU/ml or more [7, 8].

Microbiological and bacterial typing

Hands of HCW were studied by the rinse sampling method [9]. All surfaces studied and taps were studied by the wipe-rinse sampling method or the swab-rinse sampling method [9]. Samples were plated in cetrimide agar plates and in nonselective broth. Pharyngeal swab, gastric aspirate, subglottic secretions, tracheal aspirate, endotracheal tube, and rectal swab of each patient were sampled on cetrimide agar plates and chocolate agar plates, and the cultures were reported semiquantitatively. Whenever possible at least four colonies representative of the different morphological types of PA present on the culture plate were picked for subsequent characterization beyond species level.

Pulsed-field gel electrophoresis (PFGE) was performed using a previously described method [10]. Electrophoresis was performed with a Chef DRIII System apparatus (Bio-Rad, Richmond, Calif., USA) under conditions appropriate for the enzymes. Analysis of PFGE profiles was made with the software Bio Image Whole Band Analyzer (Genomic Solutions, Ann Arbor, Mich., USA).

Each strain was first compared with all others to calculate similarity using the Dice correlation coefficient (S_D). S_D was defined as twice the number of shared bands divided by the total number of bands. Isolates were defined as belonging to the same clonal lineage if their patterns exhibited less than six band differences (two genetic events) [11]. The most common profile among the isolates of each lineage was designated as the parental profile. Numbers designated strains corresponding to each major macrorestriction type, and a letter suffix indicated each of their subclonal variants (subtypes). The patterns were ordered chronologically according to time of collection. All isolates were first digested with *Xba*I. Moreover, a representative strain of the different PTs and subtypes were subjected to further digestion with a second enzyme (*Dra*I). The final types and subtypes are the result of the combined two-enzyme analysis.

Statistical analysis

Descriptive analysis was performed. Continuous variables were expressed as means (±SD). Associations of categorical variables were assessed with the χ^2 test or with Fisher's test.

Results

In 39 (54.2%) PA colonization could be proven during the study period (Fig. 1). Ten patients (13.9%) were already colonized at intubation. Two of these acquired different PA strains during the study period. Thus 31 patients (43%) were colonized during the period of MV and were classified as ICU acquired.

Genotyping

When available, four colonies were subcultured and independently typed, and a total of 1,612 isolates were obtained; 5 of these could not be studied by PFGE. Thirty-five different PFGE patterns or pulsotypes (PTs) were found. Among 279 cultures performed from the five environmental surveillance cultures 41 (14.7%) were positive for PA, and 15 different PTs were identified: the most frequent were PT1 (31.7%), PT7 (17.1%), and PT8 (14.6%). The cultures from the inanimate surfaces of the ICU were positive for PA in 34.3% of samples and the hands of HCW in 6% of cases. On the other hand, 93 of 149 (62.4%) cultures from the tap water of patients' rooms were positive for PA. Two PTs were identified in 26 cultures. Eleven different PTs were identified among PA cultures isolated from tap water, PT2 (74.2%) and PT1 (32.3%) being the most frequently isolated.

In the 39 colonized patients 288 positive cultures for PA were obtained. In this case, 22 different PTs were obtained, PT2 (51.3%) and PT1 (41%) being the most frequently isolated. The distribution of PTs differed significantly in the stomach than in other sites (Fig. 2).

Of the cultures analyzed 13% were polyclonals, 24.8% of environmental and tap water cultures, but only 7.3% of those from the various patient sites ($p<0.05$). The stomach (19.1%) and rectum (14.3%) had the highest incidence of polyclonals. Only one of the lower respiratory tract cultures was polyclonal.

Pseudomonas aeruginosa colonization

Ten of the 39 patients with PA (25.6%) were already colonized at intubation. Eight had prior hospitalization, and three were intubated after the day of admission in the ICU. Two had multiple PTs. Seven of the 13 strains of PA isolated in this subset (53.8%) had primary colonization (single PFGE pattern). The remaining six strains (46.2%) were considered exogenous colonization (three from tap water). Detailed information on distribution is shown in Table 2. Only four strains were initially isolated from the digestive tract (stomach or rectum).

Most of the 31 patients who suffered ICU-acquired PA colonization during ventilation presented simultaneous colonization at multiple sites. Distribution of colonization and days of colonization is shown in Table 3. Among the 31 patients with ICU-acquired colonization 47 strains of

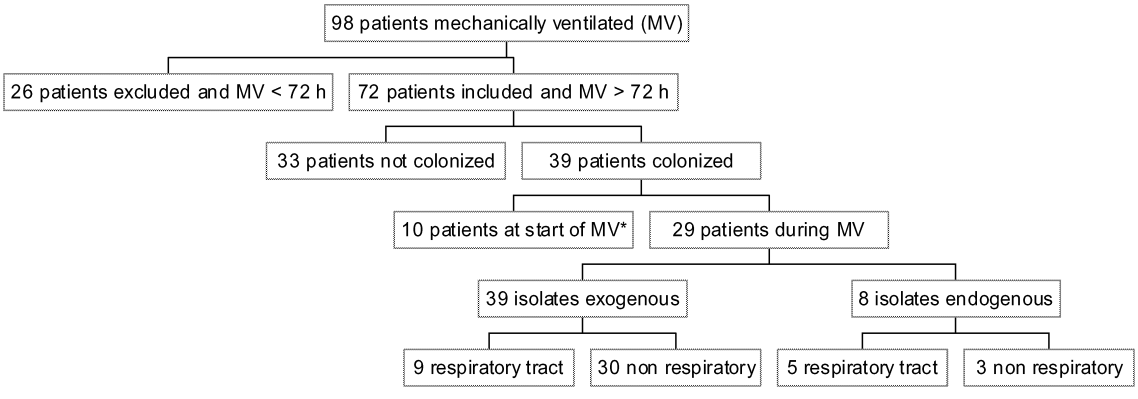

Fig. 1 Patients colonized by *P. aeruginosa* during the period of the study (*Two patients colonized by different strains during ICU stay)

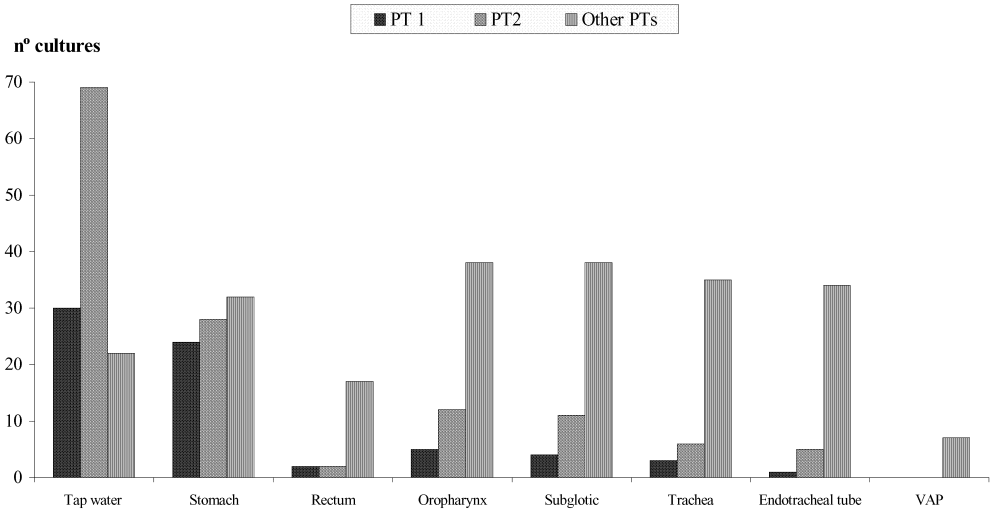

Fig. 2 Distribution of pulsotypes 1 and 2 and the remaining pulsotypes according to localization

$p < 0.01$ for pulsotypes 1 and 2 when comparing stomach with other sites from colonized patients

Table 2 Pattern of colonization among 10 patients colonized at onset of mechanical ventilation; first day of isolation in each site (*S* stomach, *Ophx* oropharynx, *SG* subglottic, *ET* endotracheal tube, *T* trachea, *R* rectum, *VAP* ventilator-associated pneumonia, *E* exogenous, *P* primary, *PT* pulsotype)

Patient no.	Intubation day	PT	Tap water	Localization (first day detected colonization)						VAP	Source
				S	Ophx	SG	ET	T	R		
8	1	1	1	–	–	–	–	–	–	–	P
		5	1	–	–	–	–	–	–	–	
		6	–	1	–	1	1	1	–	3	
31	1	1	1	1	–	–	–	–	–	–	E
53	1	1	1	–	–	–	–	–	–	–	P
		2	1	–	–	–	–	–	–	–	
		16	–	1	1	1	1	1	–	5	
55[a]	1	18	–	–	–	–	–	–	1	–	P
57	3	22	1	1	1	3	3	3	3	–	E
		19	–	–	–	–	–	–	1	–	P
		21	–	–	–	–	–	–	1	–	P
75	6	22	–	1	1	1	3	1	–	–	E
88	4	2	1		1	6	–	9	–	–	E
		34	–	3	3	1	1	1	–	–	P
90	1	1	1	–	–	–	–	–	–	–	P
		35	–	1	1	1	1	1	–	–	
92	1	1	1	–	–	–	–	–	–	–	E
		2	1	–	–	–	–	–	–	–	
		7	–	3	3	3	1	3	3	6	
94[a]	1	1	1	–	–	–	–	–	–	–	E
		6	–	3	1	1	3	3	–	5	

[a] Patients also colonized with different pulsotypes during the period of mechanical ventilation

PA were isolated. Eighteen patients were colonized with a single PT. Of the 47 strains of PA 39 (83%) were classified as exogenous. The stomach was the site with the highest incidence of exogenous colonization (94% of strains). PT1 and PT2 isolated from the tap water and environment were the most frequent PTs colonizing patients with ICU-acquired colonization. Excluding these PTs, the majority of the remaining PTs were considered endogenous. Detailed information on body sites colonized and PTs of PA in this group of patients is shown in Fig. 3 and Table 3.

Most of patients included in the study (94.4%) received antibiotic treatment prior to PA colonization. Sixteen patients (22.2%) were treated with third-generation cephalosporins, and 35 of 72 patients (48.6%) received antibiotics affecting PA. Only the prior treatment with

Table 3 Sequences, first day of colonization and sources of strains of *P. aeruginosa* in 31 patients with colonization acquired during mechanical ventilation; first day of isolation in each site (*S* stomach, *Ophx* oropharynx, *SG* subglottic, *ET* endotracheal tube, *T* trachea, *R* rectum, *VAP* ventilator-associated pneumonia, *E* exogenous, *P* primary, *PT* pulsotype)

Patient	Intubation day	PT	Tap water	S	Ophx	SG	ET	T	R	VAP	Source
2	2	1	1	3	3	–	3	3	–	3	E
		2	1	3	3	–	–	–	–	–	E
4	1	1	1	6	–	–	–	–	–	–	E
		2	1	6	–	–	–	–	–	7	E
		3	–	–	–	–	–	–	–	–	P
5	1	1	–	3	–	–	–	–	–	–	E
		2	3	–	3	3	–	–	3	–	E
		4	–	–	–	–	–	–	–	–	P
6	1	1	6	15	–	–	–	–	–	–	E
		2	6	12	–	–	–	–	–	–	E
7	1	2	3	3	–	–	–	–	–	–	E
11	1	1	–	–	3	3	–	3	–	–	E
		2	1	–	–	–	–	–	–	–	
13	4	1	–	3	3	3	–	–	3	–	E
		2	1	–	–	–	–	–	–	–	
14	1	2	1	3	–	–	–	–	–	–	E
16	1	2	1	–	6	–	–	–	–	–	E
18	1	1	1	–	–	–	–	–	–	–	P
		10	–	–	6	6	–	–	9	–	
21	1	2	1	–	–	–	–	–	–	–	P
		11	–	–	–	9	9	9	–	–	
22	1	2	1	3	3	3	–	–	–	–	E
26	3	12	–	–	–	–	9	9	–	–	P
32	1	1	–	3	–	–	–	–	–	–	E
36	1	2	3	6	6	–	–	–	–	–	E
43	1	1	–	9	–	–	–	–	–	–	E
		14	1	3	9	9	9	9	9	–	E
		15	–	–	–	–	–	–	–	–	
55[a]	1	2	1	15	–	–	–	–	–	–	E
		17	1	15	18	18	21	21	18	–	E
66	1	2	3	3	–	–	–	–	–	–	E
68	2	1	1	3	–	–	–	–	–	–	E
		2	1	9	–	–	–	–	15	–	E
		23	1	–	–	–	–	–	–	–	
69	2	1	1	–	–	–	–	–	–	–	–
		2	3	6	6	9	12	6	–	–	E
71	1	1	1	3	–	3	–	–	–	–	E
72	1	2	1	3	–	–	12	–	–	–	E
		24	3	–	–	–	–	–	–	–	
		25	9	3	6	3	6	3	–	–	P
73	1	1	–	3	–	–	–	–	–	–	E
		2	1	6	–	–	–	–	6	–	E
		26	–	–	–	–	–	–	–	–	P
76	1	1	1	12	–	–	–	–	–	–	E
		2	1	9	–	–	–	–	–	–	E
77	1	1	–	6	3	3	–	6	–	–	E
		2	1	–	6	3	–	–	–	–	E
		27	1	–	–	–	–	–	–	–	
78	1	28	1	3	6	9	9	9	6	–	E
82	1	2	1	12	–	–	–	–	–	–	E
83	3	1	–	–	3	–	–	–	–	–	E
		2	1	–	–	–	–	–	–	–	
		27	9	–	–	–	–	–	–	–	
		29	–	3	3	6	6	6	–	17	P
86	3	2	1	–	–	–	–	–	–	–	E
		7	–	–	3	3	6	6	–	8	
		17	–	6	–	–	–	–	–	–	E
89	1	2	1	3	9	3	6	3	–	–	E
		24	1	–	–	–	–	–	–	–	
94[a]	1	1	1	3	–	–	–	–	3	–	E

[a] Patients also colonized with different pulsotypes at beginning of mechanical ventilation

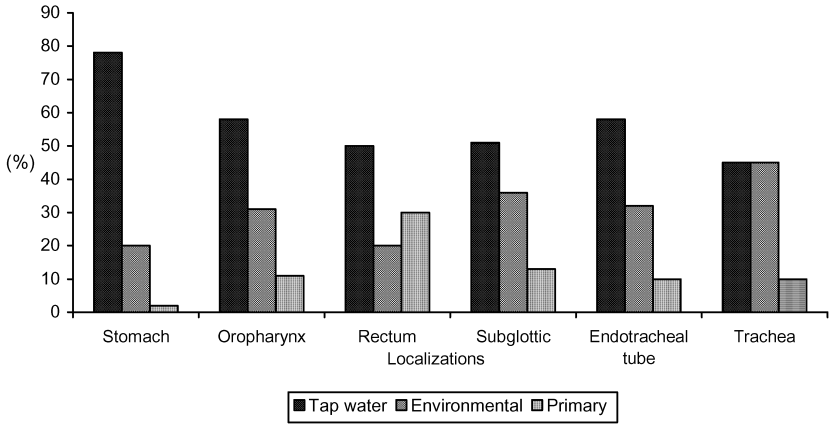

Fig. 3 Origin of *P. aeruginosa* strains according to localization among 31 patients with ICU-acquired colonization

Trachea vs. stomach between strains originated from tap water ($p< 0.01$)

third-generation cephalosporins was associated with a risk of colonization with PA (p=0.03).

Respiratory colonization

In our study 22 of 72 patients (30.5%) had colonization of the lower respiratory tract, 5 (6.9%) at intubation. Among the five patients colonized at the onset of MV four were primary. In contrast, 14 of 17 PTs isolated from the 17 patients with ICU-acquired tracheal colonization were of exogenous origin. Eight of these 17 PTs simultaneously colonized airways and gut; in three cases the first site colonized was the stomach. Only one patient had two different PTs.

Ventilator-associated pneumonia by *Pseudomonas aeruginosa*

Eight patients (11.1%) presented VAP caused by PA. Seven were confirmed by protected specimen brush and one by quantitative tracheal aspirate. The mean period of MV prior to the VAP onset was 7±4.7 days (range 3–17). Only two patients presented VAP within the first 4 days of MV. Among the five patients with upper respiratory tract colonized at onset of MV, four had VAP with 4±2 days (range 3–7) of intubation. The remaining four episodes of VAP were diagnosed in patients with ICU-acquired colonization, and the mean period of MV prior to the diagnosis of VAP was 10±5 days (range 6–17). The respiratory tract in seven of eight patients with VAP had been colonized previously by the same strain causing the pneumonia. The initial site of colonization for these strains was the upper airways in seven of eight patients (87.5%), but in four cases simultaneous digestive tract colonization was found. PFGE typing identified neither the stomach nor the rectum as the only initial site of colonization in any case. The mean period of previous tracheal colonization before the diagnosis of pneumonia was 4.3±3.4 days (range 2–11). In four cases (50%) the origin of strains causing VAP was considered exogenous (one tap water). In the remaining four patients with VAP the strains causing infection were considered primary.

Discussion

This study is unique in evaluating more than 1,600 isolates over a long period or time. More than one-half of intubated patients were colonized with PA, and this colonization was not limited to the respiratory tract. An important finding was that the source of strains causing ICU-acquired colonization was predominantly environmental, in most cases the tap water from the patient's room. However, this colonization was mainly in the gastrointestinal tract and did not affect the clinical course of the patients, because our results showed that the strains causing respiratory infections were endogenous in one-half of VAP episodes, emphasizing the limitations of infection control measures to prevent VAP by PA.

The incidence rate of colonization by PA in a range the ICU ranges between 4.5% and 37% [5, 12, 13, 14]. Our incidence was higher, although comparison of incidence rates with those other ICUs is difficult, mainly because of differences in patient populations and in the samples analyzed. Indeed, in our study among only the patients with prolonged MV (>72 h) the incidence rate of colonization by PA was 54.2%. Differences in patients who were already colonized at intubation, colonization pressure, and number of samples may explain these differences [15].

The stomach showed the highest incidence (84%) of ICU-acquired colonization, and this location was the earliest site of colonization in most of the patients. Previous studies [5, 16] have suggested that the origin of digestive colonization was endogenous, but they did not

simultaneously analyze patients' samples and environmental and tap water cultures, nor did they subculture and independently type four colonies. In our study more than 60% of the tap water in the ICU was contaminated by PA, mainly PT1 and PT2, the PTs that predominated among strains isolated from the stomach. These results suggest that the origin of gastric colonization by PA is predominantly exogenous, and that PA was transmitted directly through the water infused in the stomach by gastric tube or indirectly via handling by the HCW after handwashing. Tap water and sinks have been recognized as the origin of outbreaks of respiratory and urinary infections in pediatric and adult ICUs due to PA [17, 18, 19].

Our findings agree with a study carried out in a surgical ICU [17]. A follow-up study in the same surgical ICU [20] confirmed that all taps in the patients' rooms were contaminated with PA, and in 32% of cases a tap water isolate from the room was shown to be of the same genotype as the patient's isolate. Our findings suggest that in our ICU the tap water was the source of acquisition because in most cases the detection of PA took place prior to the detection of a strain in the patient, and because the tap water was colonized mainly by the same PTs (1 and 2) whereas the pattern of cultures of other environmental surfaces of the ICU and patient samples was more heterogeneous.

Our results indicate that the clinical importance of gastrointestinal colonization by PA in mechanically ventilated patients is low. Indeed, although most of our ventilated patients presented a gastric ICU-acquired colonization, we found that tracheal ICU-acquired colonization was present in only 23.6% of patients and was due predominantly to strains with PTs other than those found in tap water and in the stomach. Moreover, when we analyzed only patients with VAP caused by PA, no strain was found previously in the stomach. These results agree with a study on the pathogenesis of VAP [21] and demonstrate that a gastric origin is unlikely in strains of PA causing VAP.

This study is also unique in subculturing at least four colonies that were representative of the different morphological types of PA present on each culture plate. It allowed the origin of the strains to be identified and reduced the risk of underestimating exogenous colonization. Unlike environmental strains, isolates from airways were mainly monoclonal. The implications for diagnosis and therapy in clinical practice are evident.

One particularly interesting finding was that strains present in tap water or in the environment were associated with colonization, but only infrequently with infection. Indeed, our results show that more than 70% of strains causing gastric or respiratory colonization come from tap water or environmental surfaces of the ICU, whereas among strains causing pulmonary infection, 50% were of endogenous or primary origin, and only one of the remaining strains come from tap water. Further studies analyzing exoproducts or different virulence proteins secreted by different strains of PA may help to explain these findings. In fact, recent studies [22, 23] have reported that isolates of PA able to secret type III proteins are of increased virulence, and pulmonary infections caused by these strains are associated with higher mortality or recurrence. Further studies should investigate whether intubated patients colonized with type III secreting isolates are at a higher risk of developing pneumonia than patients colonized by nonsecretory strains of PA.

Our findings suggest that efforts to prevent colonization by PA should be directed toward decontamination of tap water and infection control measures that reduce cross-contamination from exogenous sources. However, recent reports [17, 20] have noted how difficult it is to reduce colonization in tap water. Cross-contamination from exogenous origin was involved in one-half of our episodes of pneumonia, and our findings support the use of alcoholic solutions for hand antisepsis [24]. In fact, our findings changed the infection control strategies in our ICU: only mineral water to administer drugs through the gastric tube, and alcohol solution was used before contact with a new patient.

Important limitations of our study were that not all intubated patients were consecutively included, and that samples for culture were obtained each 72 h. Cross-contamination from other colonized patients could thus not be detected, and in the cases with simultaneous detection of PA at different sites the true sequence of colonization remains uncertain. Other studies applying similar methods for sample collection (twice weekly) have had the same problem of interpretation [25]. Another limitation is the that only a reduced number of surveillance cultures of the environmental surfaces and hands of HCW were carried out. Although the incidence rate of hands colonized by PA was similar to that in a study [26] carried out in a neonatal ICU with endemic PA infection, increasing the number of cultures from environment and HCW should allow us to determine more exactly the importance of cross-colonization. Finally, our findings should not be generalized to ICUs with different colonization pressures and case-mixes.

In summary, although the origin of PA colonization in intubated patients is often exogenous, in patients who developed pulmonary infection the origin can be either exogenous or endogenous. Our results confirm that the ICU environment is a major source of micro-organisms colonizing critically ill patients and in particular emphasize the importance of exogenous colonization/infection of PA. A combination of early identification (and eradication) of airways colonization by PA plus infection control measures targeted to reduce cross-contamination should be the basis to prevent pulmonary infection.

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Outbreaks

Genomic epidemiological analysis of a single-centre polyclonal outbreak of *Serratia marcescens*, Belgium, 2022 to 2023

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Serratia marcescens **is an opportunistic pathogen with a propensity to cause nosocomial outbreaks, particularly in neonatal intensive care units (NICUs). We present a sustained outbreak spanning over 18 months (1 January 2022–29 August 2023) in a NICU in Antwerp, Belgium, affecting 61 neonates, identified through samples taken for diagnostic purposes and by rectal screening. Ten neonates were infected: five with lower respiratory tract infection, four with conjunctivitis and one fatal case with sepsis. In a logistic regression analysis, nursing in an incubator was significantly associated with acquisition of *S. marcescens* (odds ratio (OR): 2.99; 95% confidence interval (CI): 1.14–8.25; p<0.05). Whole genome sequencing-based multilocus sequence typing (wgMLST) and core genome single nucleotide polymorphism (cgSNP) analysis of isolates from clinical (n=4), screening (n=52) and environmental samples (n=8), identified eight clusters and five singletons not associated with the clusters. Although outbreak measures were successful in containing further spread within the ward during sudden surges when > 4 cases per week were identified (peak events), several peaks with different clonal clusters occurred. The emergence of similar outbreaks in Belgian hospitals underscores the need of continuous surveillance and NICU-specific infection prevention and control (IPC) measures.**

Introduction

Serratia marcescens, the most important opportunistic human pathogen in the *Serratia* genus, often causes outbreaks of hospital-associated infections, particularly in neonatal intensive care units (NICUs) [1-3]. The incidence of late-onset neonatal sepsis caused by *S. marcescens* has been estimated to 2.3 infections per 1,000 preterm infants. The infection is associated with significant morbidity and a reduced rate of survival (adjusted relative risk (RR): 0.88) [4]. Risk factors among neonates for acquisition of or infection with *Serratia* are low birth weight (<1,500 g), use of broad-spectrum antimicrobials, complex chronic conditions and indwelling catheter lines [3,5,6]. Outbreaks of *S. marcescens* are challenging to control and require early detection and rapid implementation of strict infection prevention and control (IPC) measures [7]. Although environmental sources of infection can usually not be identified and cross-transmission via hands of healthcare workers is assumed to be the main mode of transmission, water sources (e.g. wash-basins, air conditioning), liquid nutrition (e.g. breast milk, baby formula, total parenteral nutrition), soaps, disinfectants and medication have been implicated in *Serratia* outbreaks [3,8,9].

In Belgium, surveillance of infectious diseases is performed by the Scientific Institute of Public Health, Sciensano, with support by the National Reference Centers. No surveillance is performed for infectious diseases in neonatal (intensive care) units.

Outbreak detection

The index case (P5) was detected in the end of February 2022 when a preterm neonate born at

KEY PUBLIC HEALTH MESSAGE

What did you want to address in this study and why?

Serratia marcescens is a bacterium found in a large variety of environments, from soil and water to insect guts or hospital wards. It is a frequent cause of outbreaks in neonatal intensive care units (NICU). We investigated an outbreak with *S. marcescens* in a Belgian NICU to identify the transmission sources, assess the risk factors for infection and evaluate the effectiveness of implemented infection prevention strategies to contain the outbreak.

What have we learnt from this study?

We detected *S. marcescens* from 61 newborns and 71 environmental samples from the NICU. One case died. The bacterium was more often detected from newborns nursed in an incubator. Several variants of the bacterium were found in the patient and in the environmental isolates. We could not identify the source of the outbreak.

What are the implications of your findings for public health?

Control measures including intensive screening of patient and the environment, patient isolation and enhanced cleaning prevented further spread within the department after an increase of cases but did not prevent a new surge of cases. As these outbreaks can have severe consequences, continuous surveillance of neonates and the inanimate environment should be introduced in the NICUs.

26 weeks of gestation rapidly developed a fatal septic shock, and *S. marcescens* was detected from blood cultures (Figure 1). Contemporary detection of *S. marcescens* from a clinical specimen, a conjunctival swab, from another neonate (P4) in the NICU (Figure 1) led us to perform a retrospective review of the laboratory information system database. This revealed that *S. marcescens* had been identified from various clinical specimens from three other NICU patients (P1–P3) (Figure 1) in the preceding 3 weeks. In contrast, *S. marcescens* had been detected in only one neonate hospitalised in the NICU during 2021. An outbreak was declared when rectal screening of the neonates in NICU in early March identified three colonised patients (P6–P8) and one additional patient with conjunctivitis (P9).

In this report, we describe a prolonged *S. marcescens* outbreak in a NICU in Belgium and the impact of sequencing analysis on the understanding of strain epidemiology throughout the outbreak period.

Methods

Outbreak setting

The outbreak occurred in a 580-bed secondary care hospital, a member of the GZA hospital network that houses the largest maternity services in Antwerp with › 5,000 births recorded annually. The NICU has 27 beds separated into two physically distinct areas: the intensive care area (N1, 15 incubators) for very premature (‹ 32 weeks of gestational age) or critically ill neonates and the medium care area (N2, 12 cots) for neonates in less critical condition, as presented in Supplementary Figure 1. During periods with no outbreaks, staff and medical equipment (diaper scales, temperature probes, monitors etc.) are shared between the two areas. Within the NICU, one common area (N) is used as an entrance with a desk space and several washbasins for parents and staff to wash their hands before entering the NICU, as presented in Supplementary Figure 1.

Case definitions

A case was defined as any neonate hospitalised in the NICU and colonised or infected with *S. marcescens* between 1 January 2022 and 29 August 2023. Infected cases were defined as patients with a *S. marcescens*-associated infection according to the diagnostic criteria by European Centre for Disease Prevention and Control (ECDC) [10]. All other cases were considered as colonised cases. Peak events were defined as time periods in which ≥ 4 new cases were identified in a week. The attack rate was defined as the cumulative incidence proportion over a specified period.

Environmental sampling of the neonatal intensive care unit

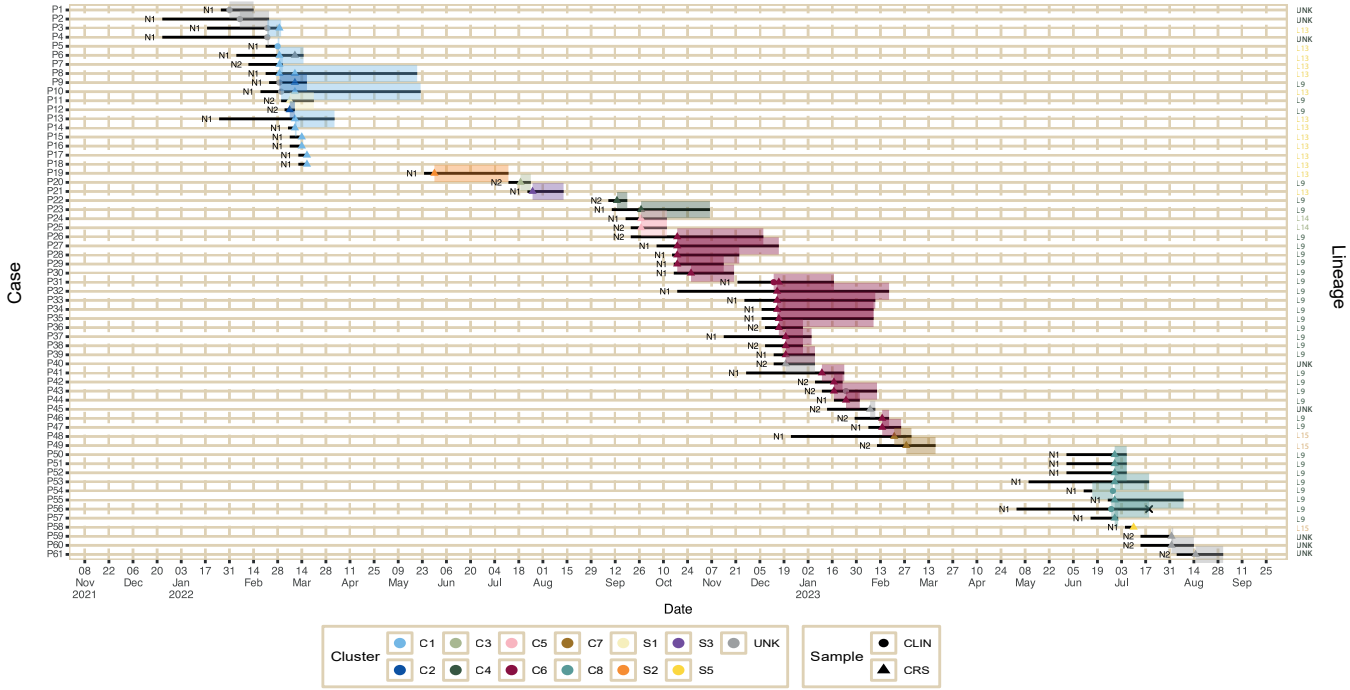
Drains (washbasins, baths) in different parts of the NICU were sampled twice weekly. Additionally, other environmental samples (feeding tubes, keyboards, water taps, outer surfaces of neonatal incubators, diaper scales, breast pumps, eye drops, liquid soaps, parenteral nutrition and prepared formula milk) were taken on 2 March 2022 and on 23 December 2022 to identify possible sources of the outbreak.

Microbiological methods

Rectal swabs, obtained once a week from all patients admitted to the NICU, were plated onto a

Figure 1

Timeline of detection of cases with *Serratia marcescens* in an outbreak in a neonatal intensive care unit, Belgium, 2022–2023 (n = 61)



C: cluster; CLIN: detection of *S. marcescens* from a clinical specimen; CRS: screening sample; L: lineage; N1: intensive care; N2: medium care; P: patient; *S: Serratia*; S: singleton cluster; UNK: unknown cluster type, *S. marcescens* isolate not sequenced.

In the graph, start and end dates of hospitalisation stay of all identified cases are presented (P1–P61). Hospital stay is indicated by horizontal lines, where different colour-markings indicate the *S. marcescens* strain cluster.

chromogenic agar specific for *Serratia* spp. (Serratia Colorex, bioTrading, Mijdrecht, the Netherlands) and incubated at 37°C overnight. Detection of *S. marcescens* from clinical specimens was performed using standard bacteriological procedures according to the Clinical Microbiology Procedures Handbook [11]. Environmental samples were obtained by swabbing surfaces using eSwab (Copan, Brescia, Italy). Isolates of *S. marcescens* were identified using MALDI-TOF (Bruker, Billerica, the United States (US)). Susceptibility testing was performed by disk diffusion according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (version 13.1) [12].

Whole genome sequencing

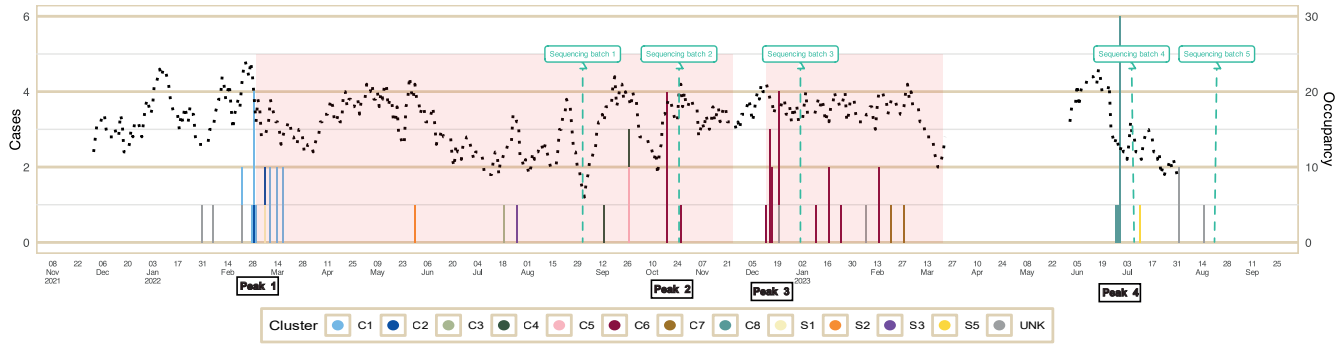
All *S. marcescens* isolates were stored at −80°C and analysed with whole genome sequencing (WGS) in four batches, on 1 September 2022, 25 October 2022, 2 January 2023 and 21 August 2023. Genomic DNA was extracted using MasterPure Complete DNA kit (LGC Biosearch Technologies, Hoddesdon, the United Kingdom (UK)). Multiplexed Nextera XT libraries were prepared and sequenced using 2 × 250 bp paired-end sequencing on a MiSeq instrument (V2 500 cycles, Illumina Inc., San Diego, US). Trimming (Trim Galore [13] version 0.6.6), assembly (SPAdes [14] version 3.13.1) and annotation (prokka [15] version 1.12) was performed using BacPipe version 1.2.6 [16]. Whole genome based multilocus sequence typing (wgMLST) was performed using a gene-by-gene approach-based allelic loci comparison (chewBBACA [17] version 2.5.5) by generating a customised study-specific scheme. The wgMLST allelic loci distances were calculated (chewBBACA), and the generated MSTree visualised (GrapeTree [18] version 1.5.0). Nine reference strains, shown in Supplementary Table 1, were used to assign *S. marcescens* lineages 9–16 as defined by Williams et al. [1]. A core genome single nucleotide polymorphism (cgSNP) alignment was generated (parSNP [19] version 1.7.4) and used to construct a maximum-likelihood tree (IQ-TREE [20] version 2.0.6) and visualised (ggtree in R version 2022.07.2 [21]).

Analytical epidemiological investigations and statistical analysis

In a retrospective case–control study, we compared cases detected between 1 March 2022 and 1 January 2023 with patients with no *S. marcescens* detected (infection or colonisation). The controls were selected from time periods with a higher incidence of cases: 1–17 March 2022, 8–23 October2022 and 13–28 December 2022. These time periods were selected to avoid having a control group of patients when there was either no or low recorded presence of *S. marcescens*. We included variables nursing in an incubator, gestational age and length of stay.

Figure 2

Epicurve of cases with *Serratia marcescens* and control measures taken in an outbreak in a neonatal intensive care unit, Belgium, 2022–2023 (n = 61)



C: cluster; *S: Serratia*; S: singleton cluster; UNK: unknown cluster type, *S. marcescens* isolate not sequenced.

Time of sequencing and start and stop of outbreak measures (red boxes) are indicated. Total occupancy in the ward is represented by a dotted line on the epicurve. Following outbreak control and response measures were taken during the marked time periods (red boxes): systematic rectal screening once a week, contact isolation (cohort), dedicated nursing, environmental screening (washbasin drains) and enhanced cleaning and disinfection.

A test for multicollinearity was performed with the selected variables (olsrr-package in R, https://www.r-project.org/). Multiple logistic regression was performed for nursing in an incubator and a lower mean gestational age at birth (glm, stats-package in R). A p value of‹0.05 was considered statistically significant.

Results

Descriptive epidemiology

In total, we identified 61 cases between 1 January 2022 and 29 August 2023. An overview of cases is presented in the epicurve within Figure 2, including a timeline for each individual case. Fifty neonates were identified as a case by rectal screening only. Four cases (P1–4) were retrospectively detected between 1 January and 1 March 2022, P3 also had a positive rectal screening early March. Ten neonates presented with a clinical infection caused by *S. marcescens*: five with lower respiratory tract infection, four with acute conjunctivitis and one with bloodstream infection. The index case died from the infection; the other cases survived. All isolates had a wild-type antimicrobial susceptibility pattern for amoxicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, ceftriaxone, ceftazidime, cefepime, meropenem, amikacin, tobramycin, ciprofloxacin and sulfamethoxazole-trimethoprim and none showed acquired resistance. During peak events (1–17 March 2022, 18 October 2022, 13–20 December 2022, 27–29 June 2023), attack rates varied between 10 of 27 patients (13–20 December 2022) and 16 of 32 patients (1–17 March 2022).

Environmental investigations

Serratia marcescens was detected from 71 of approximately 1,380 drain swab samples but not from any other environmental samples (n = 32) (Figure 3). Of the 16 drains in the NICU, *S. marcescens* was isolated from 11 of them (N1: 4/7, N2: 4/5, common space: 3/4) (Figure 3). When considering positivity per week, the positivity ratio of all screened drains was 5.8% (71/1,224).

Whole genome sequencing analysis

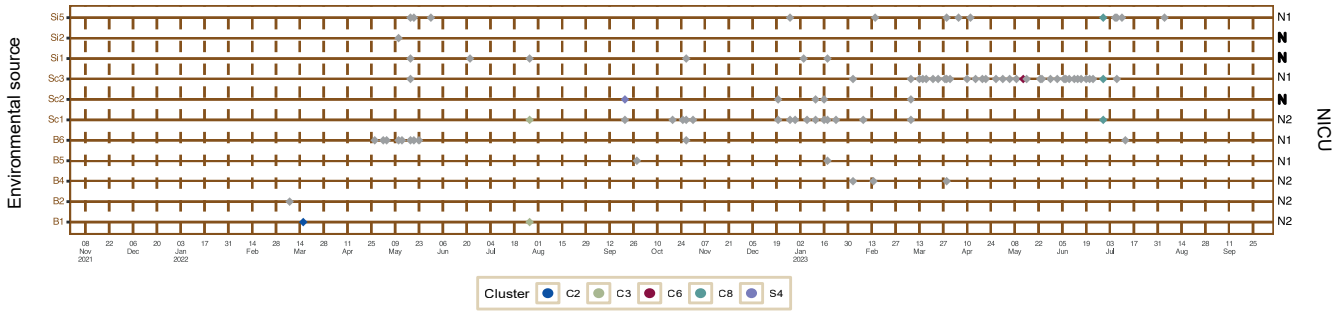
We sequenced 56 *S. marcescens* isolates retrieved from the 61 cases. The isolates were from rectal swabs (n = 51), blood culture (n = 1), endotracheal aspirates (n = 2), sputum (n = 1) and conjunctival swab (n = 1). Eight isolates from the drain swabs were sequenced: bath1 (B1, n = 2), washbasin1 (n = 2), washbasin2 (n = 1), washbasin3 (n = 2) and washbasin5 (n = 1). We identified eight distinct clusters with at least four isolates of *S. marcescens* (Figure 4), where each cluster was separated by›3,200 allelic loci differences. Remarkably during peak events, two or more patients developed clinical infection due to *S. marcescens* belonging to the predominant clonal clusters. In contrast, isolates collected during time periods between peak events resulted in smaller clusters with only two isolates or singletons and did not involve patients presenting with clinical *S. marcescens* infections.

Further, cgSNP analysis revealed that the analysed *S. marcescens* isolates belonged to separate lineages, pointing to a broad diversity (Figure 5). Within-lineage distance was less than 7,500 SNPs, except for P48, P49 and P58, which differed by less than 5,000 SNPs with each other, but differed by 12,919 SNPs from the closest reference strain (rL15). Reference strains are listed in the Supplementary material. Between lineages, there were a minimum of 14,000 SNPs. The highest SNP distance was eight SNPs (C8), but most clusters had no SNP difference (C3–C7). Cluster C1 had a maximum of one SNP distance and C2 had two SNPs. The distance matrix is presented in Supplementary Table 2.

A total of 34 sequenced patient isolates and all eight isolates from the drains belonged to a

Figure 3

Timeline of detection of *Serratia marcescens* from drain samples in an outbreak in a neonatal intensive care unit, Belgium, 2022–2023 (n = 1,380)[a]



B: bath drain; C: cluster; N: NICU common space between N1 and N2; N1: intensive care; N2: medium care; *S: Serratia*; S: singleton cluster; Sc: drain of a large washbasin; Si: drain of a small washbasin.

[a] The number of drain samples taken is an approximation. *Serratia marcescens* was detected from 71 drain samples.

hospital-associated *S. marcescens* lineage (lineage 9) [1]. All the other isolates, including those isolated from the initial outbreak cluster (C1), belonged to lineages not associated with hospitals (lineage 13–15).

Sequencing of the isolates demonstrated concurrent presence of *S. marcescens* in both patients and drains in four of five distinct clonal clusters: C2 on 15 March 2022, C3 on 27 July 2022, C6 on 13 May 2023 and C8 on 29 June 2023 (Figure 1, Figure 3). *Serratia marcescens* isolated from the drain of a large washbasin, washbasin2 (S4), on 21 September 2022, did not belong to any cluster. On 13 May 2023, *S. marcescens* (Sc3a, C6) was isolated from a large washbasin, washbasin3, 2.5 months after the discharge of the last known patient (P47) with an isolate belonging to this cluster (Figure 1).

Analytical epidemiology

During peak events with larger clonal clusters, most cases were nursed in N1 where all infants are in incubators. Length of stay was excluded from the regression analysis as the calculated tolerance was‹0.6. Nursing in an incubator was associated with detection of *S. marcescens* (odds ratio (OR): 2.99; 95% confidence interval (CI): 1.14–8.25; p‹0.05) (Table). An illustration of the NICU can be seen in Supplementary Figure 1.

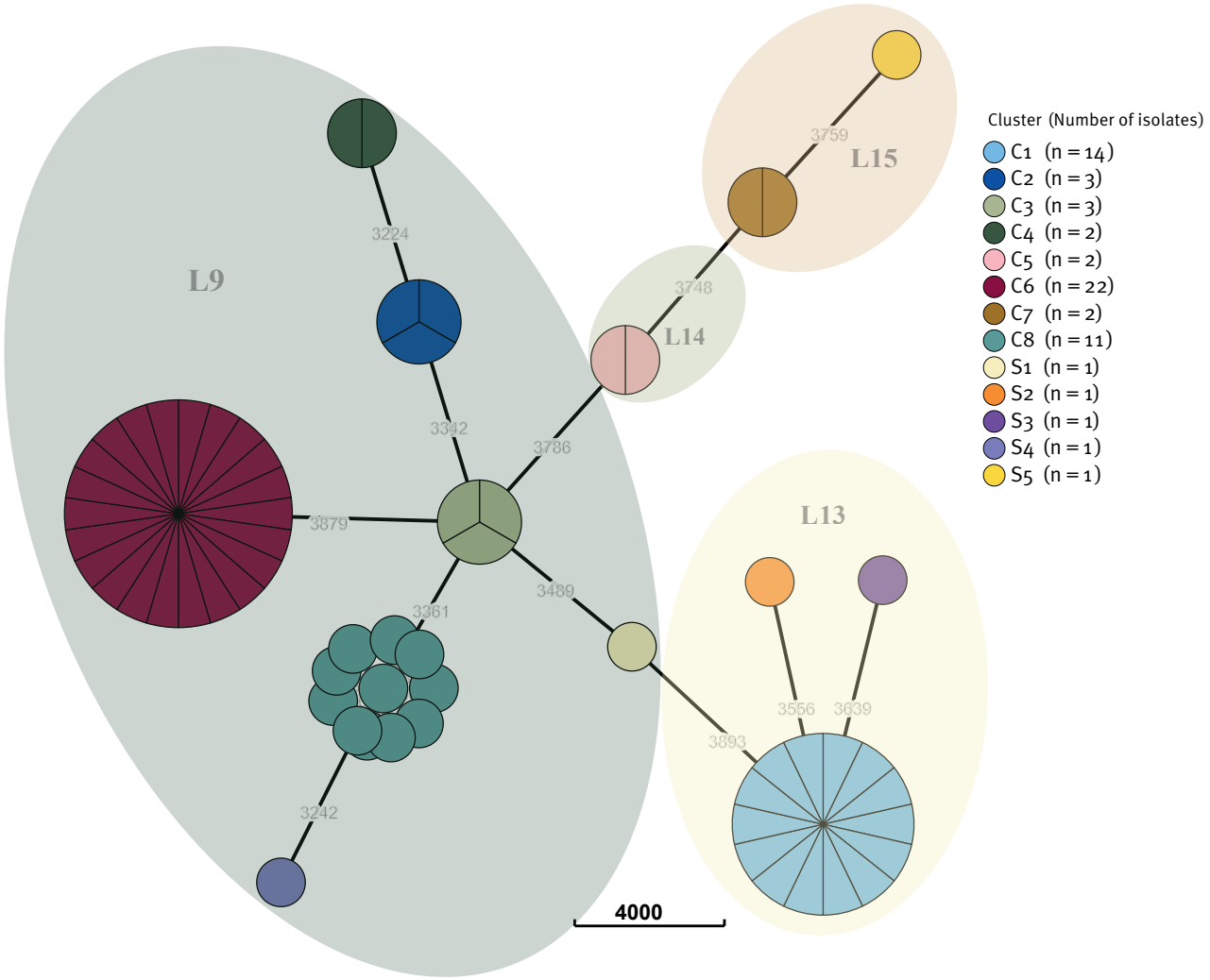
Outbreak control measures

After the detection of the outbreak, a multidisciplinary outbreak control team was assembled consisting of the heads of departments (physician and nurse), a microbiologist, a paediatric infectious disease specialist, infection control and prevention specialists and a representative of the board of directors of the hospital. At the start of the outbreak, we decided not to close the ward but to limit new admissions, initiate contact isolation measures by rearranging the location of the cases and labelling a case visible for staff but not for the parents or other visitors and start systematic rectal screening of neonates in the ward (Figure 2). The limitation on new admissions was implemented, as during the outbreak, the occupancy in the ward was high which could have had a limiting effect on the correct implementation of IPC measures. As a full closure would have a considerable impact on the activity of the maternity centre of our hospital and the surrounding hospitals, we decided to limit new admissions than force a full closure. During the first week of the outbreak declaration, daily on-site visits by the outbreak control team took place to investigate possible outbreak sources and transmission routes. Dedicated nursing staff was assigned and cohorting of the affected neonates was initiated. Cleaning and disinfection practices within the ward were reinforced by assigning specialised personnel. Following the initial meeting on 1 March 2022, in which outbreak control measures were set, weekly team meetings were held to re-evaluate the situation. The admission limitation was discontinued on 31 March 2022 once a case-free interval of 2 weeks was observed and after 13 cases had been discharged from the ward.

After the first sequencing batch was analysed on 1 September 2022, which revealed polyclonality of the outbreak and that origin from a single environmental source was unlikely, we decided to adapt our guidelines aiming to terminate all measures after a case-free interval of 2 weeks, including rectal screening. We decided to continue the systematic screening of drains. When a new case was detected on 13 February 2023, the previously applied outbreak control measures were re-introduced, except for restriction on admissions to the ward, which was not reinstated due to its significant impact on the activities of the maternity services of the hospital. Also, a new protocol for incubator disinfection was introduced as from this date, in which the incubator of a discharged neonate was disinfected with nebulised hydrogen peroxide before a new admission.

Figure 4

Minimal spanning tree of *Serratia marcescens* isolates in an outbreak in a neonatal intensive care unit, Belgium, 2022–2023 (n = 64)



Cluster and singleton names are based on the isolation date. Numbers represent distances in allelic loci from a total of 5,033 identified loci. Clusters are collapsed to pie charts when there were < 12 loci difference between the isolates. Lineage L9 is a hospital-associated lineage, while lineages 13–15 are mostly related to water, plants and insects [1].

On 1 March 2023, four heated drains were installed (large washbasins: washbasin1, 2 and 3 and a small washbasin: washbasin5). These drains have a once daily thermo-disinfection protocol wherein the drain is heated to 85°C for 5–6 cycles. However, *S. marcescens* was repeatedly isolated from samples from an incorrectly installed heated drain. As a precautionary measure, all four heated drains were removed on 18 July 2023.

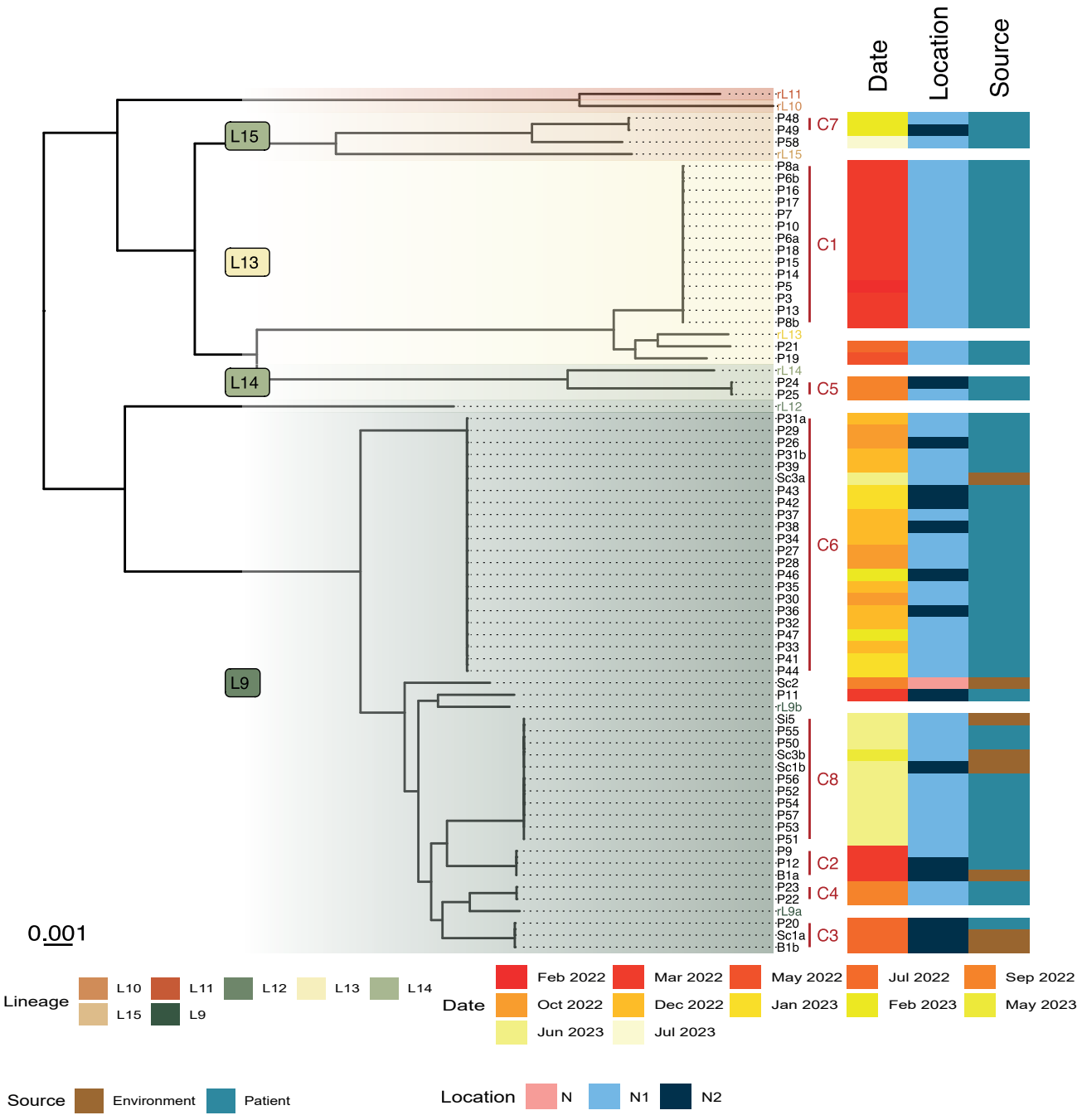
All outbreak measures, including rectal screening and sampling of drains were discontinued on 29 August 2023. It was decided that moving forward, all measures (disinfection by specialised personnel, contact isolation, nebulisation of the incubators, rectal screening, environmental screening) would be reinstated if *S. marcescens* was detected from clinical samples of two patients within a 2-week period.

Discussion

During this extended outbreak, various outbreak control measures were implemented to decrease and contain the high rate of cases. As many NICUs, including ours, are open wards, an effective contact isolation can be difficult. By rearranging the location of cases and using all available spaces, in combination with a label of a case, an effective contact isolation could be achieved. However, this implicitly requires an extensive case-finding strategy. We chose to use rectal swabs as these have been shown to be the most sensitive sample and detection site [22]. Other sample types commonly employed are conjunctival swabs, samples from the respiratory tract (mainly nasal) and swabs of the

Figure 5

Phylogenetic tree based on core genome single nucleotide polymorphism (SNP) analysis of *Serratia marcescens* isolates in an outbreak in a neonatal invasive care unit (n = 64) and reference strains (n = 9), Belgium, 2022–2023



B: bath; C: cluster; N: common space between N1 and N2; N1: intensive care; N2: medium care; rL: reference strains of *Serratia marcescens*; Sc: large washbasin; Si: small washbasin.

Heatmaps of sampling date, sampling site and sampling source are shown. Lineages with patient strains are labelled within the tree. The tree is rooted with a reference of lineage 16 (not depicted). For case isolates, a and b represent different isolates from the same patient or site. Two reference strains for lineage 9 were used (rL9a, GCF_001294565.1_ASM129456v1 and rL9b, GCF_000739215.1_ASM73921v1) to include a broader coverage of this lineage.

Table

Number of cases and controls in an investigation of an outbreak of *Serratia marcescens* in a neonatal intensive care unit, Belgium, 2022–2023[a]

Variables	Cases n = 49	Controls n = 44	OR	95% CI	p value
Nursing in an incubator	39	24	2.99	1.14–8.25	0.04
Mean gestational age at birth	31 weeks 6 days	34 weeks 1 day	0.98	0.95–1.00	0.03
Mean length of stay	37.7 days	29.4 days	0.99	0.97–1.01	0.4

CI: confidence interval; OR: odds ratio.

[a] Cases were patients with *S. marcescens* and hospitalised 1 March 2022–1 January 2023. Controls were patients with no *S. marcescens* detected and hospitalised during time periods with higher case incidence: 1–17 March 2022, 8–23 October 2022 and 13–28 December 2022.

umbilicus [22,23]. We preferred to have an extensive screening using only rectal swabs rather than samples from multiple sites from a single patient.

Given that *S. marcescens* was detected from only washbasin drains and not from other environmental samples during the initial environmental screening, we implemented a systematic screening of all drains on the ward. We observed that the number of positive drains reflected the number of cases in the ward. Our data and other recent studies argue for the removal of drains from NICU patient rooms and favour water-free care for neonates that could reduce NICU patient colonisation rates with Gram-negative bacteria like *S. marcescens* [24-26]. Eventually, we opted to remove most washbasins, which until now, proved effective in avoiding a resurgence.

Intensive screening of patients and hospital environment can be costly and labour intensive for the hospital and the microbiology laboratory. A continuous monitoring outside of an outbreak setting could enable an early detection of a silent spread within a ward and possibly predict the outbreak potential of a genetically well-characterised pathogen. Such a continuous monitoring might be feasible using a targeted approach i.e., tracking strains from clinical samples and a periodic screening of the environment, of which washbasin drains appear to have the most reservoir potential, and thus creating a view on the NICU pathobiome.

In a previous NICU outbreak of *Enterobacter* spp., incubators were one of the major contributing factors in transmission [27]. We found several clusters and different incubators involved in the present outbreak. However, the setup of incubators in N1 and cots in N2 limited our ability to effectively define direct causation between being nursed in an incubator and *S. marcescens* colonisation. In N2, isolates from cases colonised with *S. marcescens* belonged to the same clusters as environmental isolates and case isolates from N1, but cases in N2 were fewer than in N1. Although we also found an association between *S. marcescens* and nursing inside an incubator, we do not consider this to be the primary source of the outbreak, but rather a facilitator of rapid transmission. The use of invasive procedures as the real risk factors of colonisation also could not be ruled out [6].

Several distinct clonal clusters of *S. marcescens* belonging to four different lineages could be identified using WGS. Most of the sequenced *S. marcescens* patient isolates and all environmental isolates belonged to lineage 9 which is the major human, hospital-associated lineage. The shift of the specific clusters during the outbreak period might be linked to the control measures taken, as metabolic pathways differ between different *S. marcescens* lineages and even within lineages [1]. As a result, the use of certain antiseptics could select for specific lineages or clonal clusters. Alternatively, increased pressure on medical staff due to higher bed occupancy rates, which tend to reduce hygiene compliance, or other unmeasured factors related to the total disease burden in the ward might also have contributed to the propagation of certain strains present in the ward at that time, of which some may have had a greater potential to spread within a NICU environment [28].

Polyclonal outbreaks with *S. marcescens* are not rare in NICUs, and the source of these outbreaks often remains unclear [28-30]. To effectively prevent a resurgence or avoid an outbreak all together, will require a better understanding of the *S. marcescens* biology, its niches and its metabolic potential.

Conclusion

The outbreak measures outlined in this report were effective in controlling the surge of *S. marcescens* of the protracted *S. marcescens* outbreak in our NICU. Based on our experience, we consider that a systematic, continuous epidemiological surveillance of neonates admitted in NICUs, as well as of the inanimate environment should be introduced to inform on various possible sources and transmission pathways of environmentally transmitted pathogens like *Serratia* during non-outbreak periods. Given that more hospitals in Belgium have recently reported similar *S. marcescens* outbreaks in NICUs, we see a value in starting a structured surveillance system at national level, as none currently exists in Belgium.

Ethical statement

Ethical approval was obtained from the GZA hospital network ethics committee (study number 221213RETRO). No patient consent forms were obtained as it was not required by the ethics committee.

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Use of artificial intelligence tools

None declared.

Data availability

Generated sequencing data are available under NCBI BioProject PRJNA1013022.

Conflict of interest

None declared.

Authors' contributions

Conceptualisation: SVG, YG, SMK; outbreak investigation: SVG, PW, KP, DVB; microbiological analysis: SVG, BVH, KH; sequencing and data analysis: SVG, BBX, MB; original draft preparation: SVG, YG, SMK; writing, revision and editing: YG, SMK, SVG, HG. All authors read, gave input and approved the final manuscript.

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BRIEF REPORT

Faucet aerators: A source of patient colonization with *Stenotrophomonas maltophilia*

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Background: Multiple nosocomial outbreaks have been linked to contaminated water sources within the hospital. We report in this article a cluster of patients in a surgical intensive care unit who were colonized or infected with *Stenotrophomonas maltophilia*.
Methods: This study was conducted at an acute care academic hospital. Patients colonized or infected with *S maltophilia* were identified by prospective surveillance. Environmental isolates were obtained by culturing multiple water sources by using standard techniques. Patient and environmental isolates were examined by pulsed-field gel electrophoresis.
Results: Patients were colonized with 2 isolates of *S maltophilia,* which were found by pulsed-field gel electrophoresis to be identical to strains isolated from the faucet aerators present in sinks in the patients' rooms. Multiple different strains, as defined by pulsed-field gel electrophoresis, were isolated from patients during this outbreak.
Conclusions: We believe that low level contamination of our potable water led to contamination of the faucet aerators with subsequent bacterial amplification on the aerator, which led to contamination of water after aeration. Cultures should be performed on faucet aerators when water sources are suspected as the reservoir for a nosocomial outbreak. If additional clusters of infected or colonized patients are linked to contaminated aerators, consideration should be given to routine disinfection or removal of the aerators. (AJIC Am J Infect Control 1999;27:59-63)

Multiple nosocomial outbreaks have been linked to contaminated water sources, including potable water, ice, dialysis water, hydrotherapy tanks, water baths used for thawing medications or blood products, and water used in humidifiers and nebulizers.[1] Pathogens associated with potable water have included *Pseudomonas aeruginosa, Burkholderia cepacia, Serratia marcescens, Acinetobacter calcoaceticus, Flavobacterium meningosepticum, Aeromonas hydrophila,* and certain nontuberculous mycobacteria. Faucet aerators have been reported to be contaminated with gram-negative bacilli, including *P aeruginosa, Klebsiella pneumoniae,* and *Escherichia coli*.[2] Faucet aerators contaminated with *P aeruginosa*[3,4] or *Pseudomonas* spp[5] have been epidemiologically linked to colonized or infected patients.

We report in this article, a cluster of patients in a surgical intensive care unit (ICU) colonized or infected with *Stenotrophomonas maltophilia* in which the pathogen acquired by 2 patients was traced by molecular analysis to contaminated faucet aerators.

METHODS

This study was conducted at the University of North Carolina Hospitals, a 650-bed tertiary care university hospital complex. Comprehensive surveillance at UNC Hospitals is conducted by 4 full-time infection control professionals. A modified version of the Centers for Disease Control and Prevention's criteria are used to define nosocomial infections. The major modifications are that asymptomatic bacteriuria is not reported and a chest radiograph with a new or increased infiltrate is required to meet the pneumonia definition. Since 1977, surveillance data has been coded and entered into a computerized database.

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Water samples were evaluated by collecting water in a sterile container. The water was then aseptically passed through a 0.45 µm filter. The filter was cultured on sheep blood agar. Bacterial colonies were identified by standard techniques. The faucet aerators used at UNC Hospitals are stainless steel wire mesh structures that screw into the distal end of the sink faucet. These faucet aerators were cultured by swabbing them with a sterile cotton swab premoistened with trypticase soy broth (Becton Dickinson, Cockeysville, Md) and by aseptically removing the aerator and placing it in 5 mL to 10 mL of trypticase soy broth.

Stenotrophomonas maltophilia isolates were compared by using DNA microrestriction analysis by pulsed-field gel electrophoresis (PFGE). All isolates were run on the same gel and each isolate was compared with all other isolates. The DNA fingerprint of each isolate was visually scored for the presence or absence of individual bands by 2 independent observers. Isolates were considered different if they differed by 3 or more bands. In this cluster, all isolates described as identical had corresponding bands.

DESCRIPTION OF A CLUSTER

This cluster involved patients primarily in the surgical ICU, an 8-bed unit primarily used by the trauma and general surgical services. The surgical ICU is adjacent to an 8-bed neurosurgical ICU. Other intensive care units on the same floor, but not physically adjacent, include a cardiothoracic ICU, a medical ICU, and a respiratory ICU. All rooms in the surgical ICU share a common water supply.

In late November, an epidemiologic investigation was initiated after surveillance data revealed that 7 patients in the surgical ICU were colonized or infected with *S maltophilia* in the preceding 3 months. The investigation consisted of a careful review of the medical charts of all colonized/infected patients, which evaluated the frequency of risk factors for gram-negative bacterial infection and potential sources (eg, medical devices, procedures) common to the patients; an environmental assessment, including cultures of all potential sources and reservoirs of *S maltophilia*; evaluation of hospital personnel for potential skin or respiratory colonization, which might have led to employee-to-patient transmission; and molecular analysis of patient and environmental isolates. The surgical ICU was initially visited on December 12, 1995, to evaluate possible common sources of infection. No multidose medication vials were used, and no common equipment was discovered (eg, blood pressure cuffs or respiratory care equipment). No staff members had evidence of skin or respiratory tract infections. A preliminary environmental evaluation was conducted. One hundred milliliter samples of water were cultured from 4 sinks in the surgical ICU (rooms 2736, 2737, 2738, and the handwashing sink at the nurses station). No microorganisms were cultured. In addition, 1 cup of ice from the ice machine was collected, melted, and a culture was performed in a manner similar to that used for the water samples. This culture was negative for *S maltophilia*.

On December 14, a comprehensive environmental evaluation was performed. Two-liter water samples were collected from several sources in the surgical ICU, including the sink in room 2737, the handwashing sink in the staff lounge, ice water output from the ice machine, and the handwashing sink at the nurses station. In addition, a culture was performed on the ice machine drain. All of these cultures were negative for *S maltophilia*. Cultures were performed on faucet aerators from multiple locations, which included the clean utility room sink, the sink at the nurses station, the staff lounge sink No. 1, the staff lounge sink No. 2, and the sinks in patient rooms (2734, 2735, 2736, 2737, and 2738). The faucet aerators in rooms 2735 and 2736 yielded *S maltophilia*.

After the isolation and identification of *S maltophilia* on faucet aerators, all aerators were removed, cultures were performed, and the aerators were replaced with new aerators on December 18. The faucet aerator in room 2735 was again positive.

Follow-up cultures of the aerators in 7 of the 8 surgical ICU rooms were performed on March 20, 1996. All cultures were negative with the exception of the aerator from room 2735. When the culture result became positive, a follow-up culture of 10 L of water from the sink in room 2735 (surgical ICU) was obtained on March 26, 1996. This culture also yielded *S maltophilia.*

Overall, *S maltophilia* was isolated from 7 patients in the surgical ICU during a 5-month period. None of the patients had community-acquired infections as a result of *S maltophilia.* Infectious syndromes associated with *S maltophilia* included 2 patients with *S maltophilia* nosocomial pneumonia, 1 patient with community-acquired pneumonia whose respiratory tract was later colonized with *S maltophilia*, 1 patient with respiratory tract colonization, 1 patient whose bile stent became colonized, 1 patient admitted for peritonitis whose Jackson-Pratt drain was later colonized with *S maltophilia*, and 1 patient with multiple nosocomial infectious sites.

A review of our surveillance records before the outbreak revealed the following incidences of *S maltophilia* nosocomial infections in the surgical ICU during past years: 1990, 1 infection; 1991, 4 infections; 1992, 7 infections; 1993, 4 infections; and 1994, 2 infections. After this outbreak, from mid-January 1996 through December, 1996, only 3 unrelated cases of nosocomial infections as a result of *S maltophilia* occurred in the surgical ICU, a rate similar to the previous baseline.

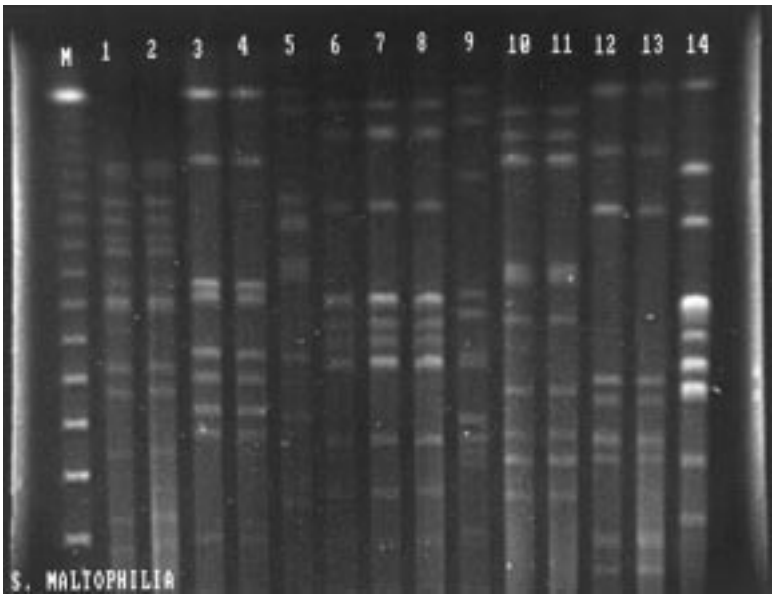

Fig 1. Pulsed-field gel electrophoresis of patient and environmental strains of *S maltophilia*. Lane M, lambda ladder PFGE marker (New England Biolabs); Lane 1, pattern A (handwashing sink surgical ICU, from Dec 14, 1995); Lane 2, pattern A (handwashing sink room 2735, from Dec 18. 1995); Lane 3, pattern B (handwashing sink room 2735, from Dec 12, 1995); Lane 4, pattern B (patient MC); Lane 5, pattern C (aerator, sink room 2735, from Mar 20, 1996); Lane 6, pattern D (aerator, sink room 2736, from Dec 14, 1995); Lane 7, pattern D (patient DM); Lane 8, pattern D (patient NC); Lane 9, pattern E (patient DN); Lane 10, pattern F (patient MM); Lane 11, pattern F (patient CA); Lane 12, pattern G (patient OS); Lane 13, pattern G (patient not housed in surgical ICU); Lane 14, pattern H (patient not housed in surgical ICU).

RESULTS

The location of colonized/infected patients, environmental sources tested for *S maltophilia,* and the results of molecular typing are displayed in Table 1 and Fig 1, respectively. PFGE revealed 5 different strains colonizing or infecting patients and 4 different environmental isolates. S *maltophilia* was isolated from the faucet aerators in 2 patient rooms (rooms 2735 and 2736) and the handwashing sink at the nurses station. Environmental samples from the water or ice did not initially yield *S maltophilia*. A large volume culture of water, 10 L, from the sink in room 2735 in the surgical ICU later yielded *S maltophilia.* A follow-up culture of the aerator in room 2735 had a different PFGE pattern unrelated to any pattern found earlier.

In 2 cases, the strain of *S maltophilia* isolated from the faucet aerator in the patient's room matched the strain colonizing the patient (ie, MC, DM). Two patient isolates (ie, MM, CA) matched each other but did not match any environmental isolates (Fig 1).

DISCUSSION

S maltophilia, a gram-negative bacillus, is an unusual nosocomial pathogen. Data from the National Nosocomial Infection Surveillance system, 1990-1992, have revealed that *S maltophilia* accounts for less than 1% of nosocomial pathogens.[6] Nevertheless, multiple nosocomial outbreaks of *S maltophilia* have been reported.[7-9] *S maltophilia* has recently emerged as an important nosocomial pathogen in patients with severe underlying diseases or immune dysfunction[9-11] and patients receiving intravenous antibiotics.[8-12] It has been documented as a cause of bacteremia, infections of the respiratory and urinary tracts, skin and soft tissue infections, biliary tract infection, meningitis, serious wound infections, mastoiditis, conjunctivitis, and endocarditis.[11]

Recently, several molecular typing schemes have been used to aid in outbreak evaluation including DNA microrestriction analysis by PFGE,[9,13,14] contour-clamped homogeneous electric field gel electrophoresis of chromosomal DNA, polymerase chain reaction with arbitrary primers (random amplified polymorphic DNA),[14,15] polymerase chain reaction with enterobacterial repetitive intergenic consensus sequences as primers,[14] and ribotyping by using the restriction enzymes EcoRI and BamHI. PFGE was chosen for its simplicity, reproducibility, and discriminatory value.

Nosocomial infections have been linked to contaminated potable water.[11] Several studies have epidemiologically linked contaminated faucet aerators to colonization or infection of patients. Recently, PFGE was used to demonstrate that a strain of *S maltophilia* isolated from the throat of a patient matched that isolated from the shower head in the bathroom, and a strain isolated from

Table 1. Epidemiologic and clinical features of patients colonized or infected with *S maltophilia*

Patient	ICU (room No.)	Admit date, discharge date	Date of isolation	Site(s) of isolation	Infectious syndrome	Patient PFGE pattern	Environmental culture(s) from Dec 12 and Dec 14
MM	S-ICU (2740)	Aug 27, 1995-Sep 25, 1995	Sep 13, 1995	Sputum	Pneumonia	F	Sink aerator negative
CA	S-ICU (2737)	Aug 11, 1995-Nov 27, 1995	Oct 11, 1995	Sputum	Pneumonia	F	Sink aerator negative
MC	S-ICU (2735)	Nov 5, 1995-Nov 28, 1995	Nov 11, 1995	Sputum	Colonization	B	Sink aerator, isolates A and B
DN	S-ICU (2738)	Nov 10, 1995-Nov 11, 1995	Nov 10, 1995	Bile stent	Colonization	E	Sink aerator negative
DM	S-ICU (2736)	Nov 22, 1995-Dec 15, 1995	Nov 28, 1995	Jackson-Pratt drain	Peritonitis,* late colonization	D	Sink aerator, isolate D
OS	S-ICU (2737)	Nov 27, 1995-Dec 16, 1995	Dec 9, 1995	Sputum	Peritonitis,* late colonization	G	Sink aerator negative
NC	S-ICU (2734)	Dec 19, 1995-Jan 20, 1996	Jan 6, 1996, Jan 11, 1996,	Blood, sputum, urine	Bacteremia, pneumonia, urinary tract infection	D	Sink aerator negative

S-ICU, Surgical ICU.
*Non–hospital-acquired infection not involving *S maltophilia*.

a different patient matched that isolated from the kitchen sink.[13] *S maltophilia* has been associated with other reservoirs, including a cardiopulmonary bypass pump,[16] chlorhexidine-cetrimide disinfectant,[17] ethylenediaminetetraacetic acid anticoagulant in vacuum blood collection tube,[18] transducer dome and calibration devices,[19] and brushes used for preoperative shaving.[20] Despite reports that highlight reservoirs for *S maltophilia*, the source of *S maltophilia* in most nosocomial infections remains unknown.

Previous studies have linked colonized faucet aerators to patients colonized or infected with the same bacterial pathogen either epidemiologically[3,5] or by using a relatively nondiscriminatory testing method (ie, pyocin typing).[4] Our study is the first study to link colonized faucet aerators with colonized or infected patients by using a highly discriminative molecular epidemiologic method (ie, PFGE). Our data demonstrate that 2 patients were colonized by strains of *S maltophilia* contaminating the faucet aerators in the sink in their room. These strains were probably carried to the patient via transient colonization of the hands of health care providers or during sponge bathing of the patient by using the tap water. As with other investigations, multiple strains of *S maltophilia* were isolated. Two patients (MM, CA) whose ICU stay overlapped had pneumonia with an identical strain of *S maltophilia*, which suggests cross-contamination or common source exposure.

Faucet aerators are commonly used to diffuse the water stream, which leads to decreased splashing. We believe that low-level contamination of our potable water led to contamination of the faucet aerator with bacterial amplification on the aerator and subsequent increased contamination of water after aeration. This study currently provides the best evidence that contamination of faucet aerators may represent a nosocomial hazard. Hospital epidemiologists evaluating nosocomial outbreaks or an increased incidence of endemic infections by organisms capable of multiplying in potable water should consider culturing faucet aerators. If either endemic or epidemic nosocomial infections continue to be linked to faucet aerators, then additional infection guidelines may be required, which could include removal of the aerators or routine disinfection. Decontamination could be achieved by removing the aerators and immersing them in a 1:10 to 1:100 solution of diluted household bleach[21] and then rinsing them in tap water before reinstallation. However, because only a few reports have linked patient infection/colonization to colonized faucet aerators and only our report substantiated the linkage by using a discriminative method of molecular epidemiology, such steps are not warranted at the current time.

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Polymicrobial outbreaks

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Long-term intensive care unit outbreak of carbapenemase-producing organisms associated with contaminated sink drains

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SUMMARY

Background: Between 2018 and 2022, a Belgian tertiary care hospital faced a growing issue with acquiring carbapenemase-producing organisms (CPO), mainly VIM-producing *P. aeruginosa* (PA-VIM) and NDM-producing Enterobacterales (CPE-NDM) among hospitalized patients in the adult intensive care unit (ICU).
Aim: To investigate this ICU long-term CPO outbreak involving multiple species and a persistent environmental reservoir.
Methods: Active case finding, environmental sampling, whole-genome sequencing (WGS) analysis of patient and environmental strains, and implemented control strategies were described in this study.
Findings: From 2018 to 2022, 37 patients became colonized or infected with PA-VIM and/or CPE-NDM during their ICU stay. WGS confirmed the epidemiological link between clinical and environmental strains collected from the sink drains with clonal strain dissemination and horizontal gene transfer mediated by plasmid conjugation and/or transposon jumps. Environmental disinfection by quaternary ammonium-based disinfectant and replacement of contaminated equipment failed to eradicate environmental sources. Interestingly, efflux pump genes conferring resistance to quaternary ammonium compounds were widespread in the isolates. As removing sinks was not feasible, a combination of a foaming product degrading the biofilm and foaming disinfectant based on peracetic acid and hydrogen peroxide has been evaluated and has so far prevented recolonization of the proximal sink drain by CPO.

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Conclusion: The persistence in the hospital environment of antibiotic- and disinfectant-resistant bacteria with the ability to transfer mobile genetic elements poses a serious threat to ICU patients with a risk of shifting towards an endemicity scenario. Innovative strategies are needed to address persistent environmental reservoirs and prevent CPO transmission.

Introduction

The prevalence of clinically relevant carbapenemase-producing organisms (CPO), such as *Pseudomonas aeruginosa* and Enterobacterales, has increased worldwide [1,2]. Genes encoding for carbapenemases, such as the Verona integron-encoded metallo-β-lactamases (VIM) and the New Delhi metallo-β-lactamases (NDM), coexist with many other resistance determinants and are often transmitted between organisms by mobile genetic elements, such as transposons and/or plasmids, contributing to their spread [3]. Healthcare-associated infections caused by CPO are particularly worrying since they are associated with an increased financial burden, prolonged hospital stays, and increased mortality [4–8]. In this context, the prevention of the acquisition and spread of these strains is a priority. Current infection prevention and control interventions include screening, hand hygiene promotion, barrier precautions, enhanced surface disinfection, waste management, and contaminated source identification and elimination [9,10].

Between 2018 and 2022, our healthcare facility was confronted with a rising number of CPO acquisitions, mainly VIM-producing *P. aeruginosa* (PA-VIM) and NDM-producing Enterobacterales (CPE-NDM) among hospitalized patients in the adult intensive care unit (ICU). We report the investigation of a CPO long-term outbreak in ICU and the identification of an environmental aquatic source with genomic analysis confirming the horizontal transfer of mobile genetic elements. The aim was to provide insight into the complexity of outbreak management in this specific type of outbreak involving a persistent reservoir and multiple species, which may be encountered in ICU settings worldwide, and to demonstrate the need for combined measures over time.

Methods

Setting

Cliniques universitaires Saint-Luc is a tertiary care hospital in Belgium, with approximately 1000 beds. The adult ICU includes 14 single-bed rooms. Each room contains a sink and a bedpan washer (Figure 1A and B).

Case definitions

Cases were defined as ICU colonized or infected patients identified with acquired CPO between January 2018 and December 2022. Colonized patients were defined as patients in whom CPO was identified only on screening samples (endotracheal aspirate, rectum and urine samples were collected upon admission and twice weekly). Infected patients were defined as patients with at least one clinical sample CPO positive. The acquisition was defined when CPO were identified in the patient ≥48 h after hospital admission.

CPO microbiological investigations

Routine rectal swabs were recovered with Copan ESwab® (Brescia, Italy) and inoculated on ChromID ESBL (bioMérieux, Marcy l'Etoile, France) medium.

Bacterial isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-Biotyper; Bruker Daltonics, Bremen, Germany). When CPO was suspected on rectal swabs or clinical samples based on antimicrobial susceptibility testing, the identification of carbapenemase type was confirmed by an in-house multiplex polymerase chain reaction or by immunochromatographic assay (K-SeT; Coris BioConcept, Gembloux, Belgium) [11].

Infection control measures

Specific detection of CPO acquisition prompted enhanced infection control procedures, maintained daily until patient's death or discharge and including alert in the electronic health record, contact precautions and environmental chlorine dioxide cleaning. Complete room disinfection was performed with Tristel Fuse® (Tristel, Anvers, Belgium) and misting with hydrogen peroxide, upon discharge of each CPO-positive patient. In February 2019, additional preventive interventions were implemented to mitigate contamination of sink drains and reduce CPO transmission. The ICU siphons were replaced by the HygieneSiphon® (Aquafree, Hamburg, Germany), consisting of a permanent drain valve with a replaceable inlet. The inlet was replaced once every three months and upon discharge of each CPO patient. Starting from September 2021, the inlet was replaced monthly, combined with daily cleaning with 1 L of 0.5% Incidin Pro® (2-phenoxyethanol, *N,N*-bis-(3-aminopropyl) dodecylamine, benzalkoniumchloride; Ecolab, Groot Bijgaarden, Belgium). The sink drains of the 14 rooms (from the drain valve to the wall including bottle trap (P1) and the pipe (P2) (Figure 3A)) were changed in November 2019, February 2021 and January 2022.

Environmental sampling

Between December 2018 and January 2023, intermittent environmental sampling of sink drains (inlet and/or drain valve) was performed. Environmental samples were recovered with ESwab, inoculated on ChromID Carba Smart (bioMérieux), and incubated for 48 h. Since January 2022, the remaining suspension of each sink drain swab was incubated in a Letheen broth at 37 °C for seven days before plating on ChromID Carba Smart to detect low concentrations of CPO.

Figure 1. (A) Map of the adult intensive care unit, which includes 14 single-bed rooms. (B) Each room contains a sink and a bedpan washer. Sinks in patient rooms were located <5 feet (<2 m) from patient beds. The sink is systematically on the side of the patient's feet. The pipes of the different rooms are connected to a horizontal drainage system within the unit.

To evaluate the colonization rate of the sink drain, samples were collected from the inlet or the drain valve of four ICU rooms before the inlet replacement and twice a week during four weeks after replacement. ESwabs were serially diluted and plated on Columbia blood agar (Becton Dickinson, Cockeysville, MD, USA) and ChromID CARBA to quantify the total number of bacteria and carbapenem-resistant bacteria, respectively. In parallel, a pre-enrichment in a Letheen broth of each swab was incubated at 37 °C for seven days before plating on ChromID Carba. Bacterial and CPO identification were assessed as described above.

Foam cleaning protocol evaluation

A new protocol combining enziSurf™ (OneLife, Louvain-la-Neuve, Belgium) and Phago'Spore® (Christeyns, Gent, Belgium) was evaluated on four contaminated ICU sink drains (without inlet) in November 2022. The enziSurf protocol is composed of two foaming products: enziSurf Descale (descaling agent containing phosphoric acid, lactic acid and anonic and non-ionic surfactants; applied 5 min) and enziSurf Drain (solution containing five enzymes known to degrade biofilm matrix (including protease, lipase, amylase, and DNase), applied for 15 min. The Phago'Spore is a foam non-quaternary ammonium-based detergent/disinfectant composed of peracetic acid 0.034% and hydrogen peroxide 3.26% applied 15 min after the enziSurf protocol. The water was run for a few seconds between each product, until complete flushing of foam. Two treatments were applied successively: a curative protocol (enziSurf and Phago'Spore every day for four days) followed by a preventive protocol (enziSurf and Phago'Spore twice weekly for four weeks). Prior to each application and twice a week, samples were collected from the proximal sink drain to a depth of 10 cm and were assessed as described above to estimate the cfu/mL of carbapenem-resistant bacteria and evaluate the presence of CPO.

In February 2023, the 14 ICU HygieneSiphons were replaced by standard chrome-plated brass sink drains and cleaned twice weekly with the preventive protocol combining enziSurf and Phago'Spore. Samples were collected from the proximal sink drain once per month and assessed as described above.

Whole-genome sequencing

Isolates were analysed by whole-genome sequencing (WGS). Libraries were constructed with Illumina DNA prep kit (Illumina, San Diego, CA, USA) and were sequenced on the Illumina MiSeq or NextSeq1000 platform according to the manufacturer's protocol. Sequence reads, whole-genome multi-locus sequence typing (wgMLST) and single-nucleotide polymorphism (SNP) were analysed using BioNumerics (version 8.0; Applied-Maths, Sint-Martens-Latem, Belgium). wgMLST was analysed with a scheme containing 15,143 loci for *P. aeruginosa*. SNP analysis was performed using as reference the contig harbouring

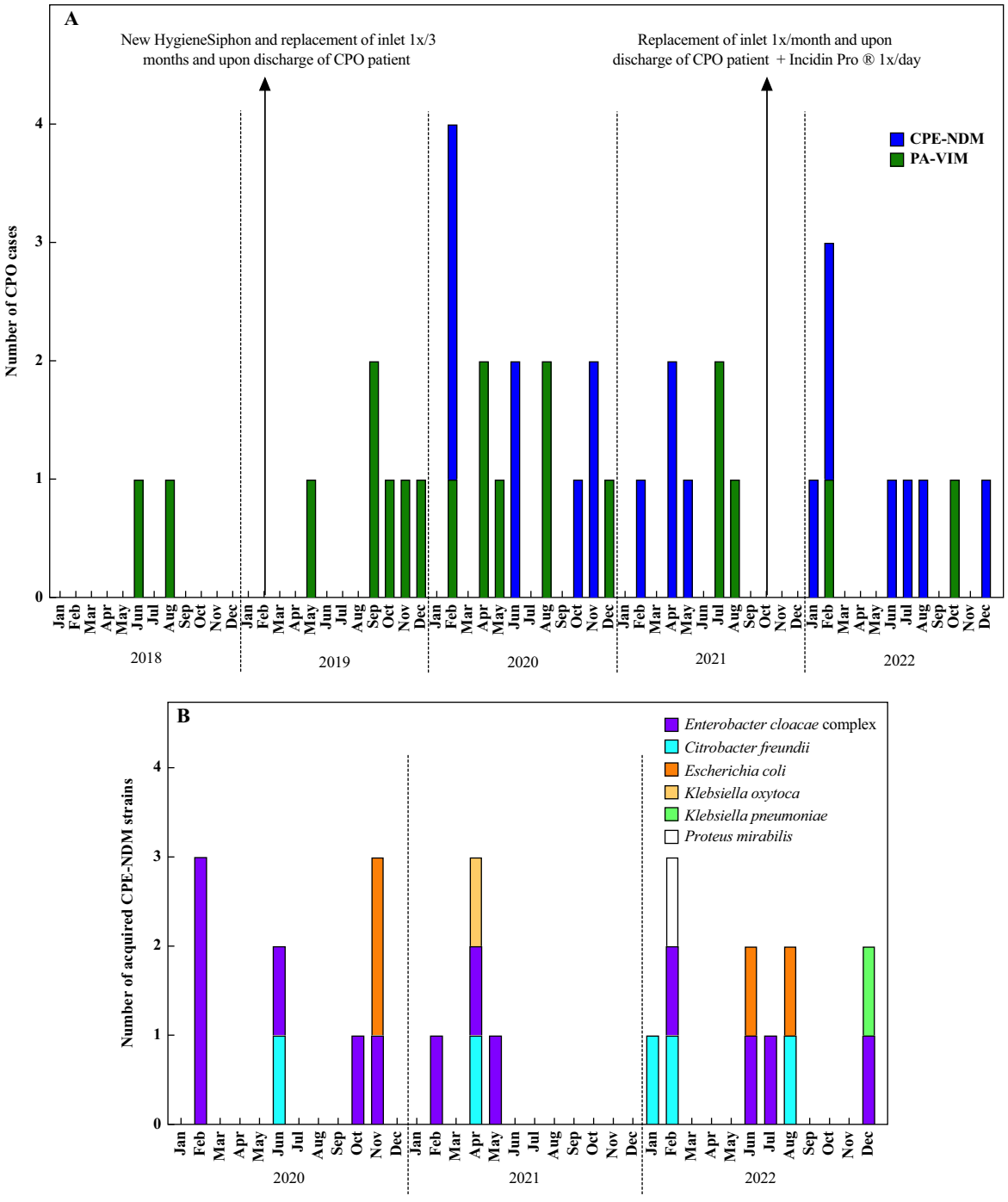

Figure 2. (A) Epidemic curve of VIM-producing *Pseudomonas aeruginosa* (PA-VIM) and/or NDM-producing Enterobacterales (CPE-NDM) patient acquisitions in the intensive care unit (ICU). (B) Epidemic curve of CPE-NDM strains acquisition in ICU.

bla_{NDM-1} isolated from the first CPE-NDM strain of the outbreak CPE275 (ST395; 136,152 pb; October 2019) for Enterobacterales and PA1936 the first PA-VIM strain of the outbreak (ST111; 7,001,756 pb; June 2018) for PA-VIM ST111.

Results

Outbreak description

CPO acquisition incidence per 1000 hospitalization-days increased in ICU from 2019. Notably, PA-VIM increased from 0.49 in 2018 to 1.72 in 2020 and CPE-NDM increased sharply from 0 in 2018 to 1.97 in 2020. The combined attack rate of PA-VIM and CPE-NDM increased from 0.17% in 2018 to 0.49% in 2019, 1.89% in 2020, 0.72% in 2021, and to 0.87% in 2022. From 2018 to 2022, 37 ICU patients were newly colonized or infected with acquired CPE-NDM and/or PA-VIM. The median ICU length of stay was 43 days, and 51% of patients died. Prior to CPO detection, the median ICU length of stay was 19 days, and all patients received anti-Gram-negative antibiotics. A total of 19 PA-VIM and 25 CPE-NDM were detected, including 13 *Enterobacter cloacae* complex, five *Citrobacter freundii*, four

Escherichia coli, one *Klebsiella oxytoca*, one *Proteus mirabilis*, and one *K. pneumoniae* (Figure 2A and B). One patient acquired both PA-VIM and two CPE-NDM isolates, and five patients harboured two different CPE-NDM isolates (Supplementary Table A1). Patients with acquired CPO were not related to one room, suggesting several persistent environmental reservoirs of PA-VIM and CPE-NDM during the five years.

Environmental investigations/sink colonization

The sink drain of ICU rooms (Figure 3A) has been suspected to be an environmental reservoir since 2019. Indeed, we investigated several environmental sources (sink drains, sink, faucet jetbreaker, water and bedpan washer) and no CPO was found in any of these samples except those from the sink drains. Between June 2018 and January 2023, intermittent sampling of the 14 sink drains (210 environmental samples from the inlets and 70 from the drain valves) confirmed their colonization with PA-VIM and/or CPE-NDM (Figure 3B). In October 2019, the sink drain of the rooms M4 and M10 were positive with CPE-NDM whereas no CPE-NDM-positive patients had previously been hospitalized in these rooms. Likewise the sink drain of room M2 was repeatedly positive for PA-VIM without any known PA-VIM-infected/colonized patients in this room. These observations suggested a contamination route of some sink drains independent of CPO-positive patients. The rate of colonization upon inlet replacement and despite daily cleaning was rapid. After one month, the four inlets were colonized by 10^3–10^4 cfu/mL of carbapenem-resistant bacteria, including PA-VIM, *E. cloacae* complex NDM, and *C. freundii* NDM. The permanent drain valves were colonized with 10^7–10^8 cfu/mL of carbapenem-resistant bacteria (Figure 4A). In January 2023, sampling of different siphon parts revealed a colonization of the whole siphon with CPO (Figure 3).

Sequencing results

One hundred and twenty-six isolates were sequenced, 40 (18 PA-VIM and 22 CPE-NDM) from patients and 86 (28 PA-VIM and 58 CPE-NDM) from environmental sampling.

VIM-producing P. aeruginosa

Polyclonality was observed among PA-VIM, including ST111 (N=32) as the predominant clone, ST179 (N=7), ST175 (N=2), ST245 (N=2), and ST235, ST253, and ST395 with one isolate of each (Supplementary Figure A1). Nevertheless, all isolates (except ST235) harboured bla_{VIM-2} and shared a large array of associated resistance genes (Supplementary Figure A2). In 93.3% (42/45) of isolates, the bla_{VIM-2} gene was found within a class I integron, inserted in a Tn21-like transposon. No plasmid was detected in PA-VIM isolates. The genetic environment of bla_{VIM-2} of ST175 and ST395 clinical isolates differs from the other strains and may thus not be linked to the same environmental reservoir. The wgMLST analysis showed the genetic proximity between the isolates within each MLST. Focusing on PA-VIM ST111, SNP analysis confirmed that most (30/32) clinical and environmental isolates originated from a common reservoir (<10 SNPs). However, isolates from room M2 differed genetically in ~37 SNPs, suggesting different origins (Supplementary Figure A3).

NDM-producing Enterobacterales

The 80 CPE-NDM isolates included 46 *E. cloacae* complex, 24 *C. freundii*, four *E. coli*, four *K. oxytoca*, one *K. pneumoniae*, and one *P. mirabilis*. Polyclonality was observed among *E. cloacae* complex, *C. freundii*, *E. coli*, and *K. oxytoca* with a clonal spread of ST595 *E. cloacae* complex (34/46) (Supplementary Figure A1). The plasmid belonging to the incompatibility (Inc)C (~140 kb) harbouring bla_{CMY-6} and bla_{NDM-1}, *sul1*, *qacEdelta1*, and *aac(6′)-Ib3* located within a class 1 integron was present in all Enterobacterales species from the outbreak, including patient and environmental strains (Supplementary Figure A4). SNP analysis confirmed that the CPE-NDM outbreak isolates differed by less than two SNPs in this genomic region regardless of species (Supplementary Figure A5). The same plasmid was found in a community-associated *E. coli* strain (CPE399), suggesting the spread of these genes within the community. The three ST544 NDM-1-*E. cloacae* complex isolates (from patients and sink drain) harboured *mcr-9* (mobilized colistin-resistance) genes carried by a different plasmid (Inc HI2/HI2A).

Interestingly, 116 isolates (72 CPE-NDM and 44 PA-VIM) from patients and sink drains harboured the gene *qacEΔ1* located in the 3′-CS of class 1 integron. The IncC plasmid also carried the *sugE* gene. These resistance genes encode for efflux pumps (small multidrug resistance (SMR) family), conferring resistance to quaternary ammonium compounds (QACs).

Mitigation strategies

Due to the detection of nine PA-VIM and CPE-NDM acquisitions in 2022 with the evidence for a water reservoir, and the ineffectiveness of measures, a new protocol combining enziSurf and Phago'Spore was evaluated on four ICU sink drains without inlet.

The efficacy of this protocol was compared during one month with the routine protocol (Figure 4B). After the four-day curative protocol, no carbapenem-resistant bacteria were detected in the drain valve. After four weeks of preventive protocol, two drain valves were colonized by 10^2–10^3 cfu/mL of carbapenem-resistant bacteria (*Pseudomonas monteilii*) but not by CPO, unlike the routine protocol where CPO quickly recolonized the four new inlets.

These preliminary results led to replacing all HygieneSiphons by standard chrome-plated brass sink drains and cleaning twice per week with the new preventive protocol combining enziSurf and Phago'Spore by the cleaning staff. The monthly control of the proximal drain showed aquatic (ex: *Pseudomonas oleovorans*) and skin bacteria but no CPO after seven months of prospective analysis, even when the distal parts (pipe connected to the wall) were colonized by CPO (Figure 3). Only one NDM-*E. cloacae* complex acquisition was detected in May 2023 in room M01 non-related with sink drain. The cleaning staff reported no fumes nor odour during the use of these products, but the application took longer and was less straightforward than the previous Incidin Pro protocol.

Training of healthcare workers on the correct use of the sinks in ICU patient rooms was performed in parallel. They focused on the appropriate use of the sinks for hand hygiene, the non-use of the sinks to pour intravenous bags and dialysis fluid down the drain, and the separation of non-contaminated and contaminated areas and tasks.

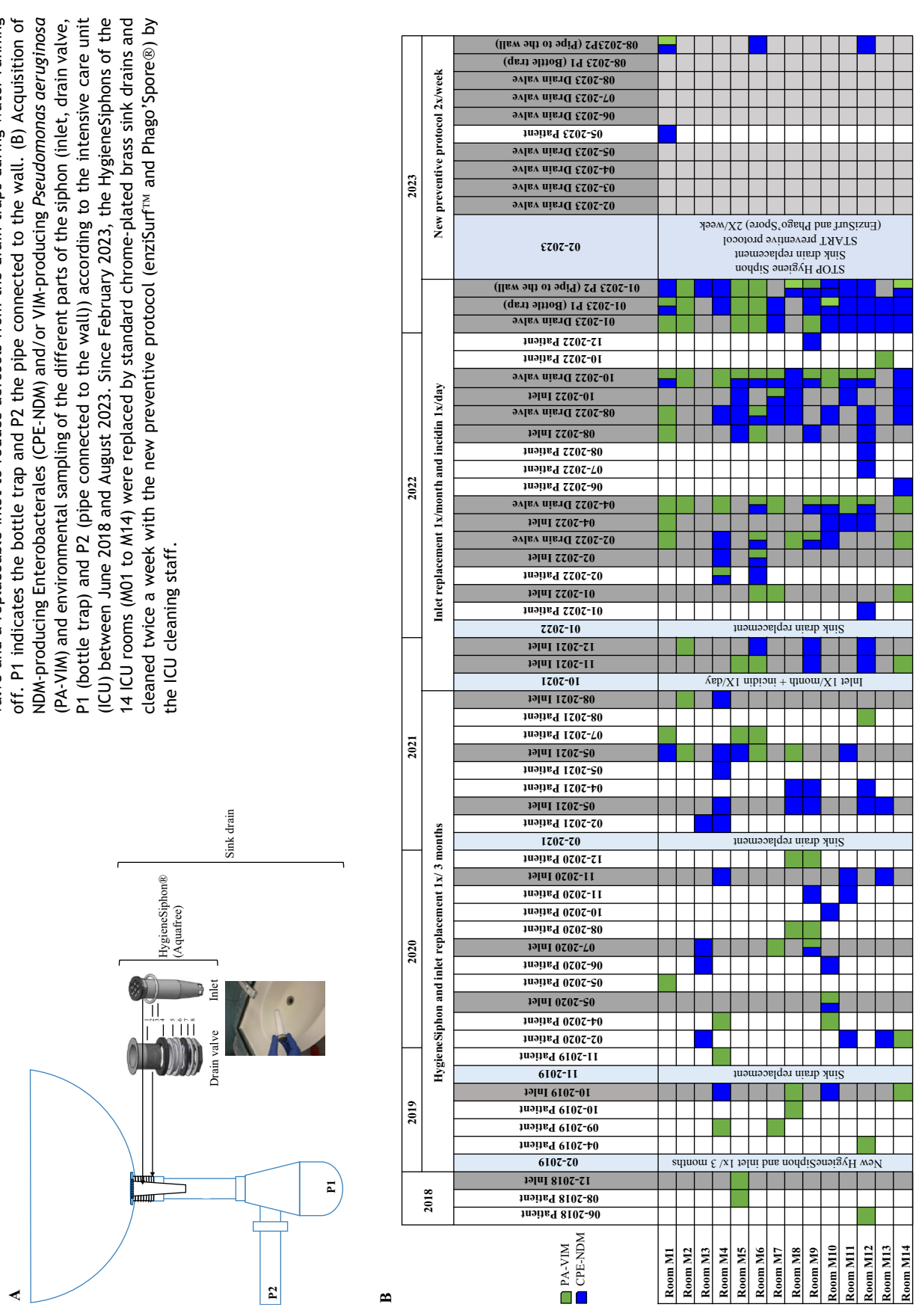

Figure 3. (A) Siphon scheme. HygienSiphon® (Aquafree) is composed of a permanent drain valve and a replaceable inlet to reduce aerosols from the drain traps during water running off. P1 indicates the bottle trap and P2 the pipe connected to the wall. (B) Acquisition of NDM-producing Enterobacterales (CPE-NDM) and/or VIM-producing *Pseudomonas aeruginosa* (PA-VIM) and environmental sampling of the different parts of the siphon (inlet, drain valve, P1 (bottle trap) and P2 (pipe connected to the wall)) according to the intensive care unit (ICU) between June 2018 and August 2023. Since February 2023, the HygieneSiphons of the 14 ICU rooms (M01 to M14) were replaced by standard chrome-plated brass sink drains and cleaned twice a week with the new preventive protocol (enziSurf™ and Phago'Spore®) by the ICU cleaning staff.

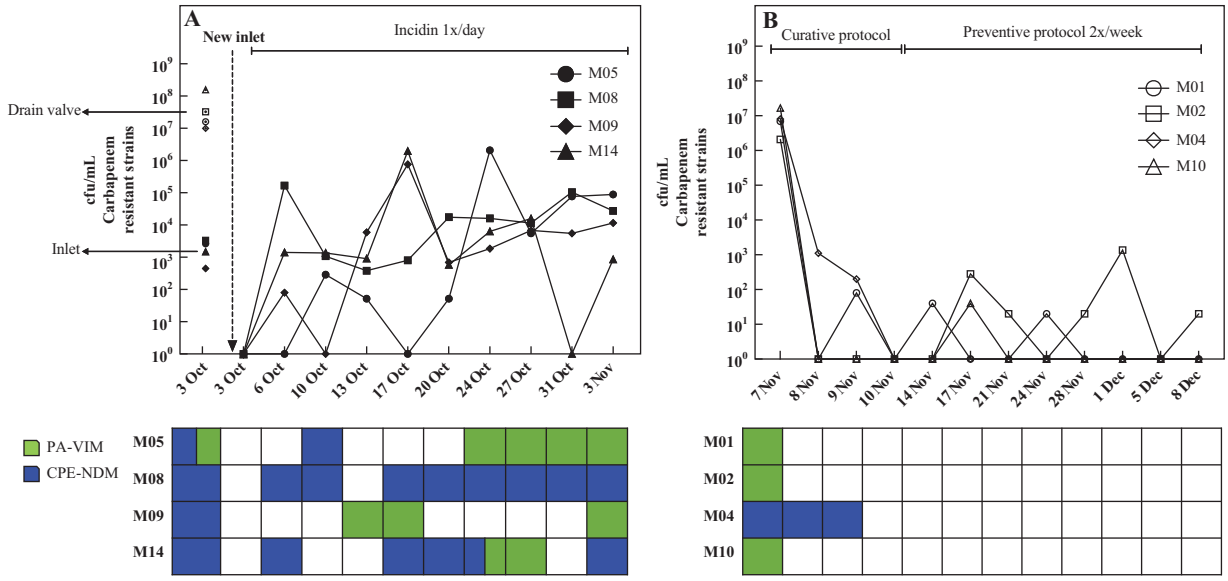

Figure 4. Colonization rate of sink drains by carbapenem-resistant Gram-negative bacteria. The routine protocol (daily cleaning with 1 L of 0.5% Incidin Pro® with new inlet replacement) (A) was compared to a new protocol combining enziSurf™ and Phago'Spore® (B) in four sinks. The number of carbapenemase-resistant bacteria was expressed in cfu/mL.

Discussion

To our knowledge, this investigation represents the first described long-term outbreak of CPO involving a diverse set of bacterial species with a common environmental reservoir.

Reported healthcare-associated CPO outbreaks are generally caused by a single, clonal strain. In this study, the WGS analysis revealed both a polyclonality among CPO strains with a clonal spread of *E. cloacae* complex ST595 and *P. aerginosa* ST111, and highly transmissible mobile genetic elements carrying a plethora of resistance. The VIM-2-producing *P. aeruginosa* ST111 is a high-risk, epidemic MDR/XDR lineage, globally widespread including Belgium and associated with high morbidity and mortality [12—15]. Unlike the latter, *E. cloacae* complex ST595 has only been previously described in two American studies carrying class A β-lactamase *Klebsiella pneumoniae* carbapenemase [16,17]. Multiple genetic mechanisms were involved in NDM or VIM transmission, including clonal spread and horizontal gene transfer mediated by plasmid and/or transposon jump. Both bla_{NDM-1} and bla_{VIM-2} genes were located within a class I integron, found in Tn*21*-like transposons integrated either in the IncC plasmid for Enterobacterales or in the chromosome for *P. aeruginosa* [18]. Some clones, such as *P. aeruginosa* ST245, *E. cloacae* complex ST513, and *C. freundii* NT, were mainly found in one room. We also observed within the ST111 group a low genetic distance between the clinical and/or environmental isolates collected within the same room. These observations may suggest a local ecological (e.g. pathogen introduction by the hospitalized patient) and evolutionary pressure within each ICU room.

Phylogenomic analysis confirmed the epidemiological link between clinical and environmental strains. Hospital sinks are well-known reservoirs for the transmission of Gram-negative pathogens in general, and CPO in particular [19—23]. Interestingly, some sink drains were contaminated by CPO without any known infected/colonized patients previously hospitalized in these rooms. The introduction of pathogens into the sink trap is multifactorial, such as the use of sinks for handwashing and disposal of waste and the transmission from neighbouring rooms via the horizontal drainage system [24]. During faucet operation, contaminated aerosols and drain contents are then dispersed to surrounding areas from the sink and bacteria may be transferred to healthcare workers and the patient [19,22,25,26]. Sub-optimal room and sink designs can put patients at risk [25—27]. In addition, it has been observed that sterile materials and devices intended for patient insertion were regularly misplaced in the very near surroundings of the sinks in the ICU [28].

A range of interventions to eradicate these reservoirs has been published, emphasizing disinfection, biofilm disruption, replacement of sink drain/plumbing, and complete removal of the reservoir [19,29]. Infection control strategies are often bundled together during outbreaks.

Disinfection alone fails to control the CPO reservoir, leading to hospital-acquired infection [19,29—31]. Biofilms may limit the penetration of disinfectants such as chlorhexidine and QACs (benzalkonium chloride) [32]. Several QAC efflux systems have been discovered in Gram-negative bacteria (*sugE*, *emrE*, *qacE*, and *qacEΔ1*), conferring resistance to QACs and multiple antimicrobials [33—36]. The *sugE* and *qacEΔ1* genes of antiseptic resistance were broadly identified in the clinical and environmental isolates of our outbreak. Although the exact role of *qacEΔ1* is still controversial, the daily use of quaternary ammonium-based disinfectant in our sink drains may have created selective pressure on CPO [37]. According to the review by Collet *et al.*, using hydrogen peroxide or peracetic acid constitutes an improbable risk for developing resistance to antimicrobials [34].

For the control of the CPO reservoir, it is therefore helpful to reduce the biofilm's density before applying the biocide. Most of the interventions described in the literature, such as pressurized steam [38,39], self-disinfecting traps with electromechanical vibration, bundled with heat or ultraviolet

radiation [21,40,41], and replacement of sinks and/or sink drains [29], showed only temporary reductions in transmission and sink colonization, as observed in our study. Removal of bacterial reservoirs with the implementation of waterless patient care was the most successful intervention in CPO outbreaks, showing an effect in all studies [25,42,43].

In our case, the combined actions of QAC daily, the design of the HygieneSiphon (inlet replaced monthly) and the sink drain replacement every year were insufficient to prevent sink drain recolonization and CPO acquisitions. Recolonization may occur after exposure to contaminated materials or retrograde growth from P-shaped traps or the water drainage network. Indeed, we observed that CPO colonized the whole siphon to the entrance in the wall. The removal of sinks and a change in the architecture of the rooms in our setting were unfortunately not feasible. We therefore looked for an efficient and inexpensive solution to limit CPO acquisition and sink drain colonization. The repeated combination of a foaming product able to degrade the biofilm and a foaming disinfectant based on peracetic acid and hydrogen peroxide, with a longer contact time, might be a promising solution based on literature review [34,44]. Additional risk mitigation strategies (enzymatic, probiotic, or phage-based approaches) to address persistent bacterial environmental reservoirs are under investigation but still need to be adequately tested in clinical environments [44]. Enzymes, such as proteases, DNAses, and polysaccharide depolymerases, may enhance the biocidal effect of chemical disinfectants or antibiotics by disrupting the biofilm matrix [45—49]. Here we evaluated the effect of an enzymatic cocktail on multi-species colonized sink drains for the first time. The new cleaning protocol allowed a 10,000-fold reduction in carbapenem-resistant bacteria and no CPO colonization was observed after seven months in the proximal sink drain of ICU rooms, although an extended observation period is required. In addition, the enzymatic cocktails may have a less negative impact on the non-targeted organisms and the environment than biocides routinely used: the enzymatic cocktail is composed of enzymes found in the environment and humans and is biodegradable (≥99%), unlike chemical disinfectants such as Incidin Pro, which is known to be highly toxic to aquatic life with long-lasting effects.

There are several limitations in our study. First, our evaluation focused on CPO acquisition. However, other carbapenem-resistant bacteria were present in the sink drain (*Stenotrophomonas maltophila*, non-carbapenemase-producing *Pseudomonas* sp. along with CPO). These bacteria might have a role in the biofilm persistence and the exchange of genetic material. Second, environmental sampling has not been done systematically, and the inoculation method has also been optimized over the years (addition of an enrichment medium), so we cannot assess whether there has been an increase in sink drain colonization. Third, because we directly evaluated the efficacy of the enzymatic cocktail combined with a peracetic acid and hydrogen peroxide disinfectant, the effect of the products separately should be investigated. Finally, we should have performed audits to confirm the correct application of hygiene instructions by the nursing team. The disposal by healthcare workers of leftover intravenous fluids or food supplements into the sink has been demonstrated to favour the durable establishment of pathogens in the latter [22,38,50].

In conclusion, hospital sinks provide a permissive environment for biofilm formation and microbial colonization and are the source of hospital outbreaks. The persistence of bacteria resistant to antibiotics and disinfectants with the ability to transfer mobile genetic elements makes the outbreak investigation and control complex. Emphasis should be placed not only on optimizing sink design and placement but also on innovative approaches to address persistent environmental reservoirs and prevent transmission of potentially dangerous pathogens from sinks.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2023.10.010.

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Original Article

Molecular epidemiology of an extended multiple-species OXA-48 CPE outbreak in a hospital ward in Ireland, 2018–2019

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Abstract

Objectives: Molecular epidemiological description of an OXA-48 CPE outbreak affecting a tertiary-care hospital ward in Ireland over an extended period (2018–2019).

Methods: Microbiological testing and whole-genome sequencing (WGS) were performed on all 56 positive OXA-48 outbreak case isolates.

Results: In total, 7 different species were identified: *Enterobacter hormaechei* (n = 35, 62.5%), *Escherichia coli* (n = 12, 21.4%), *Klebsiella pneumoniae* (n = 5, 8.9%), *Klebsiella oxytoca* (n = 1, 1.8%), *Klebsiella michiganensis* (n = 1, 1.8%), *Citrobacter freundii* (n = 1, 1.8%), and *Serratia marcesens* (n = 1, 1.8%). *E. hormaechei* ST78 was the most common genotype (n = 14, 25%). Two major pOXA-48 plasmid types were identified throughout the outbreak, 'types' 1 and 2, and 5 major *E. hormaechei* clonal groupings were identified: ST78, ST108, ST1126, ST135, and ST66. Within each of the ST108, ST1126, ST135 and ST66 groups, the pOXA-48 harbored within each isolate were the same. Within ST78, 9 isolates contained the pOXA48 'type 2' plasmid and 5 contained the 'type 1' plasmid. Environmental specimens were taken from different outbreak ward locations: handwash basins, sink and shower drains, and taps. Of 394 environmental specimens, OXA-48 CPE was isolated from 26 (6.6%).

Conclusions: This prolonged outbreak of OXA-48 CPE was confined to one ward, but it exemplifies the complexity and difficulty in the control of these organisms. With multiple species and genotypes involved, they may be better described as 'plasmid outbreaks.' WGS provided insights into this diversity and potential transmission among cases, though its usefulness would be enhanced by analysis as close as possible to real time so that interventions can be implemented as soon as data are available.

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Carbapenemase-producing Enterobacterales (CPE) infections can lead to treatment failure, extended hospital stays, increased healthcare costs, and increased mortality.[1] They represent a major public health threat worldwide, and the World Health Organization has stated that research and development of antibiotics against them is of critical international importance.[2] CPE infection has increased in prevalence globally since the early 2000s,[3–5] when these organisms first emerged and they have been associated with nosocomial outbreaks in many countries including Ireland.[6,7] As a result, CPE was declared a national public health emergency in Ireland in October 2017,[8] and a concerted national effort to reduce the incidence of CPE demonstrated some early signs of success.[9] OXA-48 CPEs have been particularly effective at spreading globally such that they are now the most common carbapenemase type in many countries, including Ireland.[9–11]

Here, we describe an outbreak of OXA-48 CPE affecting one ward in a tertiary-care hospital in Ireland over an extended period. The ward accommodated patients admitted under a variety of medical specialties and comprised a total of 35 beds, 5 of which were in single-bed rooms, whereas the remaining 30 beds were distributed among 4 six-bed rooms, 1 four-bed room, and 1 two-bed room. All patients admitted to the ward between July 2018 and December 2019 were included in this retrospective descriptive analysis. Outbreak cases were defined as patients admitted to the ward after July 1, 2018, who had a negative OXA-48 CPE admission screen (within 24 hours of admission) but subsequently had a positive microbiological specimen culture for OXA-48 CPE (n = 45). Here, we describe this lengthy

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CPE outbreak using microbiological and whole-genome sequencing (WGS) data.

Methods

Microbiological testing

All antimicrobial susceptibility testing was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Confirmation of CPE clinical and environmental isolates was conducted in the hospital clinical microbiology laboratory using: meropenem and ertapenem minimum inhibitory concentration results; CHROMID CARBA SMART screening plates (bioMérieux, Marcy-l'Étoile, France); immunochromatography using the RESIST-3 O.K.N. *K*-SeT flow assay (Coris BioConcept, Gembloux, Belgium) to detect OXA-48, KPC, and NDM carbapenemases; and PCR via the Xpert Carba-R assay (Cepheid, Sunnyvale, CA). Species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS). Any isolates that were CPE negative on RESIST-3 O.K.N. *K*-SeT and PCR but were phenotypically nonsusceptible to carbapenems underwent carbapenem inactivation method (CIM) testing.[12] Isolate species were identified using MALDI-TOF. CIM-positive isolates were referred to the National CPE Reference Laboratory Service (NCPERLS) for WGS and determination of other CPE types not tested for by the Xpert Carba-R platform.

Environmental specimens

Although it was not undertaken during the early phase of the outbreak, environmental testing was conducted from November 2018 to May 2019 as part of outbreak investigation and ongoing monitoring. Environmental specimens for CPE detection were taken from a variety of locations within the ward. The focus of environmental sampling was high-touch surfaces, sinks, showers, and drains. Environmental sampling was not random and was not systematically conducted.

WGS and bioinformatics analysis

All outbreak cases and 3 of the environmental isolates were sequenced (paired-end sequencing, read length 300 base pairs) using the Illumina MiSeq platform at NCPERLS. The resulting short reads were quality checked and assembled *de novo* using Spades within the BioNumerics (Applied Maths) genomics software platform. In the analysis, we also included 3 OXA-48 isolate genomes from the same hospital but not the same ward and with no identifiable epidemiological link to the outbreak ward. Assembled WGS data from NCPERLS were analyzed using BioNumerics to verify sequence quality, species, OXA-48 CPE gene presence, and genotype. We also described genetic relationships among isolates using a multilocus sequence type (MLST) gene-by-gene approach. Minimum-spanning trees of pOXA-48 locus (n = 71), ribosomal MLST (rMLST) locus (n = 53) and whole genome MLST (wgMLST) locus differences among isolates were constructed. Further genomic species identification was carried out using the rMLST.org website.[13]

Control measures

Outbreak control interventions implemented included admission and weekly CPE screening of patients admitted to the outbreak ward, and isolation or cohorting of patients colonized with CPE, depending on availability of single rooms on the ward. Patient care encounters were undertaken using contact precautions, which included gloves and long-sleeved gowns for routine care. Proactive antimicrobial stewardship rounds were also undertaken. Enhanced environmental cleaning and disinfection was routinely implemented, along with hydrogen peroxide vapor (HPV) treatment upon patient discharge and regular HPV treatment of patient bathrooms and the sluice room. The ward was closed on several occasions, then decanted, with enhanced environmental cleaning and HPV treatment of the entire ward prior to reopening. Additionally, refurbishment works were undertaken across the ward, including patient bathrooms. In keeping with national guidance, staff screening for CPE carriage was not undertaken.

Results

Species identification

From 45 cases, a total of 59 new CPE isolates were obtained, with specimen dates between July 2018 and August 2019: 57 were from rectal swabs obtained during active surveillance cultures; 1 was obtained from a urine sample and 1 from a blood culture specimen. Moreover, 12 cases had >1 carbapenemase-producing species isolated (11 cases had 2 species and 1 case had 3 species) (Supplementary Table S1). MALDI-TOF was used to identify the species as follows: *Enterobacter cloacae* complex (n = 36, 61%), *Escherichia coli* (n = 14, 23.7%), *Klebsiella pneumoniae* (n = 5, 8.5%), *Klebsiella oxytoca* (n = 2, 3.4%), and *Citrobacter freundii* and *Serratia marcescens* (n = 1, 1.7%, respectively).

Antimicrobial susceptibility testing

All isolates were nonsusceptible; they had intermediate or full resistance to amoxicillin, co-amoxiclav, and piperacillin/tazobactam (Supplementary Table S2). We also identified nonsusceptibility to gentamicin (n = 13, 22%), cotrimoxazole (n = 19, 32.2%), aztreonam (n = 23, 39%); and fosfomycin (n = 4, 6.8%). In total, 40 isolates (67.8%) were classified as extended-spectrum β-lactamase (ESBL) producers because they were nonsusceptible to 1 of the third- or fourth-generation cephalosporins tested for - cefotaxime, ceftazidime, or cefepime. In total, 54 isolates (91.5%) were nonsusceptible to ertapenem and 19 (32.2%) were nonsusceptible to meropenem. All isolates were susceptible to amikacin, ceftazidime/avibactam, and colistin.

Genomic analysis

All isolates contained the $bla_{\text{OXA-48}}$ carbapenemase-producing gene. Genomic species identification largely concurred with MALDI-TOF results (n = 56). However, WGS provided further definition for isolates identified as *Enterobacter cloacae* complex by MALDI-TOF (n = 35), identifying them all as *Enterobacter hormaechei*. One isolate identified as *K. oxytoca* was identified by WGS as *Klebsiella michiganensis*.

The 12 *E. coli* isolate genomes comprised 12 different sequence types (STs), one of which was a member of the globally spread multidrug-resistant clone, the ST131 complex. *E. hormaechei* isolates comprised 5 STs, of which ST78 was the most common (n = 14) and accounted for 40% of these isolates. Most ST78 isolates also appeared at a later point in the outbreak with 85.7% of this ST appearing from February 2019 onward (Table S3). *E. hormaechei*

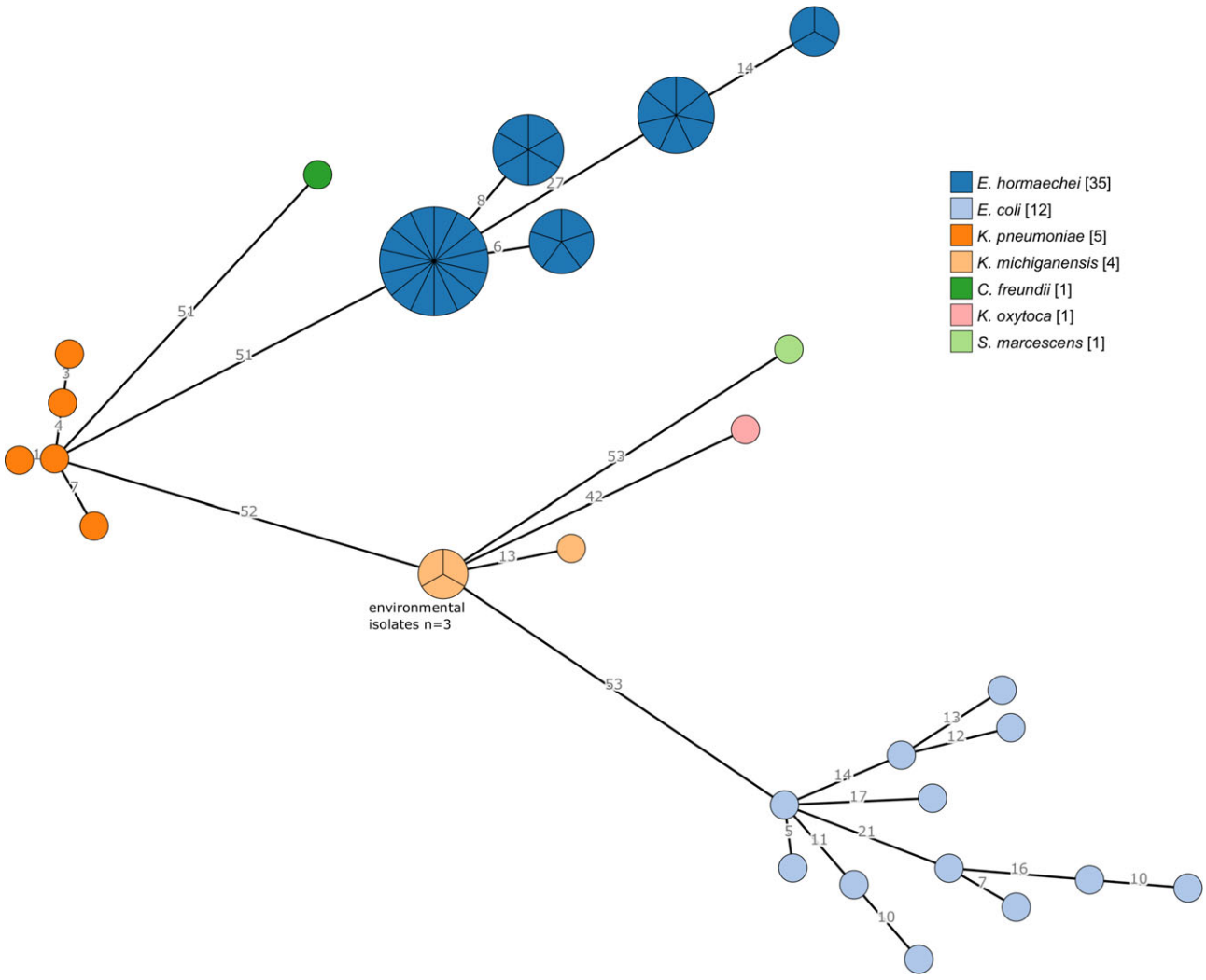

Fig. 1. Minimum-spanning tree of ribosomal MLST locus (n = 53) allele differences among 56 outbreak cases and 3 environmental isolates. Each circle (node) contains isolates that are indistinguishable at all loci. Nodes are colored by species, as determined by WGS. Numbers on lines (edges) connecting nodes indicate the number of allele differences between connected nodes. Nodes are divided in pie-chart form for individual isolates.

ST108 was the second most common (n = 7; 20%), but 4 of these isolates were sampled from 4 patients from a ward screen from the same day. All 3 environmental isolates referred for WGS were *K. michiganensis* ST143.

OXA-48 outbreak strains species genotype diversity

We identified a diverse set of genotypes amongst the species, but *E. hormaechei* was less diverse with fewer ribosomal sequence types (rSTs), even though there were more isolates of this species (Fig. 1). All ST78 isolates shared the same rST56604 (n = 14) and all ST108 isolates shared the same rST63173 (n = 7). Indeed, isolates within each 7-locus ST also shared rSTs. Each of the *E. coli* isolates had a different rST. The 3 environmental *K. michiganensis* isolates shared the same rST but differed by 13 of 53 loci with the *K. michiganensis* case isolate.

OXA-48 outbreak strain plasmid analysis

We identified 2 major pOXA-48 plasmid types among the case isolates (Fig. 2), with 2 differences of the 71 pOXA-48 plasmid loci. There were 16 isolates (328.6%) with indistinguishable type 1 plasmids, and 40 isolates (71.4%) had indistinguishable type 2 plasmids. We detected no association with species; at least 4 of the species were represented by each plasmid type. The 2 plasmid types were contained in isolates from cases that spanned the whole outbreak period (Fig. 3). The environmental isolates (n = 3) all shared the same plasmid type, but the case isolate of the same species (ie, *K. michiganensis*) was the other plasmid type. Of the 12 cases with >1 species isolate, each pair of species, or 3 species in 1 patient, shared the same plasmid type. For example, in the patient from which *E. cloacae*, *E. coli*, and *S. marcescens* were isolated, all isolates harbored the type 2 plasmid. For 10 of 12 cases, the multiple species were isolated from specimens taken on the same date. For one case the second species was isolated a week later, and for another case the second species was isolated a month later.

Outbreak Enterobacter cloacae complex and OXA-48 plasmid type

We detected 5 major clonal groupings of *E. hormaechei:* ST78 (n = 14), ST108 (n = 7), ST1126 (n = 5), ST135 (n = 3), and ST66

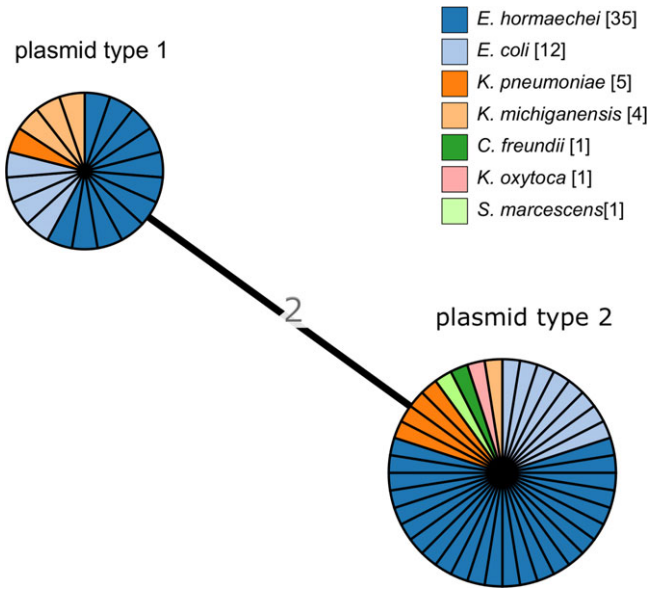

Fig. 2. Minimum-spanning tree of pOXA-48 plasmid MLST locus (n = 71) allele differences among 56 outbreak cases and 3 environmental isolates. Each circle (node) contains isolates that are indistinguishable at all loci. Nodes are colored by species, as determined by WGS. Numbers on lines (edges) connecting nodes indicate the number of allele differences between connected nodes. Nodes are divided in pie-chart form for individual isolates.

(n = 3) (Fig. 4). Moreover, <7 locus differences among 15,605 wgMLST loci were identified within each grouping, suggesting a high degree of relatedness. Within each of the ST108, ST1126, ST135, and ST66 groups, the pOXA-48 harbored within each isolate was also the same type. Within the ST78 grouping, 9 isolates contained the type 2 plasmid and 5 contained the type 1 plasmid. Also, 3 *E. hormaechei* OXA-48 case isolates from patients accommodated elsewhere the hospital, with no known link to the outbreak ward, were clustered within the outbreak isolates and therefore were considered highly related. Two ST66 isolates from October and November 2018 were 1–2 wgMLST locus differences from other outbreak isolates. One ST78 isolate from October 2018 was 4 locus alleles different from the nearest case isolates. The more diverse ST78 was the longest persisting *E. hormaechei* genotype (Table S3). Except for 1 ST135 case in April 2019, none of the other STs (ST1126, ST108, or ST66) were associated with a case after February 2019.

Environmental sampling

From November 2018 to May 2019, 394 environmental samples from the outbreak ward were tested (Supplementary Fig. S1). The earliest OXA-48–positive environmental sample was an OXA-48 *K. oxytoca* taken from the handwash basin tap in section F in January 2019. OXA-48 CPE was detected from 26 environmental samples (6.6%). The most common species detected were *E. cloacae* complex and *C. freundii*, each detected on 8 separate occasions. The last 2 positive environmental samples, on 2 separate dates in April 2019, were both *E. cloacae* complex. All positive samples were taken from handwash basins, sink drains, shower drains, or taps from various rooms or sections of the ward (Fig. S2). Of the locations in the ward with the highest number of positive environmental samples, section E was notable, with the most detections overall and detections on 9 separate occasions before and after the major decant and refurbishment in February 2019. Sink drains seemed to be particular hot spots, with OXA-48–producing isolates cultured from samples taken from sinks in several single-patient rooms as well as the treatment room (Supplementary Table S4).

Discussion

This prolonged outbreak of OXA-48 CPE on a tertiary-care hospital ward exemplifies the complexity and difficulty in the control of these organisms. Nosocomial CPE outbreaks can involve single clones,[14,15] but they can also involve multiple genotypes and species,[16] making tracking of transmission very difficult. Clonal and nonclonal spread of OXA-48 in nosocomial outbreaks have been documented.[17] Nosocomial CPE outbreaks may often also be protracted and last several years.[14,15,18] Here, the first outbreak case presented on July 31, 2018, and the last was >1 year later on August 12, 2019. The outbreak involved 7 different *Enterobacterales* spp, and within each species no single clone predominated. However, the ST78 *E. hormaechei* genotype was the most prevalent overall and is a clone associated with nosocomial outbreaks and with other carbapenemase types.[15,19,20] This ST was the most predominant in cases, despite major IPC measures, including a ward decant and refurbishment in February 2019.

Despite the complexity, genomics did provide some insight into the outbreak dynamics. Given that several patients harbored isolates that were indistinguishable by high-resolution genomics methods, there may have been short-chain transmission of the organism among patients or it may have been acquired from a common source, either environmental or another unknown and unsampled contact. For example, 5 cases shared the same ST108 *E. hormaechei* with the same pOXA-48 plasmid that were indistinguishable by wgMLST. Of these 5 cases, 4 were sampled on the same day and 1 isolate was from a sample taken ~6 weeks later. A number of these cases were known to share a common contact and/or overlapped in their stay in a section of the ward. Also, 2 of these sections, D and F, had positive environmental screens (*K. oxytoca* and *E. cloacae* complex). The direction of potential transmission between patients or between patients and environment is difficult to ascertain, particularly in a retrospective analysis. Patient-to-patient transmission is known to occur, and these organisms, particularly *Klebsiella* and *Enterobacter* spp, have become adapted to the nosocomial environment. Thus, stringent adherence to all elements of standard and transmission-based precautions is required. Retrospective genomic analysis can highlight links between apparently sporadic CPE cases in the nosocomial environment.[21]

Two closely related yet distinct pOXA-48 plasmid types were identified in this outbreak. Both of these have been found across Ireland over the past several years,[22] and one, called here 'plasmid type 2,' was associated with a prolonged outbreak in another large urban hospital in Ireland. Within-patient colonization of multiple OXA-48 species and interspecies plasmid transfer have been well documented.[23–25] For all 12 cases with >1 species isolate, each pair of species, or 3 species in the case of one patient, shared the same plasmid type. In most of these cases, specimens were taken on the same date. This finding may indicate the sharing of plasmids between species within patients. Again, these results highlight the importance of infection prevention precautions to prevent opportunities for colonization. However, no means for decolonizing CPE carriers is available yet, so measures to reduce and prevent infection (eg, wound and invasive device management) are of critical importance.

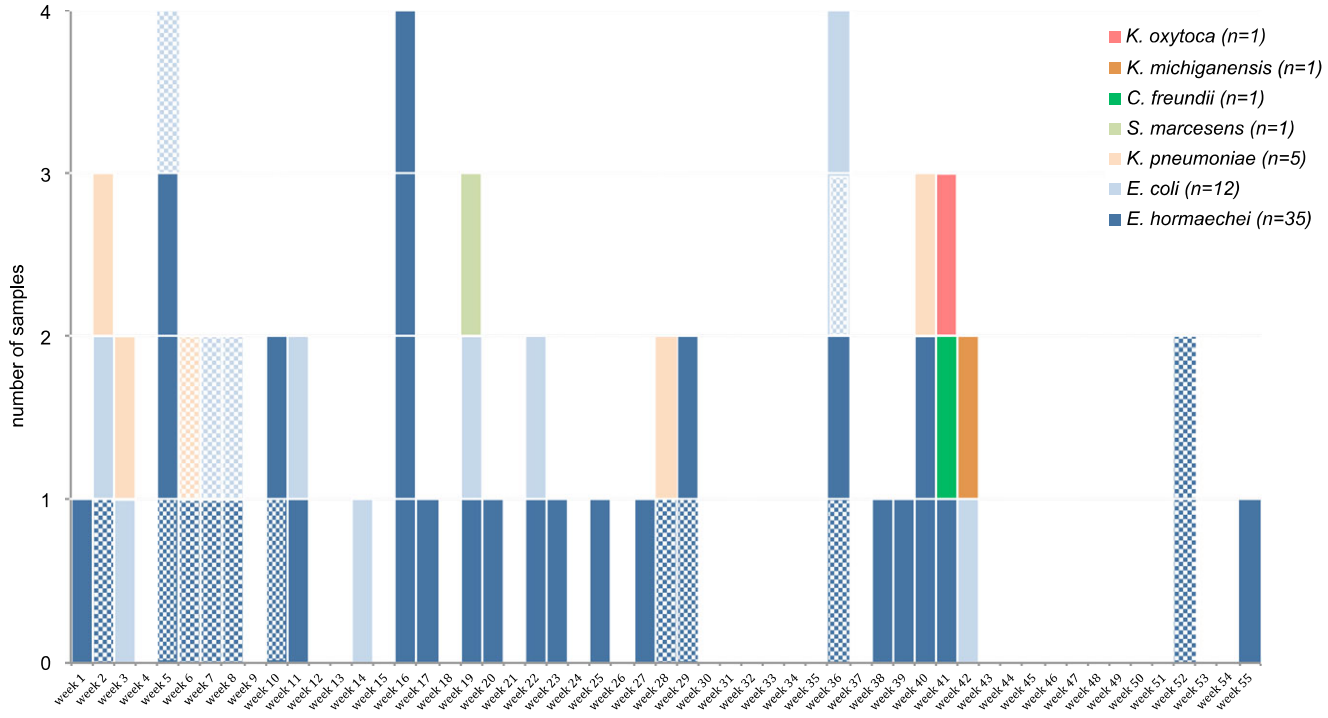

Fig. 3. Species by week of outbreak that clinical specimen was taken (the week beginning July 29, 2018, to the week beginning August 11, 2019). Plasmid type 1 is indicated by checkered bars. All others plasmid are type 2.

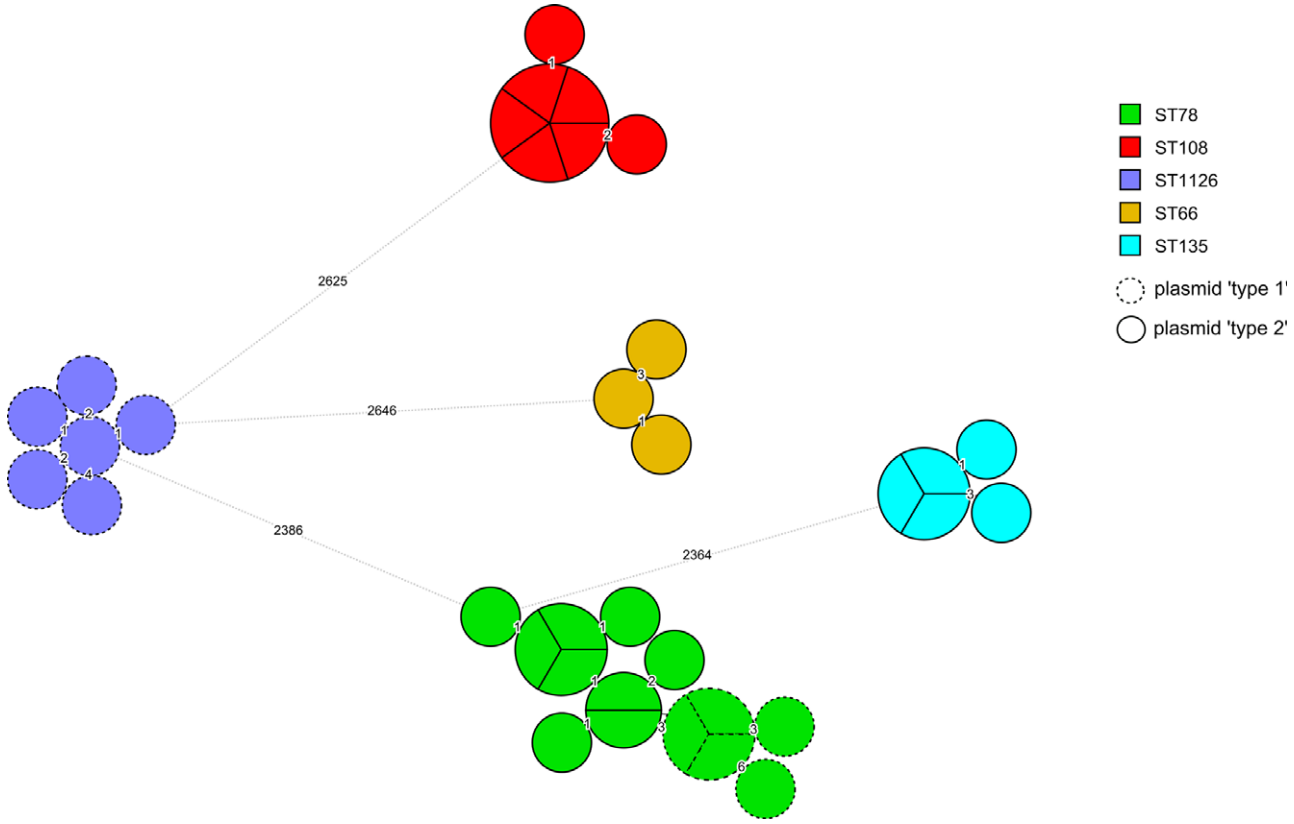

Fig. 4. Minimum-spanning tree of *Enterobacter cloacae* complex wgMLST locus (n = 15,605) allele differences among 35 outbreak isolates. Each circle (node) contains isolates that are indistinguishable at all loci. Nodes are colored by 7 locus multilocus sequence types. Numbers on lines (edges) connecting nodes indicate the number of allele differences between connected nodes. Nodes are divided in pie-chart form for individual isolates.

From the relatively short period during which environmental sampling was carried out, 5 species were detected from various locations within the ward. Despite many outbreak control interventions and extensive refurbishment of the ward, including patient bathrooms, a number of positive environmental screens were obtained. With a total of 26 environmental OXA-48–positive samples detected on the outbreak ward during the outbreak period, the most likely source of exposure was environmental, along with person-to-person transmission between patients on the outbreak ward. An additional study combining social network analysis and genomics was undertaken to fully explore transmission patterns during this outbreak.

This study had several limitations. The environmental sampling on the outbreak ward was not initiated until later in the outbreak management and was not systematic. The study was limited to mainly one period in the spring of 2019, and only 3 of the OXA-48 isolates were referred for WGS. WGS data from all positive environmental isolates on the outbreak ward could have revealed further transmission patterns and links to or among outbreak cases and various locations on the outbreak ward.

The presence of closely related OXA-48 isolates from elsewhere in the hospital without identifiable or known epidemiological links to the outbreak ward raises the possibility that the outbreak may have spread beyond the outbreak ward. Alternatively, a small number of outbreak cases may have been exposed from a common source elsewhere in the hospital, or unrecognized person-to-person or equipment-to-person transmission within the hospital. This possibility also gives rise to the question of whether the case definition could have been expanded to other areas of the hospital. The further detailed investigation and follow-up of these nonoutbreak cases was outside the scope of this study.

Recommendations from this work, which may help to prevent or control future CPE outbreaks, include (1) implementation of regular systematic environmental sampling in the hospital in outbreak and nonoutbreak periods; (2) consideration of performing periodic point-prevalence surveys for CPE carriage across the hospital, with WGS and detailed analysis carried out on any positive sample isolates in real time, along with comparison between clinical and environmental sample results; and (3) continued compliance with national CPE clinical guidelines on patient screening for CPE carriage to ensure prompt detection of carriers. Where environmental sampling results yield CPE, interventions to eradicate CPE from those areas should be implemented, with replacement of sink and shower drain pipes that may aid prolonged survival of microorganisms if CPE is persistently cultured from those sites, despite interventions. Future research opportunities could include using metagenomics to index the diversity of microbes and antimicrobial and disinfectant resistance genes across a range of locations across the hospital and comparing these to patient microbiomes.[26] Distinct ecological niches, many of which harbor stable populations over time, have been described in hospital environments such as high-touch surfaces and sink traps.[26,27] Knowledge of the hospital microbiome can aid understanding of the biology of these organisms, potential nosocomial acquisition and transmission, as well as effectiveness of IPC measures. Although some of these recommendations may incur human and monetary resources that are already stretched, particularly in the wake of the COVID-19 pandemic, prevention of morbidity and mortality from infections caused by CPE would hopefully avoid a greater economic burden in the long term.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/ash.2021.206

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Outbreak of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* bloodstream infections at an outpatient chemotherapy center

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Abstract

Background: Four patients were hospitalized July 2011 with *Pseudomonas aeruginosa* bloodstream infection (BSI), 2 of whom also had *Klebsiella pneumoniae* BSI. All 4 patients had an indwelling port and received infusion services at the same outpatient oncology center.

Methods: Cases were defined by blood or port cultures positive for *K pneumoniae* or *P aeruginosa* among patients receiving infusion services at the oncology clinic during July 5-20, 2011. Pulsed-field gel electrophoresis (PFGE) was performed on available isolates. Interviews with staff and onsite investigations identified lapses of infection control practices. Owing to concerns over long-standing deficits, living patients who had been seen at the clinic between January 2008 and July 2011 were notified for viral blood-borne pathogen (BBP) testing; genetic relatedness was determined by molecular testing.

Results: Fourteen cases (17%) were identified among 84 active clinic patients, 12 of which involved symptoms of a BSI. One other patient had a respiratory culture positive for *P aeruginosa* but died before blood cultures were obtained. Available isolates were indistinguishable by PFGE. Multiple injection safety lapses were identified, including overt syringe reuse among patients and reuse of syringes to access shared medications. Available BBP results did not demonstrate iatrogenic viral infection in 331 of 623 notified patients (53%).

Conclusions: Improper preparation and handling of injectable medications likely caused the outbreak. Increased infection control oversight of oncology clinics is critical to prevent similar outbreaks.

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Conflict of interest: None to report.

Keywords

Outpatient oncology care; Injection safety; *Klebsiella pneumonia*; *Pseudomonas aeruginosa*

On July 18, 2011, district health officials at the Mississippi State Department of Health (MSDH) were notified by a local hospital infection preventionist of a cluster of *Pseudomonas aeruginosa* bloodstream infections (BSIs) involving 4 patients hospitalized between July 9 and July 16, 2011. Two of these patients also had *Klebsiella pneumoniae* BSI. The 143-bed hospital serves as a regional referral center for several surrounding rural counties. All 4 patients had an indwelling infusion port and were receiving infusion services at the same local oncology clinic.

An initial investigation of the clinic by MSDH on July 18 did not identify a potential source for the infections; however, on that same day, 4 additional patients were admitted to the hospital with catheter-associated BSIs, all of whom received care at the same oncology center. Given the report of these additional infections, on July 20, the oncology clinic was closed under a public health order as an imminent public health threat. This report summarizes the findings of a public health investigation conducted to determine the cause and extent of the outbreak.

METHODS

For this study, a case was defined as a blood or port culture positive for *K pneumoniae* or *P aeruginosa* in a patient receiving infusion services at the oncology clinic during July 5-20, 2011. All patients actively receiving infusion services at the oncology clinic during this period were contacted and assessed for symptoms of a BSI or device-associated infection (eg, port-related infection). Regular communication was maintained with regional hospital infection preventionists to identify any hospital admissions from this group of patients. In addition, each patient's primary care physician was notified of the potential risk of infection and asked to monitor patients for relevant symptoms and to report any associated infections to public health authorities.

Patient isolates associated with the outbreak were sent to the Centers for Disease Control and Prevention (CDC) for analysis by pulsed-field gel electrophoresis (PFGE) to assess for genetic relatedness. In brief, chromosomal DNA from the *K pneumoniae* isolates was digested with the restriction endonuclease XbaI, under run conditions with switch times of 5 and 40 seconds and a total run time of 22 hours. DNA from the *P aeruginosa* isolates was digested with SpeI, and run conditions were switch times of 5 and 40 seconds for 21 hours. The genetic relatedness of the isolates was analyzed using BioNumerics software (Applied Maths, Austin, TX). Isolates were considered genetically related if their patterns were >90% similar.

After the clinic closed, MSDH conducted an extensive site evaluation, including in-depth interviews of current and former clinic staff, to identify potential modes of transmission. Infection control practices related to the storage and handling of parenteral medications,

including preparation of saline solution and heparin syringes for flushing central lines, as well as infusion techniques, were reviewed with clinic staff.

Infection control issues discovered during this investigation that might have predated the current outbreak prompted MSDH to notify all patients ever treated at the outpatient oncology center of their potential exposure to harmful practices and advising them to seek testing for viral blood-borne pathogens, including human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). A review of clinic records was matched against the Mississippi vital statistics registry to identify all living patients who had received care at the clinic since it opened in January 2008. A letter was sent to all identified patients, and free blood-borne pathogen testing was offered at MSDH county clinics.

Blood samples positive for blood-borne pathogens were sent to the CDC for verification and molecular testing. Because HCV was the sole blood-borne pathogen in which active infection was identified in more than 1 patient, molecular testing was performed for HCV-positive samples only. HCV RNA was extracted from positive samples, and the NS5b gene region was amplified as described previously.[1] Samples that were NS5b- positive were subjected to HVR1 quasi-species amplification. The E1/E2 junction region, which contains the HVR1 region, was amplified using the endpoint limiting-dilution (EPLD) real-time nested polymerase chain reaction (PCR) protocol described by Ramachandran et al.[2] Nested NS5b and HVR1 amplicons derived from the PCR amplification were purified (PCR Purification Kit; Qiagen) and sequenced with their respective nested primers using the BigDye v3.1 chemistry sequencing kit and an ABI 3130xl automated sequencer (Applied Biosystems, Foster City, CA) as described previously.[1]Maximum likelihood phylogenetic trees were then constructed using MEGA version 5 (http://www.megasoftware.net/).

RESULTS

Fourteen cases were identified among 84 patients who received infusion services at the oncology clinic between July 5 and 20, 2011. Initial dates of culture positivity ranged from July 9 to August 26, 2011 (Fig 1). Among the 14 cases, cultures identified *K pneumoniae* in 3 patients, *P aerguinosa* in 4 patients, and both *K pneumoniae* and *P aeruginosa* in 7 patients. All 14 patients had an indwelling port; 11 patients had their first positive culture from blood, and 3 had their first positive culture from an explanted port. Twelve of the patients had symptoms consistent with a BSI, including fever, nausea, vomiting, and lethargy, in addition to pain at the port implantation site. Two patients were asymptomatic and underwent elective port removal, which was found to be culture-positive. One patient with *K pneumoniae* bacteremia also had *P aeruginosa* growth from a respiratory specimen. One other patient died from sepsis before the acquisition of blood cultures; this patient had a respiratory specimen that yielded *P aeruginosa* and was classified as a probable case. No other known exposure besides the oncology clinic was identified in the 15 patients, including the probable case. Patient age ranged from 46 to 91 years. All 15 patients were hospitalized, with admission dates between July 9, 2011, and August 25, 2011.

PFGE analysis of available *K pneumoniae* and *P aeurginosa* isolates from 8 different patients demonstrated indistinguishable banding patterns (Figs 2 and 3). A *P aeruginosa*

isolate from a respiratory specimen of a patient with *K pneumoniae* BSI also matched the outbreak strain.

The oncology clinic was a freestanding facility operated by a single physician, who served as the medical director, assisted by 2 registered nurses and several administrative staff members. All port access and infusion services were rendered by the 2 nurses and by an administrator who had been trained by previous staff and the physician medical director. Both nurses were recent associate degree graduates from nursing school with no previous experience with chemotherapy or infusion services. Neither had received any specialty training in chemotherapy administration. Minimal onsite training was provided by the physician medical director and the non–medically trained administrator.

In-depth interviews identified a recent change in protocol that might have led to unsafe injection practices. As a reported costcontainment measure, starting on July 5, 2011, staff nurses were directed by the physician medical director to use common-source 1-L saline and 1-L heparin flush bags rather than single-dose vials for all port and line flushes. These bags were used over several days for multiple patients. A single syringe was dedicated to each patient to draw up the saline flush for the entire day; each syringe could be reused multiple times to access the common bag of saline solution before being discarded at the end of the day. Other syringes were dedicated to drawing up the heparin flush for all patients from the common-source heparin flush bag. These heparin syringes were shared among multiple patients over an indeterminate period of time, and in many cases were discarded only if visible blood was seen in the syringe. In addition, some syringes were dedicated for mixing nonchemotherapy medications in smaller, individual-unit doses. These syringes were used over many days and stored up to several months at a time in a nonsterile drawer. There was no indication that any of these specific syringes were used directly on patients.

Interviews with former staff members suggested that the identified injection safety lapses, particularly the overt reuse of syringes among patients, could have occurred at any time before July0 5,2011. In light of this, 623 living patients who had been seen at the clinic between January 2008 and July 2011 were notified of a recommendation for testing for blood-borne pathogens. Of the 331 of these 623 patients with available test results, 37 had evidence of current or resolved HBV or HCV infection, with 4 demonstrating evidence of previous infection with both. Twenty-eight patients had evidence of resolved HBV infection (hepatitis B surface antigen negative and core antibody positive), and 1 patient had chronic HBV infection (hepatitis B surface antigen positive). No temporal clustering of patients with resolved HBV infection was identified. Twelve patients had detectable antibodies to HCV. Five of these patients had detectable HCV RNA, of whom 4 had sufficient RNA for HVR1 quasi-species determination by EPLD. Phylogenetic analysis of the NS5b and intrahost HVR1 sequences revealed the absence of intermixing of HCV variants among individuals and no evidence of genetic relatedness to suggest iatrogenic transmission.

Additional risk factor data were available for 32 of the 37 patients who had current or resolved HBV or HCV infection, 8 of whom were previously aware of their infection. Eighteen patients had some potential additional risk factor for the acquisition of bloodborne pathogens, including blood transfusions before 1992 (n = 7), HCW (n = 5), tattoos (n = 11),

household contact with hepatitis (n = 2), and intravenous drug abuse (n = 1). No patient tested positive for HIV.

DISCUSSION

We describe an outbreak of *K pneumoniae* and *P aeruginosa* BSIs in patients undergoing infusion procedures at an outpatient chemotherapy infusion center. We identified 14 confirmed cases and 1 probable case involving a fatality in a patient with *P aeruginosa* isolated from a respiratory specimen. All available clinical isolates of *P aeruginosa* and *K pneumoniae* shared indistinguishable PFGE patterns, consistent with a common source outbreak. We identified several lapses in the preparation and handling of injectable medications that could have resulted in crosscontamination with subsequent spread of infection to multiple patients. Similar lapses in injection safety have been implicated in previous outbreaks of invasive bacterial infections,[3-6] including BSIs, meningitis, and epidural abscess, and have led to transmission of blood-borne viruses.[7-9]

Of most concern were our findings of syringe reuse among patients as well as syringe reuse to access shared medications (eg, saline bag), demonstrating a lack of adherence by clinic staff to fundamental principles of injection safety.[9,10] Because of the severity of these lapses and the associated risk of blood-borne pathogen exposure,[7,9] and because these lapses may have occurred after the clinic first opened, 623 clinic patients were notified to undergo blood-borne pathogen testing. To our knowledge, this is only the second patient notification for blood-borne pathogen testing owing to unsafe injection practices in the context of a bacterial outbreak. The first patient notification event that involved a bacterial outbreak was also prompted by the finding of syringe reuse to access shared medication vials.[5]

To prevent transmission of infections to patients, all providers should adhere to safe injection practices as part of Standard Precautions.[10] These include using a new syringe and needle for each patient and for accessing medication vials or bags, promptly disposing a syringe and needle after each use, and not using saline or heparin bags as a common source supply for multiple patients.[9-12]

This study has some limitations. Given the multiple ongoing injection safety lapses, the exact route of transmission for this outbreak cannot be determined. Among patients who were notified for blood-borne pathogen testing, results were available for only 53%; only a small number of these patients had detectable virus (ie, HCV) that could be assessed for genetic relatedness. Thus, the transmission of blood-borne pathogens in this clinic cannot be definitively excluded.

Outpatient settings are accounting for an increasing proportion of total health care delivery. In Mississippi, as in most states, there is no official oversight of infection control practices in outpatient facilities that are not certified by the Centers for Medicare & Medicaid Services, such as outpatient oncology clinics. In Mississippi, the facility medical director and nurses have direct responsibility for maintaining proper infection control procedures; however, no specific nurse training is required to provide infusion services. The state board of medical licensure and the state nursing boards serve as the only backstop to address unacceptable

practices of the individual provider, but these functions are typically exercised only after an untoward effect has occurred. In addition, most outpatient facilities lack a system for detecting infections associated with care. In many cases, outbreaks originating in outpatient settings are detected by hospitals to which affected patients have been admitted, as was the case in this outbreak. The vigilance of the hospital infection preventionist and her close relationship with the local health department was critical to the successful detection and control of this outbreak.

In response to reports of outbreaks involving outpatient oncology settings,[3,4,7,8] the CDC launched a campaign in October 2011 that featured new tools and resources to prevent infections among oncology patients.[11] These features include a basic infection control plan containing key policies and procedures that any outpatient oncology facility can implement to standardize and improve its infection prevention practices.[12] Continued efforts to increase facility awareness of these infection control resources and implementation of recommended practices are needed to protect this vulnerable subset of outpatients.

Acknowledgments

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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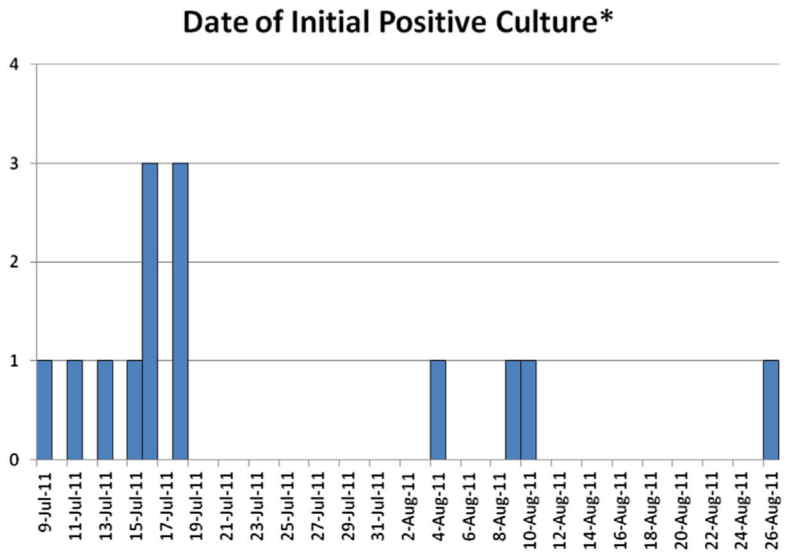

Fig 1.

K pneumoniae and *P aeruginosa* cases by date of first positive blood or port culture, July-August 2011. *Initial positive cultures obtained on August 4, August 10, and August 26, 2011, were obtained from explanted ports that had been removed electively or because of local inflammation.

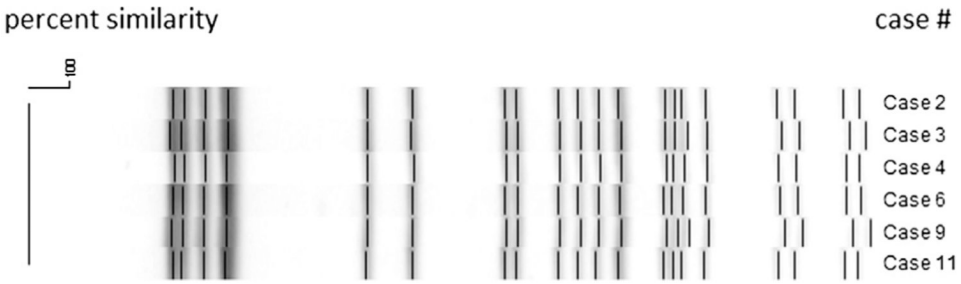

Fig 2.
PFGE of available *K pneumoniae* outbreak isolates, July-August 2011.

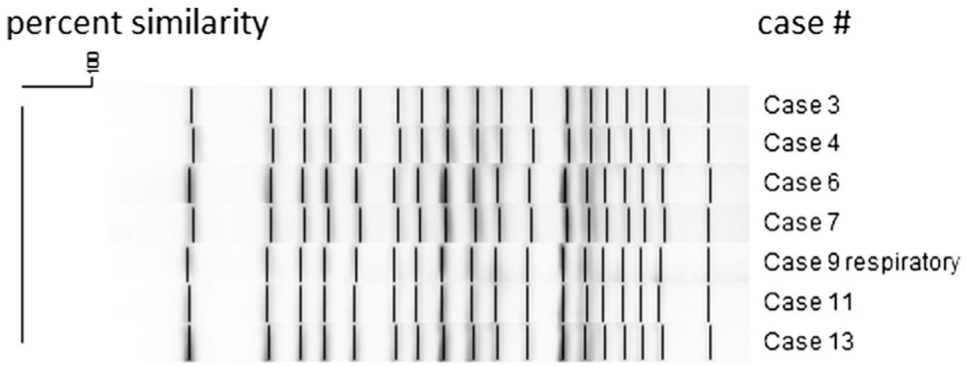

Fig 3.
PFGE of available *P aeruginosa* outbreak isolates, July-August 2011.

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Outbreaks of Gram-Negative Bacterial Bloodstream Infections Traced to Probable Contamination of Hemodialysis Machines -- Canada, 1995 United States, 1997; and Israel, 1997

During 1996, approximately 236,000 persons received hemodialysis in the United States; of these, an estimated 183,000 (78%) received chronic hemodialysis (1). Patients who receive chronic hemodialysis are at increased risk for bloodstream infections (BSIs) because of the need for repeated vascular access. Reported BSI rates for hemodialysis patients have ranged from 8.4 to 16.8 episodes per 100 patient-years (2), and BSI has been identified as the cause of death in 6%-18% of hemodialysis patients (2). Outbreaks of BSIs in hemodialysis units usually have been caused by inadequate disinfection of 1) water treatment or distribution systems (3,4) and 2) reprocessed dialyzers (5-8). This report summarizes the investigations of three clusters of gram-negative bacterial BSIs at hemodialysis centers in Canada, the United States, and Israel. The findings indicate that all three outbreaks probably resulted from contamination of the waste drain ports in the same model of hemodialysis machine.

Canada

From June 17 through November 15, 1995, nine adult patients at an ambulatory hemodialysis center in Montreal, Canada, had Enterobacter cloacae BSIs. All patients at the hemodialysis center were dialyzed on COBE {Registered} Centrysystem 3 * (CS3, GAMBRO {Registered} Healthcare {TM}, Lakewood, Colorado) hemodialysis machines. Each CS3 had a Centry {Registered} Waste Handling Option (WHO {TM}), which is a waste port designed to dispose of the saline used to flush a dialyzer before the machine is used for a patient Figure_1. The WHO waste drain line employs two one-way valves to prevent drain line waste from refluxing into the WHO. The investigation indicated that at least one of the two one-way valves in the WHO waste drain lines of seven of 11 machines were incompetent, ** potentially allowing drain backflow and contamination of dialysis lines in contact with the WHO port.

An epidemiologic investigation demonstrated that case-patients (i.e., the nine patients at the hemodialysis center who had Enterobacter cloacae BSIs) were more likely than control-patients to have received dialysis on a machine that had at least one incompetent valve on the WHO waste drain line (all seven case-dialysis sessions versus 145 {53%} of 272 control-dialysis sessions; odds ratio: undefined; p=0.02). Case- and control-patients were otherwise similar in demographic characteristics, underlying renal disease, type of vascular access, and dialyzer type. Enterobacter cloacae isolated from all nine infected patients and from the WHOs of 10 of 11 dialysis machines were identical when examined by pulsed field-gel electrophoresis (PFGE).

United States

From December 5, 1996, through January 25, 1997, a total of 10 adult patients at an ambulatory hemodialysis center in Maryland had gram-negative bacterial BSIs. Six BSIs were caused by Enterobacter cloacae, four by Pseudomonas aeruginosa, and two by Escherichia coli; two were polymicrobial BSIs. All patients at the hemodialysis center were dialyzed on CS3 hemodialysis machines that had WHOs. Results of a cohort study of all patients receiving dialysis at the center during the 2-month epidemic period indicated that the risk for gram-negative BSI was associated with exposure to any of three particular dialysis machines (seven BSIs in 20 patients who were exposed to one or more of the three machines versus three BSIs in 64 patients who were exposed to the other machines; relative risk=7.5; 95% confidence interval=2.1-26.2). Incompetent valves on WHO waste drain lines were present in eight of 26 dialysis machines and in two of the three implicated machines. Enterobacter cloacae was recovered from the WHOs of 14 of 26 machines, and P. aeruginosa was recovered from seven of 26. PFGE patterns of available Enterobacter cloacae isolates from the dialysis machines and from three patients were identical; none of the P. aeruginosa isolates obtained from patients were available for PFGE testing.

Israel

From February 9 through September 19, 1997, eight adult patients at an ambulatory hemodialysis center in Jerusalem, Israel, had gram-negative bacterial BSIs. BSIs in four patients were caused by Escherichia coli, three by P. aeruginosa, two by Enterobacter cloacae, and one by Stenotrophomonas maltophilia; two patients had polymicrobial BSIs. All patients at the hemodialysis center were dialyzed on CS3 hemodialysis machines that had WHOs. All eight patients who had BSIs had been dialyzed on three of 13 dialysis machines. Backflow was observed in the WHOs of the three implicated dialysis machines, and cultures obtained from the WHOs of six of 13 machines were positive for gram-negative organisms. Five of the eight patients, including all four with Escherichia coli BSIs, had been dialyzed on one machine that subsequently was culture-positive for Escherichia coli and P. aeruginosa. Both patients with Enterobacter cloacae BSIs had been dialyzed on a second machine that was culture-positive for Enterobacter cloacae and P. aeruginosa. Escherichia coli isolates obtained from three patients and the WHO of the implicated machine were identical by PFGE.

Follow-Up Investigation

Daily quality-control testing of WHOs as specified by the manufacturer had not been performed at any of the three hemodialysis centers. The manufacturer specifies that preventive maintenance of the valves in the WHO waste drain line includes replacement of the two valves after every 2000 hours of use. However, personnel at the three hemodialysis centers were aware of the need to change only one valve in the WHO waste drain line, and personnel at two centers did not know a second WHO valve existed; schematic diagrams provided by the manufacturer to these two hemodialysis centers identified only one of the two valves. At one center, experimentally bending and twisting the main drain line of a machine that had incompetent valves in the WHO waste drain line demonstrated the ease with which backflow can occur in the WHO.

In one hemodialysis center, the outbreak was controlled after high-level WHO disinfection (i.e., disinfecting dialysis machines with formaldehyde on two occasions and increasing the dwell time for routine weekly machine disinfection). In the other two centers, the outbreaks were terminated by discontinuing use of the WHO. All three hemodialysis centers discontinued using the WHOs.

In June 1997, GAMBRO Healthcare sent a Medical Device Safety Alert letter to all hemodialysis centers of record that use the CS3. This letter informed users of the need to ensure proper functioning of the WHO and outlined procedures for proper disinfection and maintenance of the equipment.

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Editorial Note

Editorial Note: Bacterial BSI is a potentially severe complication associated with hemodialysis vascular access. In the United States, complications associated with vascular access represent one of the most common sources of morbidity among patients undergoing end-stage renal dialysis, with associated costs exceeding an estimated $1 billion per year (9). This report links three outbreaks of gram-negative bacterial BSIs to a unique design feature of the CS3 hemodialysis machine. The results of these outbreak investigations demonstrated that the WHO, if not properly maintained and disinfected, may be a source of bacterial contamination leading to BSIs in hemodialysis patients. Because waste backflow can occur with incompetent valves and WHO contamination can occur easily, the design of the WHO creates a mechanism for possible cross-contamination of the patient dialysis line.

In addition to the problems associated with the WHO feature, insufficient training of hemodialysis personnel about the design and proper handling and maintenance of WHOs might contribute to transmission of BSIs to hemodialysis patients. In June 1996, GAMBRO Healthcare and CDC surveyed 595 U.S. dialysis centers that use CS3 machines to characterize the methods used to clean and disinfect the dialysis machines and to characterize quality-control procedures (GAMBRO Healthcare and CDC, unpublished data). The survey indicated that personnel at most (87%) of the responding dialysis centers reported weekly disinfection of their dialysis machines as specified by COBE guidelines, although most (62%) were not disinfecting dialysate and bicarbonate sampling ports as often as recommended. Of the 290 centers that reported using the WHO, only 42 (14%) performed the recommended daily quality-control assessment of the WHO valves to determine whether drain reflux was occurring. Of the 137 centers responding to the question "If fluid can be aspirated from the WHO, what is done?," 112 (82%) indicated the need for replacing WHO valves or taking the machine off-line for servicing.

This report underscores the importance of surveillance and infection control in the ambulatory health-care setting. The detection of these outbreaks and identification of the likely cause was aided by the brief time-frame during which multiple infections were identified. The limited availability of data about infection rates in ambulatory dialysis centers impedes the identification of small or prolonged low-level outbreaks. Because of the lack of such data, inappropriate infection-control or maintenance practices that were identified in the GAMBRO Healthcare/CDC survey could not be linked to adverse patient outcomes at the dialysis centers surveyed. Outbreaks of gram-negative bacterial BSIs in hemodialysis patients that appear to be associated with use of the WHO should be reported to state health departments and to CDC's Hospital Infections Program, National Center for Infectious Diseases; telephone (404) 639-6413.

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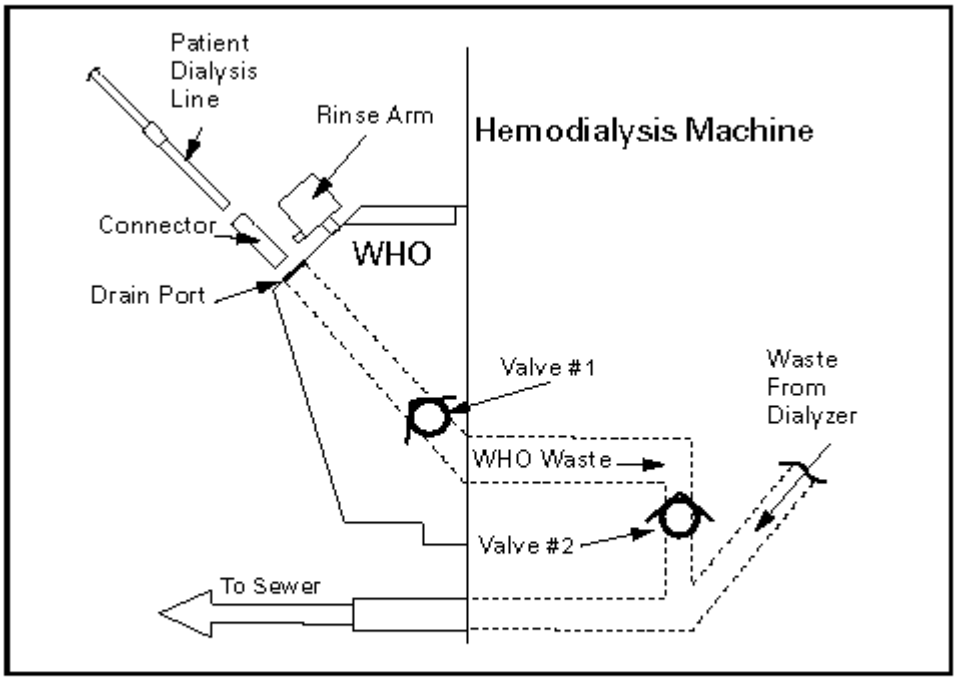
* Use of trade names and commercial sources is for identification only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

** The manufacturer recommends daily testing of the competency of WHO valves by filling a 30 cc syringe with water, injecting the contents into the WHO drain port, and attempting to draw back fluid from the WHO. Competent valves should prevent backflow.

Figure_1

FIGURE 1. Waste Handling Option (WHO™)* of a Centrysystem 3† hemodialysis machine



*A WHO is a waste port that is attached to the front of the hemodialysis machine; it is designed to dispose of the saline used to flush the dialyzer before the machine is used for a patient. The waste drain line of the WHO joins the dialyzer waste drain line inside the dialysis machine to become one main drain line that empties into the sewer. Two valves along the WHO waste drain line are designed to prevent reflux of waste to the WHO drain port.
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Outbreak of carbapenemase-producing Enterobacteriaceae associated with a contaminated water dispenser and sink drains in the cardiology units of a Korean hospital

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SUMMARY

Background: Concerns are growing over the importance of the hospital water environment for the transmission of carbapenemase-producing Enterobacteriaceae (CPE).
Aim: To report a large outbreak in the cardiology units involving intensive care units (ICUs) and wards at a tertiary-care hospital.
Methods: This was a contact tracing, case–control study to find the risk factors for acquisition of CPE and environmental sampling was performed during a CPE outbreak between July and December 2018.
Findings: A total of 87 patients with CPE infection or colonization were identified in the cardiology units of the Asan Medical Centre. Diverse organisms were identified containing bla_{KPC}, bla_{NDM-1}, bla_{VIM} or bla_{IMP}, bla_{OXA-48}, and co-producing organisms. A case–control study indicated that using the sinks in the ward patient room bathroom for teeth brushing was associated with CPE acquisition (83% vs 30%; P=0.03). The environment was cultured and *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Escherichia coli* was isolated from a water dispenser and New Delhi metallo-beta-lactamase (NDM) 1-producing *Citrobacter freundii* and *Enterobacter cloacae* from sinks in patient rooms. Pulsed-field gel electrophoresis (PFGE) analysis of KPC-producing *E. coli* from patients and the water dispenser in ICU and NDM-1-producing *E. cloacae* from the patient and sink drain showed the same pulsotypes.
Conclusions: The water dispenser and sink drain were suspected as possible reservoirs of CPE in this outbreak. Close contacts with contaminated water such as tooth brushing were identified as risk factors for CPE acquisition. Education for the adequate use of the water

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environment system as well as the control of the hospital water environment should be implemented to prevent the CPE outbreaks.

Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) are emergent pathogens in Korea, with confirmed reports growing exponentially year by year. The number of CPE cases reported from sentinel surveillance units in 2015, 2016 and 2017 were 565, 1453 and 2657, respectively [1]. Increasing CPE is a significant threat because genes encoding carbapenemase, a β-lactamase conferring resistance to certain β-lactam antibiotics, can be transmitted between organisms by mobile genetic elements. Limited options are available for the treatment of CPE infection, and mortality rates are high once it progresses to the invasive stage [2,3].

To control the spread of CPE in healthcare settings, bundle approaches, including isolating a patient cohort, contact isolation, dedicated staff for quarantine wards, educating staff, improving hand hygiene, and environmental cleaning are recommended [4,5]. However, which specific strategy is the most important for the prevention of transmission has yet to be defined. The current consensus indicates locating and removing reservoirs and a thorough understanding of CPE transmission routes.

Recently, sources for nosocomial CPE outbreaks have been occasionally identified in the hospital water environment [6–8]. A large and protracted outbreak was experienced in the cardiology units of the Asan Medical Centre, and this study set out to investigate the epidemiologic links between patients and potential environmental sources.

Methods

Hospital setting

This study was conducted at the Asan Medical Centre, a 2700-bed tertiary-care centre in Seoul, South Korea. Cardiology units comprised a 16-bed cardiology intensive care unit (CCU), 15-bed cardiothoracic surgery intensive care unit (CSICU), two 99-bed cardiology wards, and one 50-bed cardiothoracic surgery ward. In detail, there were seven and three single-isolation rooms in the CCU and CSICU, respectively. In addition, there were 13 single-patient rooms, 13 two-patient rooms, and 10 six-patient rooms in the cardiology wards and eight single-patient rooms, six two-patient rooms, and five six-patient rooms in the cardiothoracic surgery ward. In each patient room in the ward, there were toilet facilities, and there was a communal bathroom in each ward. About 11,000 patients are admitted each year to the cardiology units. Active surveillance for CPE was not performed until an outbreak was confirmed, and culture-confirmed CPE patients were isolated in a single room with contact precautions for entry (personal protective equipment (PPE); gloves and gown). The infection-control office monitored the incidence and prevalence of CPE carriers and acquisition rates in all wards and intensive care units (ICUs) weekly. This study was approved by Institutional Review Board of the Asan Medical Centre with waiver of consent (IRB no. 2019-1103).

Definitions

Cases were defined to include patients with CPE infection or colonization admitted to cardiology units, or those in CPE isolation following surveillance culture after exposure to CPE patients from 26 July to 17 December 2018. Controls were patients who had never been colonized or infected with CPE, as they had one or more negative CPE screening results, which was obtained at least 48 h after admission to the cardiology units. Acquisition locations of CPE were defined as locations where CPE-positive individuals were 48 h before the first isolation of CPE. Contact patients were defined as those who stayed in the same rooms as index patients or in the same ward as index patients in the ICU. Acquisition from cardiology units was defined as isolation of CPE 48 h after admission to cardiology units. When surveillance cultures for contact patients housed at other wards 48 h before culture were positive for CPE, it was assumed that CPE was not acquired from the cardiology units.

Case–control study

During the outbreak (week 38), we conducted a case–control study using a questionnaire for items not identifiable through chart review to home in on risk factors for the acquisition of CPE in the general wards. The questionnaire included use of the hand hygiene sink, purpose of hand hygiene sink use, use of the sink or toilet in the bathroom in the patient room, use of the sink or toilet in the shared ward bathrooms, and use of the water dispenser.

Microbiologic studies

Clinical isolates were screened for CPE, and its antimicrobial susceptibilities were determined using a Microscan NC72 panel (Beckman Coulter). For surveillance culture, we used ChromID CARBA agar (Biomerieux) followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker) for identification. We performed a modified Hodge test with combined disk inhibition test for phenotypic assay of CPE in both clinical isolates and surveillance cultures. Then, in-house polymerase chain reaction (PCR) assays were used to identify the presence of bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA48} and determine CPE genotypes. We also introduced Xpert Carba-R (Cepheid) for surveillance during the outbreak. Due to the high costs of Xpert Carba-R, we used Xpert Carba-R only on admission to the cardiology units for releasing preemptive isolation quickly. We continuously performed in-house PCR in weekly surveillance for CPE.

Environmental samples from surfaces were taken with sterile cotton swabs and from sink drains with a sterile brush.

We used the Centers for Disease Control and Prevention (CDC) broth enrichment protocol with minor modifications [9]. Briefly, swabs and brushes were placed in 5 mL of trypticase soy broth (TSB) containing a 10-μg ertapenem disk. If the TSB became turbid, we performed a subculture of the broth on to ChromID CARBA agar. If colonies with colour grew on the agar, identification and susceptibility tests were performed using the Microscan WalkAway plus system, and Xpert Carba-R (Cepheid) was used to confirm CPE genotypes. We also conducted pulsed-field gel electrophoresis (PFGE) to compare *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Escherichia coli* isolated from the patients and the water dispenser, and New Delhi metallo-beta-lactamase-1 (NDM-1)-producing *Enterobacter cloacae* from patient and sink drain in the patient room. For PFGE, genomic DNA were cut with *Xba*I (TaKaRa, Tokyo, Japan) and subjected to electrophoresis using a CHEF DR-III contour-clamped homogeneous electric field apparatus (Bio-Rad, Hercules, CA, USA). Tiff images of the gels were normalized using FPQuest ™ 4.5 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA). Categorical variables were compared using the χ^2 or Fisher's exact test, and continuous variables were compared using the Mann–Whitney *U*-test, as appropriate. A *P*-value of less than 0.05 was considered statistically significant.

Results

Description of outbreaks and patient characteristics

A total of 87 patients positive for CPE culture or infection were identified from 26 July to 17 December 2018. The incidence rate at baseline, from January to June 2018, was 0/10,000 patient-days; that during the outbreak, from July to December 2018, was 18.1/10,000 patient-days. CPE in the first two patients was identified by sputum culture performed upon suspicion of pneumonia, and in the remaining 85 patients via surveillance culture on exposed patients (N = 22) or during active surveillance (N = 63). Twenty five (29%) patients were housed in the ICU before CPE isolation, and 20 (23%) underwent cardiac surgery before CPE isolation. Median days from admission to positive CPE test and isolation was 8 days (interquartile range, 3–19 days). Fifty-five (63%) acquired CPE from cardiology units, 16% from other units, and 21% of patients were positive for CPE within 2 days after being admitted to hospital. More than half (56%) received antibiotics in the month preceding isolation of CPE, with only 7% having received carbapenem. All but two patients were positive for CPE colonization; the remaining two had pneumonia due to CPE.

A diverse array of carbapenemase-producing organisms were identified (Table I). Thirteen (15%) patients had KPC-producing organisms, mainly *E. coli*, and 55 (63%) had NDM-1-producing organisms. Of these, 17 (31%) were *Citrobacter freundii*. Other metallo-β-lactamase-producing organisms were identified in 12 (14%) patients, oxacillinase (OXA)-48-producing organisms in three (3%) patients, and co-producing organisms in four (5%) patients. Epicurves of KPC-producing and NDM-1 producing organisms are shown in Figure 1 and Supplementary Figure S1.

Table I
Microbiologic results of 87 patients with carbapenemase-producing Enterobacteriaceae

Genotype and organism	Number (%)
KPC-producing organism	13 (15)
Escherichia coli	9 (10)
Klebsiella pneumoniae	2 (2)
E. coli and *K. pneumoniae*	1 (1)
Xpert positive but not isolated on culture	1 (1)
NDM-1-producing organism	55 (63)
Citrobacter freundii	17 (20)
Enterobacter cloacae	5 (6)
K. pneumoniae	5 (6)
E. coli	5 (6)
Other [a]	10 (11)
Xpert positive but not isolated on culture	13 (15)
Other MBL (VIM, IMP-1)-producing organism	12 (14)
K. pneumoniae	7 (8)
Other [b]	3 (3)
Xpert positive but not isolated on culture	2 (2)
OXA-48-producing organism	3 (3)
E. coli and *K. pneumoniae*	1 (1)
Xpert positive but not isolated on culture	2 (2)
Co-producing (NDM-1 plus IMP and NDM-1 plus VIM)	4 (5)

IMP-1, imipenemase-1; KPC, *Klebsiella pneumoniae* carbapenemase; MBL, metallo-beta-lactamase; NDM-1, New Delhi metallo-beta-lactamase-1; VIM, Verona integron-encoded metallo-beta-lactamase.
[a] *Klebsiella oxytoca* (N = 1), *Klebsiella variicola* (N = 1), *Citrobacter braakii* (N = 1), *Enterobacter asburiae* (N = 1), *E. kobei* (N = 1), *E. cloacae* and *C. freundii* (N = 1), *K. pneumoniae* and *E. coli* (N = 3), *Raoultella ornithinolytica* and *C. freundii* (N = 1).
[b] *R. ornithinolytica* (N = 1), *K. oxytoca* (N = 1), *C. freundii* (N = 1).

Investigation of outbreak and control measures

At week 31, 2018, the infection-control team became aware of an outbreak of CPE (Figure 1). Contact isolation was conducted using gown and glove PPE in a single-patient room and dedicated staffing for patients with CPE. Contact screening was implemented, and we supplied CPE education to healthcare workers and active enforcement of hand hygiene. Patients with CPE received chlorhexidine bathing.

We conducted active surveillance tests in the ICU using conventional culture with in-house PCR and Xpert Carba-R. In the CCU and CSICU, the water dispenser for provision of water to patients was located near a handwashing sink; these were surveyed as well. Of note, used dialysing solution after haemodialysis was emptied into this handwashing sink. At week 33, we performed environmental cultures, and KPC-producing *E. coli* was isolated from the water dispenser outlet (Table II). We removed the water dispenser, provided bottled water to the patients, and treated the sink drain with bleach. On week 38, all sink drains in the ICU were replaced. PFGE analysis of strains of KPC-producing *E. coli* from patients and from the water dispenser showed the same pulsotype (Figure 2(a)) for all. Supplementary Figure S2 indicates timeline of isolation of KPC-producing *E. coli* and units for

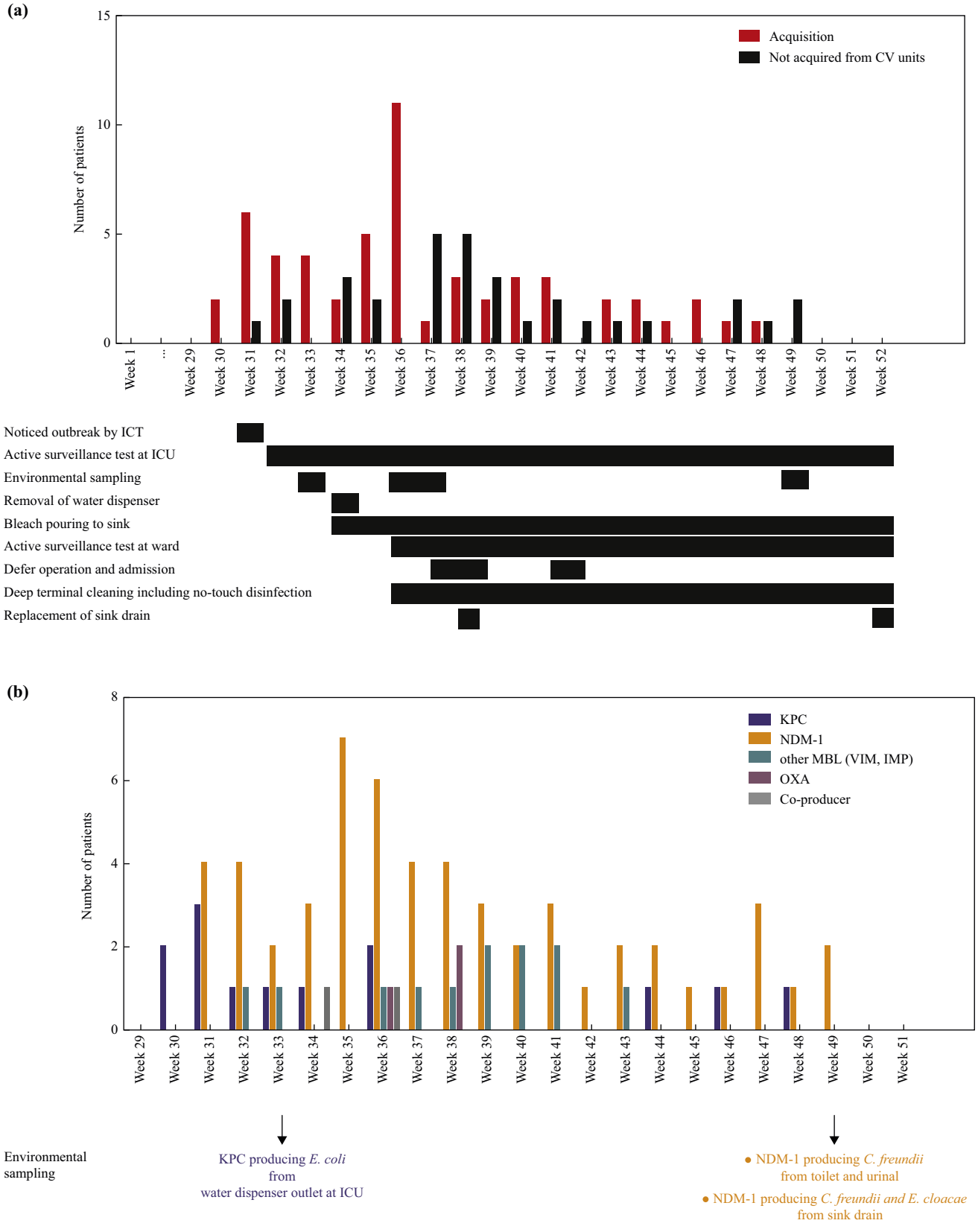

Figure 1. Epicurve and interventions to control outbreak. (a) Epicurve stratified by acquisition site (cardiology units or other units) and intervention. (b) Epicurve stratified by carbapenemase genotype and results from environmental sampling. CV, cardiovascular; ICU, intensive care unit; ICT, infection control office; IMP, imipenemase; KPC, *Klebsiella pneumoniae* carbapenemase; MBL, metallo-beta-lactamase; NDM-1, New Delhi metallo-beta-lactamase-1; OXA, oxacillinase; VIM, Verona integron-encoded metallo-beta-lactamase.

Table II
Results of environmental culture

	Location of environmental sampling	Number (%) of sampling of positive CPE results	Description of positive CPE results	
			Location	Organisms
Week 33	ICU (portable EKG machine, ultrasonography machine, computer keyboard and mouse of healthcare worker, sink U-trap and bowl, patient area, and water dispenser)	1/136 (0.7)	Water dispenser	KPC-producing *Escherichia coli*
Week 36	Water dispenser and sink around water dispenser at ward	0/19 (0)	—	—
Week 37	Eight hand hygiene sinks (faucet, bowl, and U-trap) and toilet at ward	0/40 (0)	—	—
Week 49	Ward (computer keyboard, mouse, telephone, EKG machine, nursing cart, wheelchair, toilet, urinal, sink U-trap, bowl, and faucet, water dispenser, and patient area)	6/402 (1.5)	Toilet and urinal in the shared bathroom ($N = 3$) Sink U-traps in the bathroom in patient room ($N = 3$)	NDM-1-producing *Citrobacter freundii* NDM-1-producing *Enterobacter cloacae* and *C. freundii*

CPE, carbapenemase-producing Enterobacteriaceae; EKG, electrocardiogram; ICU, intensive care unit; KPC, *Klebsiella pneumoniae* carbapenemase; NDM-1, New Delhi metallo-beta-lactamase-1.

hospitalization of nine patients with KPC-producing *E. coli*. Patients 1, 4, and 5 were admitted to CCU and patient 3 was admitted to CSICU. Their beds were near the water dispenser (Supplementary Figure S3). Also, they drank the water from the water dispenser contaminated with KPC–producing *E. coli*. After removing the water dispenser, there were no additional cases of KPC-producing *E. coli* acquired in the CCU or CSICU.

Although interventions were implemented immediately in the ICU, at week 36, 11 patients were found to have CPE acquired from general wards (Supplementary Figure S1). We performed active surveillance tests in general wards and pre-emptive isolation, along with setting up a running buffer room. Thorough daily cleaning with monitoring and deep terminal cleaning using no-touch disinfection (hydrogen peroxide vapour and ultraviolet area decontaminator; ASEPT. 2X SILVER, Sanuvox, Canada) was also conducted. In addition, we deferred the operation schedule and admission to the wards.

Due to a cluster of cases without a contact history with patients with CPE, few patients having received carbapenem, and involvement of most rooms in the wards, we suspected an environmental reservoir. We conducted environmental sampling at weeks 36 and 37 in the general wards focusing on the water dispensers and hand-washing sinks, but CPE was not identified therein. A case–control study with a questionnaire was implemented to find the source of CPE in the general wards, and it was found that using the sinks in the patient room bathroom of the ward for teeth brushing was associated with CPE acquisition (83% vs 30%; P=0.03) (Table III). In the ward handwash sink and in the sink in the bathroom, the sink drain was directly below the outlet (Supplementary Figure S4). We noticed that patients and caregivers occasionally used sinks for the disposal of drinks, although few cases answered that they throw drinks in the sink in the questionnaires. Extensive environmental sampling was conducted at week 49 based on this finding, which revealed NDM-1-producing *C. freundii* in the sink drain in the patient bathroom and in the toilet and urinal in the shared bathroom, and NDM-1-producing *E. cloacae* in the sink drain in the patient room (Table II). PFGE analysis of NDM-1-producing *C. freundii* from patients and the environment showed multiple pulsotypes, while NDM-1-producing *E. cloacae* from the patient in the identified patient room (No. 81) and sink drain in that patient room bathroom showed the same pulsotype (Figure 2(b)). We treated the patient room bathroom sink drain with bleach daily (1 L of 5.5% sodium hypochlorite

Figure 2. (a) Pulsed-field gel electrophoresis (PFGE) analysis of the eight *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Escherichia coli* from patients and water dispenser. (b) PFGE analysis of New Delhi metallo-beta-lactamase (NDM)-1-producing *Enterobacter cloacae* from patient and sink drain in the patient room.

Table III
Case–control study using questionnaire

Characteristics	Case ($N = 15$) [a]	Control ($N = 12$)	P
Clinical characteristics			
Age, years, median (IQR)	63 (55–73)	61 (43–73)	0.87
Male gender	6 (40)	4 (33)	>0.99
Underlying disease or condition			
Valvular heart disease	4 (27)	2 (17)	0.66
Myocardial infarction or angina	0 (0)	2 (17)	0.19
Heart failure	4 (27)	3 (25)	>0.99
Infective endocarditis	6 (40)	1 (8)	0.09
Aortic syndrome	1 (7)	2 (17)	0.57
Other underlying disease	0 (0)	2 (17)	0.19
End stage renal disease on haemodialysis	3 (20)	0 (0)	0.23
ICU stay (>2 days)	4 (27)	7 (58)	0.13
Underwent cardiac surgery	6 (40)	5 (42)	>0.99
Use of antibiotics	9 (60)	9 (75)	0.68
Carbapenem	1 (7)	1 (8)	>0.99
Piperacillin/tazobactam	3 (20)	5 (42)	0.40
Cephalosporin	6 (40)	6 (50)	0.60
Quinolone	1 (7)	4 (33)	0.14
Results of questionnaire			
Used hand hygiene sink	7/14 (50)	7/10 (70)	0.42
Used bathroom in the patient room	12/15 (80)	10/10 (100)	0.25
Used sink in the bathroom	12/12 (100)	9/10 (90)	0.46
For hand washing	12/12 (100)	8/10 (80)	0.19
For tooth brushing	10/12 (83)	3/10 (30)	0.03
For washing face	8/10 (67)	3/10 (30)	0.07
For throwing up into sink	1/11 (9)	0/10 (0)	>0.99
Used toilet in the bathroom	12/12 (100)	10/10 (100)	N/A
Took shower in the bathroom	1/12 (8)	0/10 (0)	>0.99
Used shared bathroom in the ward	10/15 (67)	8/11 (73)	>0.99
Used toilet in the shared bathroom	10/10 (100)	6/9 (67)	0.09
Used sink in the shared bathroom	1/10 (10)	2/6 (33)	0.52
Used shared shower room in the ward	11/15 (73)	9/11 (82)	>0.99
Used water dispenser in the ward	8/15 (53)	8/12 (67)	0.70

ICU, intensive care unit; IQR, interquartile range.
[a] Genotypes of cases were as follows: 11 carried New Delhi metallo-beta-lactamase-1; two carried Verona integron-encoded metallo-beta-lactamase; and two carried *Klebsiella pneumoniae* carbapenemase.

with concentration of 5500 ppm) and replaced the general ward sink drains. Thereafter, no additional cases were identified for 3 weeks and we closed the outbreak.

Discussion

During this outbreak, many cases presented without known risk factors for acquisition of CPE such as critical illness, ICU stay, or receipt of antibiotics [10,11], and with no epidemiologic links between cases. We thereby suspected environmental reservoirs and found that critical points in the hospital water environment, including a water dispenser, sink drains, and toilets, were CPE reservoirs as evidenced by environmental sampling and PFGE analysis.

In the ICU, human waste such as dialysis fluid that might contain glucose was frequently emptied into the handwashing sink, which was near the water dispenser. Biofilm-forming Enterobacteriaceae can form reservoirs in sink drains, and water from the faucets can be aerosolized by splashing and contaminate the basin and surrounding areas. We believe that the sink was contaminated with KPC-producing *E. coli*, and droplets or aerosol from that sink resulted in contamination of the water dispenser. Patients who drank water from this dispenser tested positive for colonization with KPC-producing *E. coli*. Using PFGE analysis (Figure 2(a)), we found that the pulsotype of the KPC-producing *E. coli* from the water dispenser and that from the patients were identical, supporting this hypothesis. Our data suggest that the pouring of human waste into the handwashing sink adjacent to a water dispenser was the direct cause of the KPC-producing *E. coli* outbreak. However, the risk of cross-contamination from healthcare workers' hands with CPE from sink drains and the water dispenser could not be ruled out, limiting our firm conclusion. It is worth noting that we used bottled water as one of several interventions for the infection control. Although bottled water can sometimes transmit *Pseudomonas aeruginosa*, we thought that the risk of acquisition of CPE from contaminated water dispenser might outweigh the acquisition of *P. aeruginosa* from bottled water. In addition, Hopman *et al*. reported that water-free patient care including provision of bottled water reduced the Gram-negative bacilli acquisition in the ICU [12]. Further studies are needed on this issue.

In the general wards, identifying the source of CPE was more difficult, because the patients with CPE had nothing in common with regard to medical team or medical practice. Caregivers, multiple-occupancy rooms, and facilities, including shared bathrooms, toilets, sinks, and water dispensers, were obstacles to controlling the outbreak. Several factors including misuse of the sink in the ward for disposal of drinks and sink drain directly below the outlet could contribute to the dispersion and contamination of CPE. We evaluated risk factors using a case–control study with a questionnaire, and teeth brushing at the patient bathroom sinks was identified as a risk factor. Because brushing the teeth could result in direct inoculation of CPE to the gastrointestinal tract, we believe that it has added risk over other acts such as face or hand washing. Multiple environmental samplings revealed the presence of NDM-1-producing *C. freundii* and *E. cloacae* in the sink drain in the bathroom of a multiple-occupancy room, and in the toilet and urinal in a shared ward bathroom (Table II). PFGE analysis revealed that at least one clone of NDM-1-producing *E. cloacae* in the sink drain of the patient's room might have been the possible source of the corresponding patient infections, although reverse causality could not be ruled out. A growing body of evidence has identified the water environment as a

reservoir for CPE [6—8,13—17], and studies also showed that the outbreak was contained by exchanging the sink drain or plumbing system. Removal of biofilms in drains is difficult, and we terminated the outbreak by removing the water dispenser and replacing sink drains as needed. As there were multiple organisms with diverse genotypes of CPE involving multiple wards, we believe that there were several outbreaks rather than one. Replacement of sink drain terminated not only KPC and NDM outbreaks, but also other metallo-beta-lactamase (MBL) (Verona integron-encoded metallo-beta-lactamase (VIM), imipenemase-1 (IMP-1)) and OXA outbreaks.

We used Xpert Carba-R to identify the patients with CPE and subsequently released those without CPE from preemptive isolation. This process minimized the number of exposed patients and saved resources expended by putting patients in preemptive isolation. There were 18 (21%) cases presenting as Xpert Carba-R positive and culture negative. We surmised that these cases were false-negative culture results and not false-positive Xpert Carba-R results based on previous reports indicating superior sensitivity of the Xpert Carba-R test over culture [18]. However, further studies are needed regarding the utility of rapid molecular tests in the control of hospital CPE infection outbreaks.

This study has certain limitations which should be acknowledged. First, as we defined the acquisition location of CPE as patient locations 48 h before the first isolation of CPE, it is possible that acquisition occurred in other wards. A substantial number of patients were moved from the ICU to a ward or from one ward to another, and as active surveillance tests for CPE were not conducted hospital-wide, the original acquisition site may have been missed. Second, environmental studies were not performed in other wards, nor did we evaluate the possibility of contamination of the hospital's horizontal drainage system. Third, as the number of cases and controls included in the case—control study were small, it is possible that using a sink in the bathroom for handwashing, or face washing could be significantly associated with patient transmission by CPE if the number of patients had been slightly higher; and we could not perform the multivariate analysis for identifying the risk factors for acquisition of CPE due to the low number of cases and controls. Fourth, although there is a report of a nosocomial foodborne outbreak originating from a hospital kitchen central food supply sector [19], we did not screen the sink in the hospital kitchen, which has the same drainage network.

In conclusion, water environments were the likely reservoirs of CPE in this outbreak. Replacement of the plumbing system, treatment with bleach, and the removal of a water dispenser were necessary to control the outbreak. These findings suggest that investigation of the water system should be conducted immediately upon the discovery of CPE. When detecting CPE outbreak, we suggest investigating the water system for adequateness of cleaning, misuse of the handwashing sink (disposal of human waste, nutrition fluid, or medication), and structure of the sink (faucet and drain position). Whenever possible, environmental sampling of the water system, and disinfection of the sink drain or replacement of the plumbing system are warranted. Furthermore, meticulous investigations of close contacts with the hospital water environment, including tooth brushing, face washing, and water drinking, are needed. In addition, appropriate hand hygiene of healthcare workers after being in contact with the water environment cannot be overemphasized.

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Conflict of interest statement
All authors report no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2019.11.015.

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Recurrent *Sphingomonas paucimobilis*-bacteraemia associated with a multi-bacterial water-borne epidemic among neutropenic patients

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Summary: A cluster of septicaemias due to several water-related species occurred in a haematological unit of a university hospital. In recurrent septicaemias of a leukaemic patient caused by *Sphingomonas paucimobilis*, genotyping of the blood isolates by use of random amplified polymorphic DNA-analysis verified the presence of two distinct *S. paucimobilis* strains during two of the separate episodes. A strain of *S. paucimobilis* identical to one of the patient's was isolated from tap water collected in the haematological unit. Thus *S. paucimobilis* present in blood cultures was directly linked to bacterial colonization of the hospital water system. Heterogeneous finger-printing patterns among the clinical and environmental isolates indicated the distribution of a variety of *S. paucimobilis* clones in the hospital environment. This link also explained the multi-microbial nature of the outbreak.

Keywords: *Sphingomonas*; bacteraemia; nosocomial infection; random amplified polymorphic DNA technique.

Introduction

Non-enteric Gram-negative bacilli and non-tuberculous mycobacteria which may induce severe nosocomial infections in patients with underlying debilitating conditions and/or preceding medical interventions are known to colonize water distribution systems in hospitals.[1,2] An unusual cluster of bacteraemic infections was observed among adult haematological patients in Kuopio University Hospital during a several months' period in 1994. Among the 195 bacterial isolates recovered from their blood cultures during the outbreak, 25% ($N = 48$) were glucose non-fermenting Gram-negative bacilli, e.g., *Pseudomonas aeruginosa, Acinetobacter* sp., *Achromobacter xylosoxidans, Sphingomonas paucimobilis* and *Stenotrophomonas maltophilia*. These species caused septicaemia in 10 patients.

The recognition of a cluster of infections associated with bacteria potentially derived from environmental reservoirs prompted a survey of the microbiological quality of the hospital water supply. Septicaemia in a leukaemia patient, caused by *Mycobacterium fortuitum*, had been linked to the hospital water distribution systems, as described

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earlier.[2] Further investigations suggested the same water source as an apparent reservoir of bacteraemic infections caused by a variety of species growing in similar conditions.

We describe here results of molecular epidemiological analyses of *S. paucimobilis* isolated from blood cultures of a neutropenic patient with recurrent bacteraemic episodes, and her hospital environment. As far as we are aware, only two previous epidemiological reports on *S. paucimobilis* have been based on molecular typing of clinical and environmental isolates in a hospital setting,[3,4] and this is the first time hospital water has been linked to nosocomial bacteraemias.

Materials and methods

Case report

A 57-year-old female presented with acute lymphoblastic leukaemia in October 1993. Induction chemotherapy was carried out through a central tunnelled catheter (Chemo-Cath®, HDC) and was followed by morphological remission. During the neutropenic periods following the induction chemotherapy and the first consolidation chemotherapy cycle, the patient suffered from three septic episodes. The first of the episodes was caused by *Streptococcus mitis* and *Enterococcus faecium*, the second by *Bacteroides fragilis* and *Lactobacillus* sp. and the third by *Streptococcus sanguis*. All episodes were successfully treated with appropriate antibiotics. There was a purulent infection on the exit site of the tunnelled catheter during the third septic episode caused by *S. sanguis*, but no cultures were performed from the exit site.

In April 1994 the patient received her fifth course of chemotherapy, which resulted in severe neutropenia (neutrophils $< 0.5 \times 10^9$/L) lasting 7 days. She presented with fever and oral mucositis, with *Candida albicans* and Herpes simplex virus I detected in the oral lesions. No signs indicating catheter infection were present. Blood cultures collected from a cubital vein on the second day after admission revealed a slowly growing non-enterobacterial Gram-negative rod. Treatment with piperacillin and netilmycin was followed by rapid clinical response without the removal of the central catheter. The blood culture isolate was initially incorrectly identified as *Sphingobacterium multivorum* and later as *S. paucimobilis*. Because of susceptibility results, piperacillin was replaced by ceftazidime while the patient was already afebrile. Prior to the first blood culture positive for *S. paucimobilis*, the patient had been hospitalized for 142 days in the preceding 6 months.

The patient returned to the hospital in the end of May 1994 to receive the final planned chemotherapy cycle. On admission, she had no signs or symptoms of infection. Laboratory tests revealed normal white blood cell and neutrophil counts. Following flushing of the Chemo-Cath she developed a fever of 40.1°C with chills. Blood cultures obtained from a cubital vein and through Chemo-Cath were again positive for *S. paucimobilis*. Treatment with ceftazidime and netilmycin was followed by resolution of fever. After a 10-day course of antibiotic treatment she received the chemotherapy cycle, and returned home with Chemo-Cath in place for removal after recovery of the bone marrow hypoplasia. She was re-admitted to hospital 29 days later with fever and sore throat. Laboratory tests showed neutropenia (below 0.5×10^9) lasting for further 19 days. Blood cultures were again positive for *S. paucimobilis*, and the infection again responded to treatment with ceftazidime and netilmycin. After this episode, the Chemo-Cath was removed and oral maintenance treatment for acute leukaemia was started. Bacterial culture of the removed Chemo-Cath was negative.

Bacterial strains

Clinical isolates

Blood cultures were performed in a semi-automated system (BACTEC 730, Becton Dickinson, Sparks, MD, USA). Non-fermentative Gram-negative rods were biotyped with API20 NE (bioMérieux, Marcy l'Etoile, France), and isolates were stored in skimmed milk at −70°C.

Environmental isolates

After recognition of a cluster of bacteraemias in April 1994, 22 environmental samples were collected from taps, showers and detergent dilutions used by the patients in the haematology ward. Water samples were collected in sterile containers after removal of the aerator or showerhead and flushing for 30 seconds. The aerators and showerheads were also sampled with sterile cotton swabs. Water samples were diluted 1:10 in sterile water and aseptically filtered through a 0.45 μm membrane (10 mL/membrane) (Schleicher and Schuell, Dassel,

Germany). The membranes were placed on CLED (cystine-lactose-electrolyte-deficient) agar plates (Becton Dickinson, Cockeysville, MD, USA) and incubated at 36°C for 2 days, and on Sabouraud medium (Becton Dickinson, Cockeysville, MD, USA) at 30°C for 4 weeks. Swab samples were streaked on to CLED agar and incubated at 37°C for 2 days. Detergent dilutions were analysed according to the method of Kelsey–Maurer.[5] The following autumn, a similar surveillance, comprising 30 environmental cultures, was performed in the haematology ward.

A total of 71 randomly selected colonies, each representing different colony types detectable on separate plates, were subcultured for further studies. Non-fermentative Gram-negative bacillary isolates, initially identified using API20 NE, were stored in skimmed milk at −70°C for later molecular analyses.

Genotyping

Two to six parallel subcultures of each isolate of *S. paucimobilis* were grown in 5 mL tryptone soy broth at 36°C for 48 h. The bacteria were pelleted by centrifugation and washed once with phosphate-buffered saline (PBS). DNA was extracted by using a commercial nucleic acid isolation kit (High Pure PCR Template kit, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Twenty commercial 10-mer primers (Operon Technologies, Inc., Alameda, California) were screened in a pilot study using two to four strains. The primers with the best discriminatory power, OPB10 (5′ CTGCTGGGAC) and OPB17 (5′ AGGGAACGAG) (Operon Technologies, Inc., Alameda, CA, USA), were selected for the final random amplified polymorphic DNA (RAPD) analyses. PCR amplification was carried out as described earlier,[6] in a final volume of 27 μL. Amplified DNA was electrophoresed on 2.0% agarose gels containing 0.5 μg/mL ethidium bromide and 1x Tris-acetic acid-EDTA running buffer, and photographed under UV light. Different RAPD types were designated by a two-letter code showing the pattern for each of the primers OPB10 (letters A–D) and OPB17 (letters a–e).

16S rRNA analysis

Selected isolates were analysed for their partial 16S rRNA sequences to verify their identification. The partial 16S rRNA gene was amplified by using primers pA and pE′.[7] PCR was performed in a 50 μL reaction mixture containing 25 pmol of each primer, 25 ng bacterial DNA, 200 μM dNTP, 1 × DynaZyme buffer, and 1 U DynaZymell polymerase (Finnzymes, Espoo, Finland). The thermal cycler was programmed as described previously.[7] The amplification products were purified for sequencing with MicroSpin S-400 HR columns (Pharmacia, Piscataway, NJ, USA) and sequenced with primers pD′ and pE′.[8] Sequencing was done by using a Dye Terminator Cycle Sequencing kit and an automated ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Clinical isolates

Eight isolates were recovered from six blood culture samples of the index patient. They were yellow-pigmented, glucose non-fermenting Gram-negative rods. All gave an API20 NE code 0463304 indicating either *Sphingobacterium multivorum* (64.8%), *S. paucimobilis* (21.1%) or *Sphingobacterium spiritivorum* (14.0%) at a low discrimination level.

Environmental isolates

Twelve yellow-pigmented isolates from eight water samples produced an API20 NE code similar to the patient's. These were selected for further comparative studies. The other species isolated from the same water systems included *S. multivorum, Stenotrophomonas maltophilia, Pseudomonas* sp., *Acinetobacter* sp. and *Mycobacterium fortuitum*. The detergents examined did not contain non-enteric Gram-negative bacilli.

16S rRNA analysis

Twelve isolates, two clinical and 10 environmental isolates, were selected for partial 16S rRNA sequencing as representatives of the different RAPD-patterns detected (see below). Nine, including the clinical isolates initially identified as *Sphingobacterium multivorum* by phenotypic characteristics, were identified as *S. paucimobilis* by gene sequencing. They all showed 100% similarity in partial 16S rDNA sequences of the first variable region. This sequence was identical to that of the *S. paucimobilis* type strain (Genebank accession number U37337). The remaining three isolates represented other distinct environmental species.

Genotyping

The results of the RAPD fingerprinting demonstrated that the index patient harboured two *S. paucimobilis* strains in her bacteraemic episodes. In the first two episodes, she had both strains simultaneously, whereas in the last one she only had one of the strains. As shown in Figure 1, the

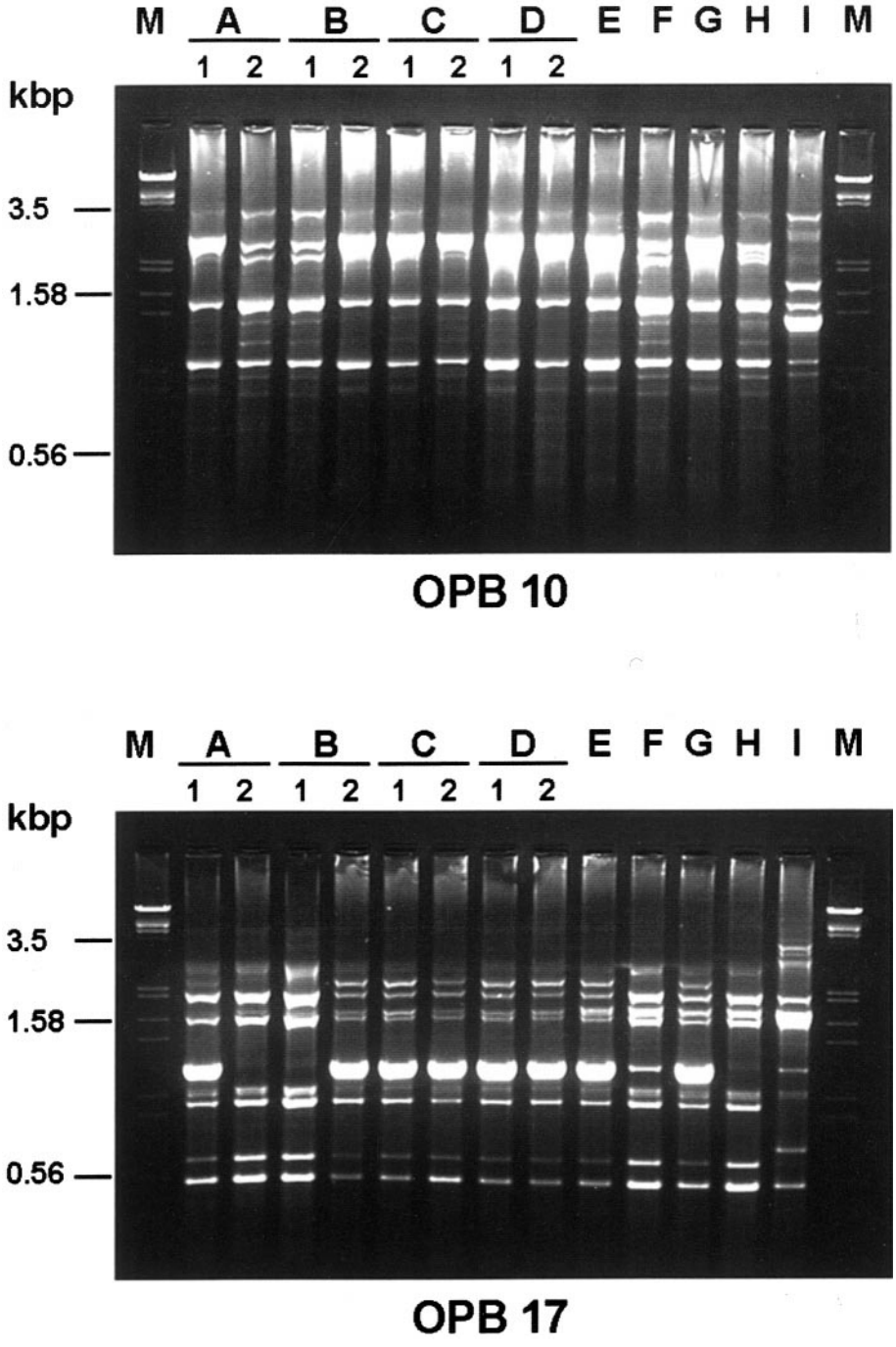

Figure 1 RAPD fingerprint patterns using primers OPB10 and OPB17 for *Sphingomonas paucimobilis* isolates from the blood cultures of the index patient obtained on four distinct dates during recurrent episodes (A–D) and from the tap water at the haematological department (E–I). For pattern and isolate descriptions refer to Table I. Lane M; molecular weight marker (Lambda DNA/*Eco*R I + *Hind* III).

first two patient isolates (lanes A–B) had distinct RAPD-patterns ('Aa' and 'Bb'), whereas the latter patient isolates (Figure 1, lanes C–D) shared the pattern 'Aa'.

In all, five different finger-printing patterns were generated by the 13 isolates using the two primers applied (OBP10 and OBP17) (Figure 1, Table 1). Two water isolates (Figure 1, lanes E and G) had a pattern identical to the patient's isolates of type 'Aa'. These water isolates were recovered at four-week intervals from a single tap of a patient bathroom at the haematological ward. Another water isolate from the same tap (Figure 1, lane F) had a pattern closely similar to pattern 'Bb' of the index patient (Figure 1, lanes A2 and B1). The patient had a direct access to this bathroom, located next to the patient room she occupied. The other two tap water isolates recovered from different rooms at the same ward had unique patterns (Figure 1, lanes H and I).

Discussion

To our knowledge, this is the first time that a nosocomial *S. paucimobilis* bacteraemia has been epidemiologically linked to the hospital water system using molecular typing methods. Although the culture of the removed central venous catheter of the patient was negative on culture, clinical findings indicated that a contaminated and colonized catheter was the likely portal of entry of the organism into the bloodstream. We demonstrated two clones of *S. paucimobilis* in the bacteraemic episodes of our patient. A similar observation of several clones in a catheter-related infection of a single patient has recently been published by Hsueh *et al.*[4] The heterogeneity of RAPD patterns of *S. paucimobilis* isolates most likely reflect the diversity of *S. paucimobilis* clones in the hospital water system, regarded as the potential source of colonization of the patient.

Contamination of faucet aerators has recently been linked to colonization or infection in patients by using molecular epidemiological methods.[9] In our study, faucet aerators and showerheads were colonized with several bacterial species, mainly non-enteric Gram-negative rods. To diminish colonization, the faucet aerators and showerheads were mechanically washed and disinfected in chlorine periodically. After initiation of these procedures, clinical isolation rates of environment related bacteria returned to the level before the outbreak described.

We have found RAPD-method a useful basic tool for epidemiological studies in clinical settings. Due to its adaptability to different species, it can easily be applied to a variety of epidemiological situations.[2,6,10] It has high discriminatory power and good reproducibility if done carefully.[6,10] Due to interlaboratory variability in banding patterns, the results of different laboratories can only be compared to the level 'identical vs. non-identical'.

Increasing clinical evidence implicates water as a source of nosocomial infections. To decrease the risk of water-derived infections in tertiary care hospitals, rational limits for acceptable quality of hospital water need to be defined, and simultaneously, reasonable and sound sanitation procedures developed.

Table 1 *Recovery of different* S. paucimobilis *strains from blood cultures of the index patient and environmental waters at the adult haematological ward in 1994*

Date of isolate	Source	Specimen type	Isolate	RAPD-pattern
19 April	Patient	Blood	A1	Aa
19 April	Patient	Blood	A2	Bb
19 April	Hall	Tap water	I	De
31 May	Patient	Blood	B1	Bb
31 May	Patient	Blood	B2	Aa
1 June	Patient	Blood	C1	Aa
1 June	Patient	Blood	C2	Aa
4 July	Patient	Blood	D1	Aa
4 July	Patient	Blood	D2	Aa
14 November	Bathroom	Tap water	E	Aa
29 November	Bathroom	Tap water	F	Bc
14 December	Bathroom	Tap water	G	Aa
14 December	Room	Tap water	H	Cd

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SHEA

Original Article

Concurrent transmission of multiple carbapenemases in a long-term acute-care hospital

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Abstract

Objective: We investigated concurrent outbreaks of *Pseudomonas aeruginosa* carrying bla_{VIM} (VIM-CRPA) and Enterobacterales carrying bla_{KPC} (KPC-CRE) at a long-term acute-care hospital (LTACH A).

Methods: We defined an incident case as the first detection of bla_{KPC} or bla_{VIM} from a patient's clinical cultures or colonization screening test. We reviewed medical records and performed infection control assessments, colonization screening, environmental sampling, and molecular characterization of carbapenemase-producing organisms from clinical and environmental sources by pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing.

Results: From July 2017 to December 2018, 76 incident cases were identified from 69 case patients: 51 had $bla_{KPC,}$ 11 had $bla_{VIM,}$ and 7 had bla_{VIM} and bla_{KPC}. Also, bla_{KPC} were identified from 7 Enterobacterales, and all bla_{VIM} were *P. aeruginosa*. We observed gaps in hand hygiene, and we recovered KPC-CRE and VIM-CRPA from drains and toilets. We identified 4 KPC alleles and 2 VIM alleles; 2 KPC alleles were located on plasmids that were identified across multiple Enterobacterales and in both clinical and environmental isolates.

Conclusions: Our response to a single patient colonized with VIM-CRPA and KPC-CRE identified concurrent CPO outbreaks at LTACH A. Epidemiologic and genomic investigations indicated that the observed diversity was due to a combination of multiple introductions of VIM-CRPA and KPC-CRE and to the transfer of carbapenemase genes across different bacteria species and strains. Improved infection control, including interventions that minimized potential spread from wastewater premise plumbing, stopped transmission.

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Among the underlying mechanisms of bacterial carbapenem resistance, carbapenemases are of significant public health concern. Carbapenemases are frequently encoded on mobile genetic elements (eg, plasmids) that often contain additional resistance determinants, can be transferred between bacterial taxa,[1] and are associated with rapid increases in carbapenem resistance.[2–4] In the United States, nearly 35% of carbapenem-resistant Enterobacterales (CRE) harbor a carbapenemase, with *Klebsiella pneumoniae* carbapenemase (KPC) most commonly identified.[5] Carbapenemases are less commonly the mechanism of carbapenem resistance in *Pseudomonas aeruginosa*; ~2% of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) harbor a carbapenemase, most frequently the Verona-integron-encoded metallo-β-lactamase (VIM).[4,6,7] Carbapenemase-producing organisms (CPOs) can cause outbreaks in healthcare facilities resulting in infections with limited treatment options.[2,8,9]

Patients most susceptible to acquiring CPOs have complex medical needs.[10,11] Most patients with CPOs are asymptomatically colonized, presenting challenges for identification and initiation of transmission-based precautions.[7] CPO transmission may occur via transient hand carriage by healthcare personnel or via contaminated shared medical equipment. Additionally, a growing body of literature describes CPO transmission from healthcare facility wastewater plumbing to patients.[3,12,13]

On July 5, 2017, the Florida Department of Health (FDOH) was notified of 2 carbapenemase-producing organisms, VIM-producing

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Pseudomonas aeruginosa and KPC-producing *Klebsiella pneumoniae*, in specimens from a patient of long-term acute-care hospital-A (LTACH A) on admission to a local acute-care hospital. In response, the FDOH conducted an onsite infection control assessment at LTACH A that identified significant gaps in hand hygiene and transmission-based precautions adherence, raising concern for transmission. In July 2017, a facility-wide point-prevalence survey (PPS) of 36 patients identified 4 patients with KPC-producing CRE and 3 patients with VIM-producing CRPA. Here, we describe epidemiologic and laboratory investigations to control transmission.

Methods

Setting

LTACH A is a freestanding facility with a 6-bed intensive care unit (ICU) and a progressive care unit that expanded from 40 to 50 private rooms in January 2018.

Case definitions and case finding

We defined a case as the detection of bla_{VIM} or bla_{KPC} in a clinical isolate or screening specimen from a patient admitted to LTACH A for ≥1 night between July 13, 2017, and December 18, 2018. An incident case was the first identification of bla_{VIM} or bla_{KPC}; case patients could have 2 incident cases (1 bla_{VIM} and 1 bla_{KPC}). Incident cases were considered to have been acquired in LTACH A if they were identified from a patient without a history of colonization or infection with that carbapenemase and with ≥1 negative screening result at least 1 week before incident specimen collection. Cases considered present on admission had specimens collected within 3 days of admission to LTACH A.

In July 2017, we requested the commercial laboratory of LTACH A to submit carbapenem-resistant organisms identified in clinical specimens to the FDOH Bureau of Public Health Laboratory (BPHL) for carbapenem resistance mechanism testing. In August 2017, we initiated admission and discharge screening and biweekly facility-wide point-prevalence surveys (PPSs) to detect carbapenemase genes. Human subjects advisors in Florida reviewed the investigation activities and determined that they constituted public health response. This research was exempt from human subjects review by the Centers for Disease Control and Prevention (CDC) and was conducted consistent with applicable federal law and CDC policy [45 C.F.R. part 46.102(I)(2)].

Case investigation

We completed medical record reviews for incident cases using a standard abstraction form to collect patient demographics, past medical history, underlying medical conditions, discharge status, presence of indwelling device(s), and antibiotic administration at the time of or in the 14 days before incident specimen collection. We calculated slopes of newly acquired cases and CPO prevalence by fitting a linear regression line in 2-week intervals (based on PPSs) over the outbreak duration using R Studio version 1.2.1335 software (R Foundation for Statistical Computing, Vienna, Austria).

Cohort study

We conducted a retrospective cohort study to assess risk factors associated with bla_{VIM} and/or bla_{KPC} acquisition during the initial months of the outbreak. All patients admitted to LTACH A from July 5 to December 7, 2017, with ≥2 colonization screenings performed were included. Information regarding maintenance of medical devices, hemodialysis procedures, enteral feedings, respiratory therapy, speech, occupational, and physical therapy as well as peripherally inserted central catheter (PICC) insertion and line maintenance were obtained from procedure log books. Medical records were not abstracted for noncases; thus, we were unable to select comorbid conditions as a confounder in our regression models. Mortality data were collected through Florida's electronic death registry.[14] Univariable analysis was conducted using the Welch unequal variance t test for continuous variables and the Pearson χ^2 test for categorical variables. Confounders were identified using prior knowledge. A multivariable logistic regression adjusting for age, sex, length of stay, and ICU admission was conducted to estimate the relative risks (RRs) and 95% confidence intervals (CIs) for acquisition of bla_{VIM} and bla_{KPC} during hospitalization at LTACH A. Statistical tests based on a 2-tailed probability and significance level of $\alpha = 5\%$ were conducted using Stata IC version 16.0 software (Statacorp LLC, College Station, TX).

Infection control observations and interventions

Scheduled and unannounced infection control assessments with observations of practice were conducted using the CDC Infection Control Assessment and Response (ICAR) Tool for Acute Care Hospitals (www.cdc.gov/hai/prevent/infection-control-assessment-tools.html). We audited adherence to the World Health Organization Five Moments for Hand Hygiene[15] and recorded hand hygiene (HH) and personal protective equipment (PPE) observations via the iScrub Lite mobile phone application (version 1.5.3, 2018, SwipeSense, Chicago, IL). We also observed environmental cleaning, respiratory care, antibiotic compounding, and device reprocessing.

Laboratory investigation

Carbapenem-resistant Enterobacterales and *P. aeruginosa* from clinical cultures were forwarded to the CDC and the BPHL for carbapenem resistance mechanism testing. Colonization screenings were conducted by testing rectal swabs for carbapenemase genes using the Cepheid Xpert CarbaR (Cepheid, Sunnyvale, CA).[16] when carbapenemase genes were detected, a swab was cultured to recover carbapenem-resistant organisms (Supplementary File 1 online).

Environmental sampling

Environmental samples were collected from sink drains, splash zone surfaces, and mobile equipment (Supplementary Table 1 online). Environmental samples underwent broth enrichment and plating onto selective media agar to screen for suspect isolates.

Molecular characterization

Pulsed-field gel electrophoresis (PFGE) was performed on clinical and environmental isolates. A subset of isolates was selected for short-read whole-genome sequencing (WGS) based on epidemiological findings and representativeness of isolates in distinct PFGE clusters. The isolates that underwent short-read WGS also underwent long-read WGS to better resolve plasmid structures (Supplementary File 2 online).

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Results

Outbreak overview

From July 13, 2017, to December 18, 2018, 76 incident cases were identified from 69 case patients: 11 had bla_{VIM}, 51 had $bla_{KPC,}$ and 7 had bla_{VIM} and bla_{KPC}. All bla_{VIM} were identified in *P. aeruginosa*, and bla_{KPC} was identified in 19 *Klebsiella pneumoniae*, 7 *Citrobacter freundii*, 5 *Enterobacter cloacae* complex, 1 *Klebsiella oxytoca*, 1 *Serratia marcescens*, 1 *Providencia rettgeri*, 1 *Providencia stuartii*, and 1 *Citrobacter farmeri*. Also, 5 patients had multiple organisms harboring bla_{KPC}, and an organism was not recovered from 18 screening tests in which bla_{KPC} was identified.

In total, 8 case patients (2 bla_{VIM}, 5 bla_{KPC}, 1 bla_{KPC} and bla_{VIM}) were identified from admission screens; 53 case patients (7 bla_{VIM}, 42 bla_{KPC}, 4 bla_{KPC} and bla_{VIM}) were identified from PPS or discharge screens; and 8 case patients (2 bla_{VIM}, 4 bla_{KPC}, 2 bla_{KPC} and bla_{VIM}) were identified from clinical cultures.

Incident cases and prevalence

From July 2017 to December 2018, a gradual decrease in incidence of bla_{VIM} (slope, −0.079 every 2 weeks; $P = .004$) and bla_{KPC} (slope, −0.135 every 2 weeks; $P = .003$) was observed (Fig. 1). Declines were sharpest during the first 6 months (July 2017–January 2018: bla_{VIM} slope, −0.220 every 2 weeks, $P = .021$ and bla_{KPC} slope, −0.353 every 2 weeks; $P = .018$). The investigation closed on December 18, 2018, after no newly acquired cases were identified in LTACH A for 2 consecutive months.

Clinical characteristics and risk factors of case patients not identified on admission to LTACH A

Patient characteristics and risk factors for the 61 case patients (88%) who did not have a CPO identified on admission are described in Table 1. The median age was 66 years (IQR, 56–73 years). Demographic risk factors were similar among patients with bla_{VIM}, bla_{KPC}, and both bla_{VIM} and bla_{KPC}. Case patients with both bla_{VIM} and bla_{KPC} had higher median Charlson scores and were more likely to have diabetes than those with bla_{VIM} alone (median score, 6 vs 3; $P = .033$; diabetes, 100% vs 33%, respectively; $P \leq .05$) or bla_{KPC} alone (median score, 6 vs 3; $P = .048$; diabetes, 100% vs 67%, respectively; $P \leq .05$).

Cohort study

From July 5 to December 7, 2017, 146 patients were hospitalized at LTACH A, of whom 98 (67%) met our cohort study inclusion criteria. Among the 98 patients in the cohort, the 22 patients with CPO acquired at LTACH A had similar demographics to the 76 who did not acquire a CPO. Healthcare risk factors differed in that a greater proportion of case patients had PICC lines (68% vs 40%; $P = .017$) and ≥3 indwelling devices (77% vs 41%; $P = .003$) (Table 2).

Patients with a feeding tube or ≥3 indwelling medical devices had an increased risk of acquiring bla_{KPC} (aRR, 1.18; 95% CI, 1.01–1.39; aRR, 1.21 95% CI, 1.02–1.43, respectively) and bla_{VIM} (aRR, 1.07; 95% CI, 1.02–1.14; aRR, 1.14; 95% CI, 1.03–1.26) relative to patients without a feeding tube or with <3 indwelling medical devices (Table 3). The risk of acquiring bla_{VIM} increased with the presence of a tracheostomy and decreased with receipt of bilevel positive airway pressure (BiPAP) or continuous positive airway pressure (CPAP).

Infection control assessments and interventions

Beginning in July, we conducted 5 announced and 8 unannounced infection control assessments. At the initial assessment, we observed appropriate hand hygiene (HH) in 61% of opportunities and glove and gown use in 61% and 67% of opportunities, respectively. Access to alcohol-based hand rub (ABHR) and personal protective equipment (PPE) was limited. Recommended interventions included increasing HH and PPE audits and access to ABHR and PPE, placing case patients in cohorts by CPO status to different wings with dedicated patient-care staff, and scheduling case-patient specialized care appointments (eg, hemodialysis and physical therapy) and daily room cleaning after patients without known CPOs. However, CPO acquisitions remained high through October 2017 (Fig. 2 and Supplementary File 3 online).

A follow-up assessment in November 2017 identified continued gaps in adherence to HH and contact precautions and multiple practices with potential to transmit CPOs from wastewater plumbing to patients, including storing medical supplies such as syringes used to flush enteral feeding tubes in the sink splash zone or above the swivette toilet, discarding nutritive materials in the hand washing sink, cleaning from the sink basin to the countertop, and compounding oral vancomycin in close proximity to a hand washing sink.

The FDOH provided HH and PPE training with return demonstration to ∼225 LTACH healthcare personnel; overall adherence at the next assessment was 90% for HH and 92% for both glove and gown use and was sustained in all 5 subsequent assessments over a 10-month period. Over several months, LTACH A implemented recommendations to mitigate spread from plumbing by assessing for patient care items in sink splash zones during daily infection control rounds. LTACH A also discontinued use of sinks for liquid waste disposal, adding reminder signage near sinks, and offsetting faucets from the drain (Fig. 2). Although not a public health recommendation, LTACH A treated drains with bleach for 4 months beginning in May 2018; this practice coincided with decreased attention to other interventions intended to reduce transmission from sink drains and correlated with a resurgence of bla_{KPC} cases.

Environmental investigation

In November 2017 and January 2018, 91 environmental samples were collected from high-touch surfaces, medical equipment, sink drains in patient rooms, the pharmacy, medicine preparation rooms, and patient toilets. In total, 5 different Enterobacterales harboring bla_{KPC} and 2 *Pseudomonas* spp harboring bla_{VIM} were recovered from high-touch surfaces, sinks, and wastewater plumbing (Table 4).

Molecular characterization of clinical and environmental isolates

A PFGE dendrogram annotated with metadata including the sequence type (ST) and carbapenemase alleles for the subset of representative isolates that underwent WGS are shown in Figure 3. Among 21 KPC-producing CRE that underwent WGS, 4 KPC alleles were identified: 7 bla_{KPC-2}, 12 bla_{KPC-3}, 1 bla_{KPC-4}, and 1 bla_{KPC-8}. Also, bla_{KPC-2} and bla_{KPC-3} were identified in different Enterobacterales and in both clinical and environmental isolates. Furthermore, 6 isolates with bla_{KPC-2} including 5 *K. pneumoniae* ST14 corresponding to the largest PFGE cluster identified, and 1 *Providencia stuartii*, harbored the gene on an IncC plasmid. Also,

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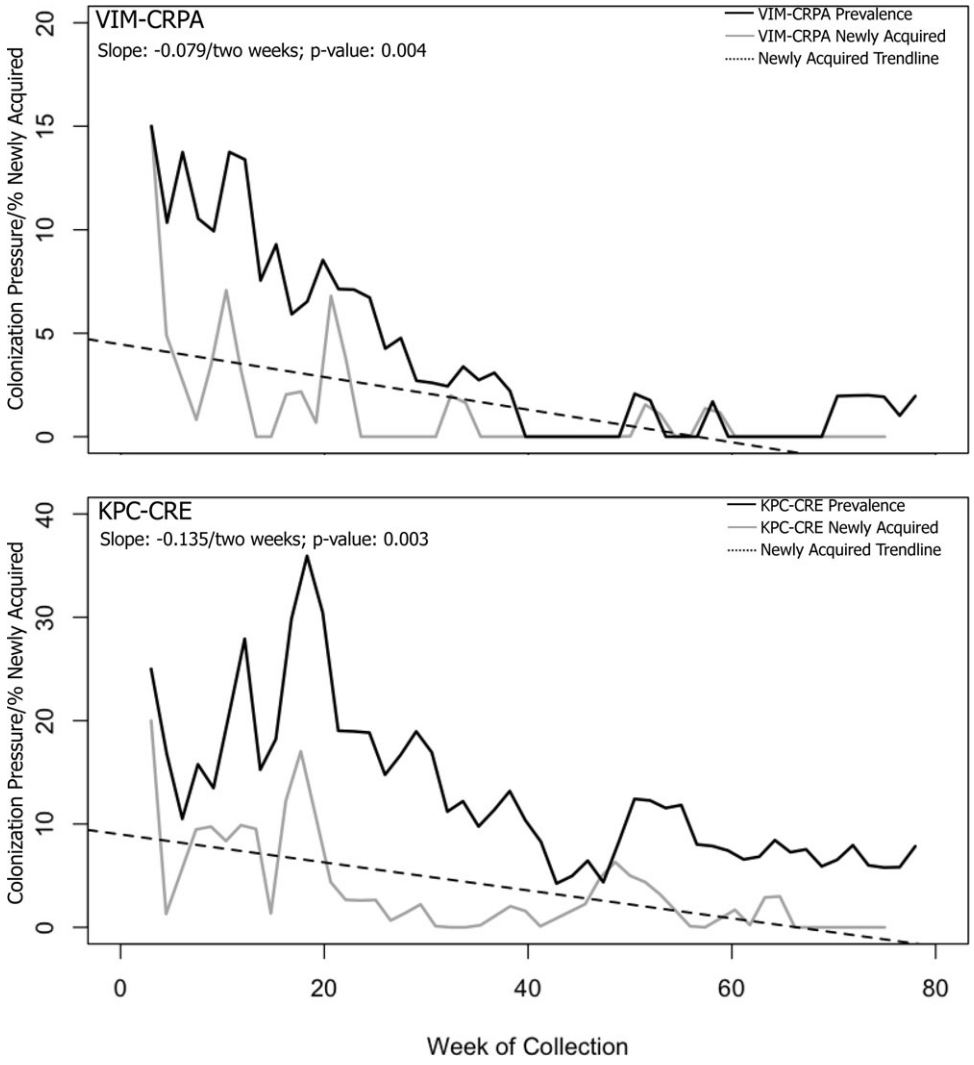

Figure 1. Prevalence and new acquisitions of carbapenemase-producing organisms detected through colonization screening at long-term acute-care hospital A, Florida, July 2017–December 2018. Prevalence (ie, colonization pressure) is the total number of cases currently hospitalized/census. Newly acquired indicates the percentage of patients with incident bla_{VIM} and bla_{KPC} among all screened patients. Note. VIM-CRPA, Verona-integron-encoded metallo-beta-lactamase–producing carbapenem-resistant *Pseudomonas aeruginosa*; KPC-CRE, *Klebsiella pneumoniae* carbapenemase–producing carbapenem-resistant Enterobacteriaceae.

12 isolates with bla_{KPC-3} represented 4 species: 5 *E. cloacae* complex, 4 *C. freundii*, 2 *S. marcescens*, and 1 *K. pneumoniae*. Among the 11 with long-read sequence data available, bla_{KPC-3} was identified on a Col (pHAD28) plasmid in 1 *K. pneumoniae* ST17 and on an IncFII plasmid in 4 *C. freundii*, 3 *E. cloacae* complex, and 1 *S. marcescens*, and on the chromosome in 2 *E. cloacae* (Fig. 3A).

No single dominant PFGE cluster was observed for VIM-CRPA (Fig. 3B). Among 6 VIM *Pseudomonas* isolates from 3 patients and 3 environmental sources that underwent WGS, 2 alleles were identified: 4 bla_{VIM-2} and 3 bla_{VIM-61} and were indentified in both clinical and environmental isolates. bla_{VIM-61} is a novel allele closely related to bla_{VIM-7} and was identified in a patient's *P. aeruginosa* ST298 isolate that was part of a PFGE cluster and in 2 environmental isolates.

Discussion

Our public health response to the identification of a patient colonized with VIM-CRPA and KPC-CRE revealed large, concurrent CPO outbreaks in an LTACH. Epidemiologic and laboratory evidence suggest that the observed diversity in organisms and mechanisms is explained by ongoing CPO importation and carbapenemase gene transfer across different species and strains. Several factors contributed to transmission. We controlled the outbreak by improving core infection control practices, intervening on spread from sink drains, and initiating admission, PPS, and discharge screening.

LTACHs in the United States play a vital role in managing critically ill patients requiring long hospitalizations. LTACHs can serve as amplifiers of multidrug-resistant organism (MDRO) transmission due to the combination of (1) the complex patient population they serve, (2) challenges with implementing infection control practices aimed at preventing transmission,[17,18] and (3) patient sharing with other healthcare facilities. In LTACH A, unrecognized importation of CPOs, combined with inadequate training and support for core infection control practices, likely contributed to spread of CPOs among patients and to the healthcare environment, creating reservoirs of resistant bacteria. Improved infection control practices andcoupled with enhanced detection of CPOs upon admission helped prevent later introductions from wider dissemination. Although the admission prevalence was relatively low (2%), LTACH A is regionally influential through patient sharing networks[19] and has continued admission screening in partnership with public health due to the perceived value of proactively identifying patients with CPOs. Outbreaks at LTACHs, as well as intensive interventions to prevent MDRO transmission in this setting, may have meaningful impacts on increasing or decreasing, respectively, regional MDRO

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Table 1. Characteristics of Case Patients with Carbapenemase-Producing Organisms, by Carbapenemase Gene Detected at Long-Term Acute-Care Hospital-A, Florida, July 2017–December 2018[a]

Characteristics	All CPOs (n=61), No. (%)[b]	bla_{VIM}, only (n=9), No. (%)[b]	bla_{VIM} and bla_{KPC} (n=6), No. (%)[b,c]	bla_{KPC}, only (n=46), No. (%)[b,d]	*P* Value[e]
Sex, male	34 (56)	7 (78)	2 (33)	25 (54)	.220
Age, median y (IQR)	66 (56–73)	60 (42–72)	67 (64–72)	66 (56–73)	.790
LOS median d (IQR)	48 (28–77)	41 (28–57)	82 (42–89)	47 (27–73)	.857
ICU stay before incident specimen	22 (36)	3 (33)	4 (67)	15 (33)	.259
Duration to incident specimen, median d (IQR)	34 (20–52)	20 (14–27)	33 (21–48)	36 (24–57)	.135
Specimen type of incident case					
Rectal screening	53 (87)	7 (78)	4 (67)	42 (91)	.166
Clinical isolate	8 (13)[f]	2 (18)	2 (33)	4 (8)	
Epidemiologic classification					
Met criteria for LTACH-A acquired[a]	40 (66)	2 (22)	5 (83)	33 (72)	.011[h]
Unable to determine if LTACH-A acquired[g]	21 (34)	7 (78)	1 (17)	13 (28)	
Death, <90 d incident specimen	20 (33)	4 (44)	4 (67)	12 (26)	.103[i]
LOS, from first-positive to death, median d (IQR)	33 (16–60)	41 (34–58)	20 (8–45)	28 (16–65)	.614
Charlson comorbidity score, median (IQR)	3 (1–5)[j]	3 (0–5)	6 (5–8)	3 (2–4)[k]	.014[h,i]
No Charlson comorbidities[k]	5 (8)	3 (33)	0	2 (4)	.006
5 Most common Charlson comorbidities					
Diabetes	31 (65)[j]	3 (33)	6 (100)	22 (67)[k]	.027[h,i]
Diabetes with complications	13 (27)[j]	1 (11)	5 (83)	7 (21)[k]	.003[h,i]
Congestive heart failure	16 (33)[j]	4 (44)	4 (67)	8 (24)[k]	.094
Chronic pulmonary disease	13 (27)[j]	1 (11)	3 (50)	9 (27)[k]	.252
Renal disease	12 (25)[j]	3 (33)	2 (33)	7 (21)[k]	.668
Current device(s) present or 14 days before incident specimen					
BiPAP/CPAP	5 (9)[l]	1 (11)	0 (0)	4 (9)[m]	.718
Feeding tube	55 (93)[l]	9 (100)	6 (100)	39 (91)[m]	.473
Mechanical ventilation	31 (53)[l]	5 (56)	6 (100)	20 (47)[m]	.048[h]
PICC line	28 (48)[l]	4 (44)	4 (67)	20 (47)[m]	.632
Tracheostomy	42 (72)[l]	7 (78)	6 (100)	29 (67)[m]	.229
Urinary catheter	18 (31)[l]	5 (56)	2 (33)	11 (25)[m]	.191
≥3 devices	43 (62)[l]	8 (73)	6 (86)	29 (57)[m]	.248
Special care services					
Hemodialysis	22 (38)[l]	4 (44)	3 (50)	15 (35)[m]	.704
Decubitus ulcers	12 (27)[n]	3 (38)[o]	2 (40)[p]	7 (22)[q]	0.519
Antibiotic therapy, 14 d before incident specimen					
Meropenem	16 (36)[r]	4 (50)[n]	3 (60)[o]	9 (29)[s]	.277
Vancomycin	23 (52)[r]	4 (50)[n]	4 (80)[o]	15 (48)[s]	.418
Cefepime	9 (20)[r]	3 (38)[n]	1 (20)[o]	5 (16)[s]	.409

Note. VIM, Verona-integron-encoded metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; LOS, length of stay; ICU, intensive care unit; BiPAP/CPAP, bilevel/continuous positive airway pressure; PICC, peripherally inserted central catheter. *P* values were calculated using the Pearson χ^2 test for categorical variables and the Welch unequal variance *t* test for continuous variables.
[a]Excludes 8 case patients who had CPO identified from admission screening.
[b]Units unless otherwise specified.
[c]For patients with both bla_{VIM} and bla_{KPC}, 5 of 6 had both carbapenemase genes identified on the same date (n=3) or within 7 days (n=2); date-specific analyses were performed using the date of specimen collection for the first incident case.
[d]One of the 46 KPC cases was identified as KPC-CRPA, the remainder were KPC-CRE.
[e]Pairwise comparison between bla_{KPC} and bla_{VIM} was <.05.
[f]Clinical specimen sources include sputum (n=2), urine (n=4), and wounds (n=2).
[g]Unable to conclusively assign time-point of CPO acquisition of no admission screening established before our first PPS and for those case-patients identified before when admission screening was implemented.
[h]Pairwise comparison between bla_{VIM} vs. bla_{VIM} and bla_{KPC} was <.05.
[i]Pairwise comparison between bla_{KPC} vs. bla_{VIM} and bla_{KPC} was <.05.
[j]n=48.
[k]No Charlson comorbidity index includes patients who may have had other comorbid conditions but did not have conditions included in the Charlson comorbitiy index.
[l]n=58.
[m]n=43.
[n]n=45.
[o]n=8.
[p]n=5.
[q]n=32.
[r]n=44.
[s]n=31.

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Table 2. Demographic and Clinical Characteristics of Patients with and without Hospital-Acquired Carbapenemase-Producing Organisms (CPOs) During Initial Months of an Outbreak, Long-Term Acute-Care Hospital A (LTACH A), Florida, July 13–December 7, 2017

Characteristic	No CPO (n=76), No. (%)[a]	Any CPO (n=22), No. (%)[a]	*P* Value	*bla*$_{VIM}$[b] (n=6), No. (%)[a]	*P* Value	*bla*$_{KPC}$[b] (n=20), No. (%)[a]	*P* Value
Sex, male	37 (48)	12 (55)	.628	3 (50)	.979	10 (50)	.959
Age, median y (IQR)	67 (59–73)	66 (55–72)	.874	71 (65–80)	.581	66 (59–71)	.957
LOS, median (IQR)	32 (23–50)	43 (34–58)	.190	46 (39–62)	.373	43 (35–58)	.121
Any ICU stay at LTACH A	14 (18)	6 (27)	.364	1 (17)	.814	6 (30)	.233
Duration to first positive or last negative swab, median d (IQR)	30 (21–42)	29 (23–41)	.392	22 (20–32)	.097	31 (24–41)	.813
Negative colonization swab(s), median (IQR)	3 (2–3)	1 (1–2)	<.001	2 (1–2)	.002	1 (1–2)	<.001
Location at time of first positive or last negative swab							
Unit A	31 (41)	8 (36)	.814	3 (50)	.691	6 (30)	.510
Unit B	37 (49)	11 (50)		1 (17)		3 (15)	
ICU	31 (41)	8 (36)		2 (33)		11 (55)	
Oral vancomycin	4 (5)	4 (18)	.051	2 (33)	.020	4 (20)	.030
Device(s) present at time of or before incident specimen collection							
BiPAP/CPAP	10 (13)	2 (9)	.608	0 (0)	.342	2 (10)	.731
Feeding tube	61 (80)	21 (95)	.090	6 (100)	.264	19 (95)	.124
Hemodialysis vascular access device	19 (25)	9 (41)	.146	2 (33)	.790	9 (45)	.068
Mechanical ventilation	34 (45)	13 (59)	.235	5 (83)	.073	12 (60)	.227
PICC line	30 (40)	15 (68)	.017	4 (67)	.293	14 (70)	.015
Tracheostomy	34 (45)	14 (64)	.118	6 (100)	.010	12 (60)	.269
≥3 indwelling devices[c]	31 (41)	17 (77)	.003	6 (100)	.010	15 (75)	.009

Note. VIM, Verona-integron-encoded metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; LOS, length of stay; ICU, intensive care unit; BiPAP/CPAP, bilevel/continuous positive airway pressure; PICC, peripherally inserted central catheter. *P* values were calculated using the Pearson χ^2 test for categorical variables and the Welch unequal variance *t* test for continuous variables.
[a]Units unless otherwise specified.
[b]4 patients had both VIM and KPC detected; referent group for VIM is no VIM detection; referent group for KPC is no KPC detection.
[c]Device cutoff was determined a priori.

Table 3. Association of Medical Exposures and Acquisition of Carbapenemase-Producing Organisms During Initial Months of an Outbreak, by Carbapenemase Gene Detected, Long-Term Acute-Care Hospital A (LTACHA), Florida, July 13–December 7, 2017

	bla$_{VIM}$			*bla*$_{KPC}$		
Medical Exposures	Adjusted RR[a]	95% CI	*P* Value	Adjusted RR[a]	95% CI	*P* Value
Oral vancomycin	1.22	0.92–1.63	.170	1.37	0.98–1.93	.066
Feeding tube	1.07	1.02–1.14	.022	1.18	1.01–1.39	.041
BiPAP/CPAP	0.94	0.89–0.98	.015	0.96	0.76–1.22	.744
Hemodialysis	1.02	0.93–1.12	.695	1.14	0.93–1.40	.195
Mechanical ventilation	1.09	0.97–1.23	.163	1.05	0.89–1.25	.519
PICC line	1.05	0.93–1.18	.437	1.21	1.02–1.45	.030
Speech therapy	1.12	0.97–1.29	.127	1.08	0.64–1.85	.752
Tracheostomy	1.13	1.03–1.24	.010	1.07	0.91–1.25	.408
≥ 3 indwelling devices[b]	1.14	1.03–1.26	.014	1.21	1.02–1.43	.025

Note. VIM, Verona-integron-encoded metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; LOS, length of stay; ICU, intensive care unit; BiPAP/CPAP, bilevel/continuous positive airway pressure; PICC, peripherally inserted central catheter. *P* values were based on a 2-tailed probability and a significance level set at $\alpha < .05$.
[a]All models were adjusted for age, sex, length of stay, and intensive care unit stay.
[b]Device cutoff was determined a priori; the referent group for medical exposures was the absence of the device and the referent group for ≥3 devices was 0–2 devices. Device history was collected through procedure logs provided by LTACH-A on a monthly basis.

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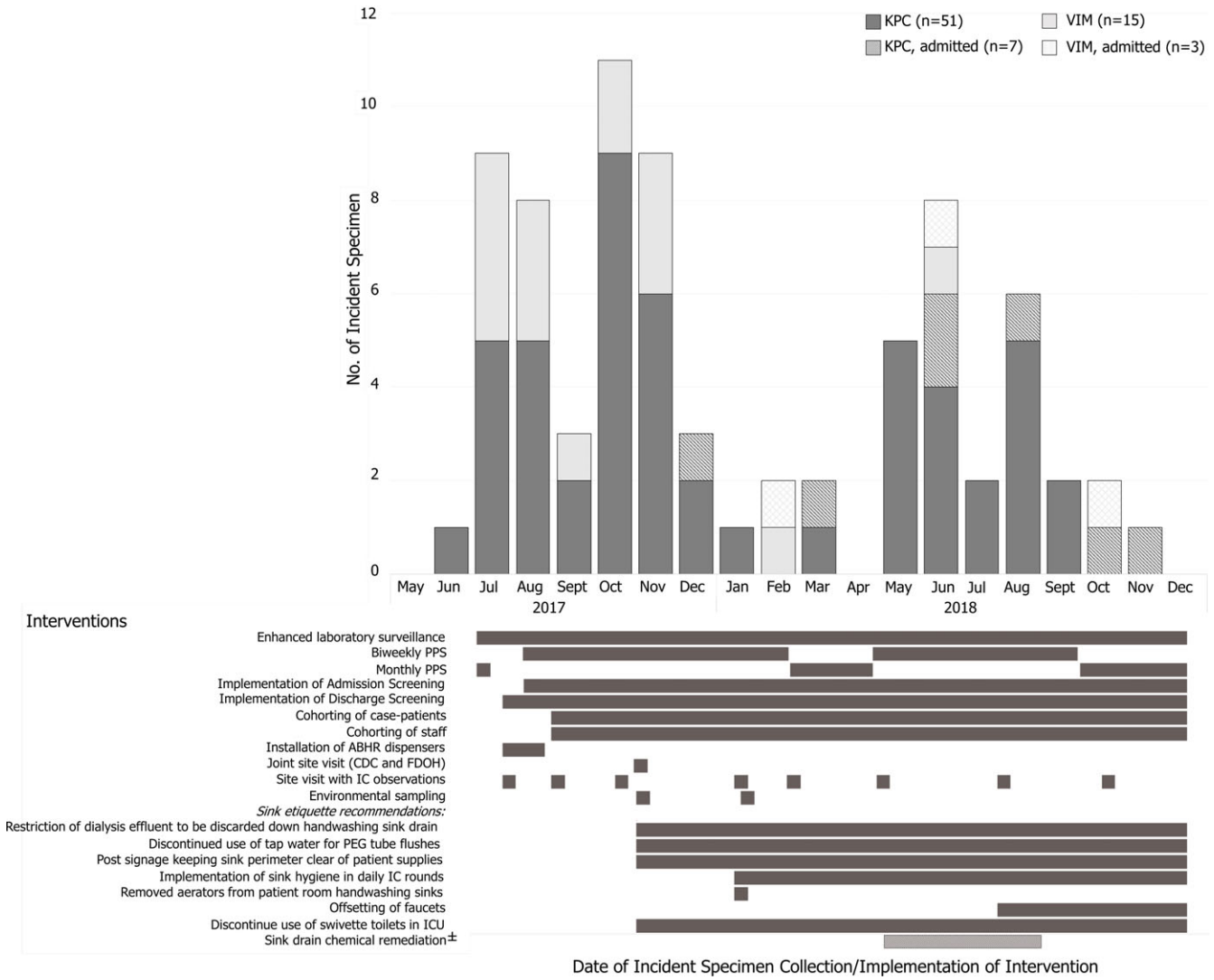

Figure 2. Epidemic curve and timing of infection control interventions to control new acquisitions of carbapenemase-producing organisms detected at long-term acute-care hospital (LTACH) A, Florida, July 2017–December 2018. "±" denotes action taken by LTACH A, but not recommended by public health officials. "KPC, admitted" represents patients identified with KPC at the time of admission. "VIM, admitted" represents patients identified with VIM at the time of admission. The incident specimen is the specimen that yielded the patient's first identified organism and mechanism combination. Note. CDC, Centers for Disease Control and Prevention, Division of Healthcare Quality and Promotion; FDOH, Florida Department of Health; IC, infection control; PPS, point-prevalence screenings.

spread.[20–24] Thus, these sustained efforts at LTACH-A may provide considerable benefits to the broader region.

Most case patient isolates with $bla_{\text{KPC-2}}$ belonged to large PFGE clusters of *K. pneumoniae* that were identified early in the investigation (September 2017–January 2018), when adherence to core infection control practices was poor. Isolates harboring $bla_{\text{KPC-3}}$ corresponded to the 2 largest PFGE clusters of *E. cloacae* and 3 other Enterobacterales and were identified for the duration of the outbreak. Although the diversity of PFGE patterns and periodic identification of case patients on admission could have led to the conclusion that KPC cases were due to multiple introductions followed by small clusters of transmission, added resolution from WGS suggests horizontal transfer of plasmids among species may have contributed to some of the observed diversity. As short- and long-read WGS become increasingly available, their integration into public health responses may improve identification of plasmid outbreaks.

CPO outbreaks attributed to hospital wastewater plumbing have been increasingly reported, with sink drains being the most recognized reservoir.[25–28] Wastewater plumbing is readily contaminated with CPOs during patient care; the biofilm omnipresent in plumbing structures provides a fertile environment for plasmid exchange.[2,3] Although recovery of CPOs from wastewater plumbing does not indicate directionality of spread,[26] several factors increase the plausibility of CPO transmission from wastewater plumbing to patients at LTACH A. These include cleaning practices that disseminated contaminants from the sink basin to surrounding area, recovery of CPOs from the sink splash zone where supplies were stored, and control of transmission following improved adherence to sink hygiene. Additionally, risk factors identified in the cohort study, the presence of a feeding tube and receipt of oral vancomycin, were linked to observed sink hygiene gaps: storage of syringes for feeding tube flushes within the sink and swivette toilet splash zones and compounding of oral vancomycin adjacent to a pharmacy sink drain from which KPC-CRE was recovered. Although wastewater plumbing is hypothesized to have been the source of many transmissions, person-to-person CPO transmission also contributed, as

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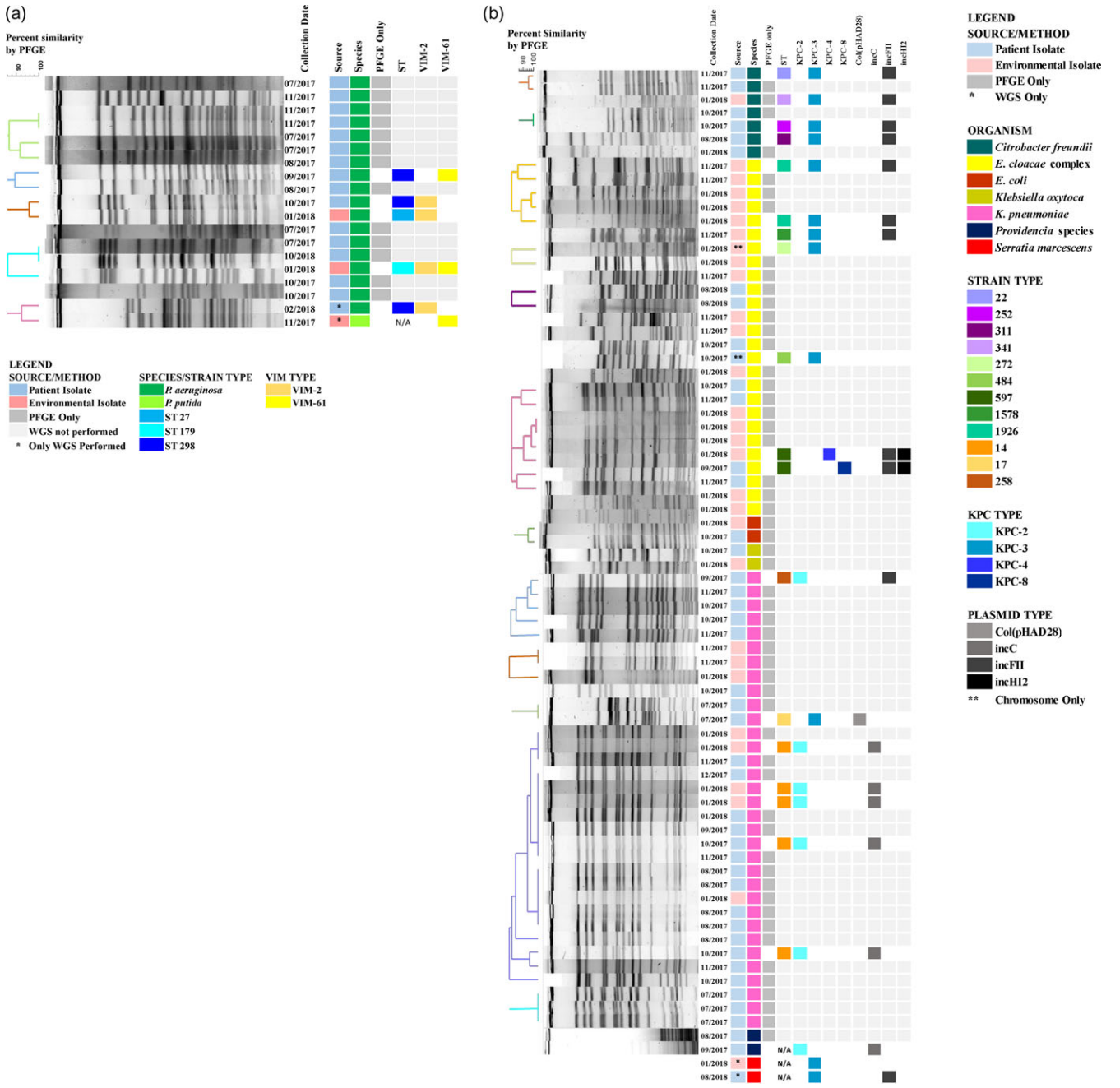

Fig. 3. Pulsed-field gel electrophoresis and whole-genome sequencing results for clinical and environmental isolates with (A) VIM-CRPA and (B) KPC-CRE detected at long-term acute-care hospital (LTACH) A, Florida, July 2017–December 2018.

demonstrated by some geographic clustering of cases (data not shown) and epidemiologic links to specialty care services (eg, hemodialysis), particularly early in the outbreak when adherence to hand hygiene and PPE use was low.

The index case was the first VIM-CRPA reported in Florida.[13] LTACH-A VIM-CRPA isolates, however, showed surprising diversity in alleles and strain types, indicating that VIM-CRPA may be more common in central Florida than previously recognized. Supporting this finding, ~1% of patients screened at admission carried bla_{VIM}. Furthermore, some patient and environmental isolates harbored an allele, $bla_{\text{VIM-61}}$, that appears to be unique to central Florida and has been identified in patients without epidemiologic linkages to LTACH-A. These findings emphasize the value of admission screening and collaboration with other healthcare facilities and public health partners to prevent further spread of CPOs in the central Florida region.

Our investigation had several limitations. First, we used paper procedure logs to identify exposure risk factors in the cohort study, and we were unable to evaluate some potential confounders, such as comorbid conditions and antibiotic receipt for noncases. For many cases, we were unable to conclusively determine whether they were acquired at LTACH A because they were identified before implementation of admission screening. Finally, we selected a subset of isolates representing varied PFGE patterns and specimen sources for WGS to infer isolate relatedness; however, it is possible that isolates with related PFGE patterns could harbor

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Table 4. Carbapenemase-Producing Organisms Detected from Environmental Samples Collected at Long-Term Acute-Care Hospital-A, Florida, November 2017 and January 2018[a]

Location/Site	Mechanism	Organism
ICU swivette toilet	bla_{VIM-61}	*Pseudomonas putida*
Patient room handwashing sink drain	bla_{VIM-2}, bla_{VIM-61}	*P. aeruginosa*
Patient room handwashing sink surface area	bla_{VIM-2}, bla_{VIM-48}	*P. aeruginosa*
ICU patient room handwashing sink drain	bla_{VIM}[a]	*P. putida*
ICU swivette toilet	bla_{KPC-3}	*Enterobacter asburiae*
Patient room handwashing sink drain	bla_{KPC-3}	*E. cloacae* *E. kobei* *E. asburiae*
Patient room handwashing sink drain	bla_{KPC-3}	*E. cloacae*
Patient room handwashing sink drain	bla_{KPC-3}	*Citrobacter freundii*
Patient room handwashing sink surface area	bla_{KPC-4}	*E.cloacae*
ICU patient room handwashing sink drain	bla_{KPC-3}	*E. asburiae*
Medication dispensing room sink drain	bla_{KPC-2}	*Klebsiella pneumoniae*
Pharmacy sink drain	bla_{KPC-3}	*Serratia marcescens*

Note. ICU, intensive care unit.
[a]All environmental samples collected from long-term acute care hospital-A are provided in Supplementary Table 1 (online). In total, 91 environmental samples were collected. Whole-genome sequencing was not performed.

different carbapenemase alleles or plasmid markers. Isolates with plasmids sharing the same replicon and carbapenemase allele could represent plasmids from different sources and may indicate evolution of plasmid genes during the outbreak or unique plasmids introduced to LTACH A.

Through epidemiologic and molecular investigations, we identified concurrent outbreaks of carbapenemase-producing organisms. The primary reservoirs and modes of transmission may have varied among the different alleles and organisms and at different stages of the outbreak. This investigation illustrates how sustained public health and healthcare facility collaboration can control spread of emerging resistance in high-acuity postacute care facilities.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/ice.2023.231

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Competing interests. All authors report no conflicts of interest relevant to this article.

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MICROBIOLOGY SOCIETY

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Genomic surveillance, characterization and intervention of a polymicrobial multidrug-resistant outbreak in critical care

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Abstract

Background. Infections caused by carbapenem-resistant *Acinetobacter baumannii* (CR-Ab) have become increasingly prevalent in clinical settings and often result in significant morbidity and mortality due to their multidrug resistance (MDR). Here we present an integrated whole-genome sequencing (WGS) response to a persistent CR-Ab outbreak in a Brisbane hospital between 2016–2018.

Methods. *A. baumannii, Klebsiella pneumoniae, Serratia marcescens* and *Pseudomonas aeruginosa* isolates were sequenced using the Illumina platform primarily to establish isolate relationships based on core-genome SNPs, MLST and antimicrobial resistance gene profiles. Representative isolates were selected for PacBio sequencing. Environmental metagenomic sequencing with Illumina was used to detect persistence of the outbreak strain in the hospital.

Results. In response to a suspected polymicrobial outbreak between May to August of 2016, 28 CR-Ab (and 21 other MDR Gram-negative bacilli) were collected from Intensive Care Unit and Burns Unit patients and sent for WGS with a 7 day turnaround time in clinical reporting. All CR-Ab were sequence type (ST)1050 (Pasteur ST2) and within 10 SNPs apart, indicative of an ongoing outbreak, and distinct from historical CR-Ab isolates from the same hospital. Possible transmission routes between patients were identified on the basis of CR-Ab and *K. pneumoniae* SNP profiles. Continued WGS surveillance between 2016 to 2018 enabled suspected outbreak cases to be refuted, but a resurgence of the outbreak CR-Ab mid-2018 in the Burns Unit prompted additional screening. Environmental metagenomic sequencing identified the hospital plumbing as a potential source. Replacement of the plumbing and routine drain maintenance resulted in rapid resolution of the secondary outbreak and significant risk reduction with no discernable transmission in the Burns Unit since.

Conclusion. We implemented a comprehensive WGS and metagenomics investigation that resolved a persistent CR-Ab outbreak in a critical care setting.

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Keywords: carbapenem resistance; *Acinetobacter baumannii*; CR-Ab; whole-genome sequencing; WGS; intensive care unit; burns ward; metagenomics; genomics; surveillance.

Abbreviations: CPE, carbapenemase-producing Enterobacteriaceae; CR-Ab, carbapenem-resistant Acinetobacter baumannii; ESBL, extended-spectrum beta-lactamase; GC, global clone; ICU, intensive care unit; LB, Luria-Bertani; MAG, metagenome assembled genome; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; PacBio SMRT, pacific biosciences single molecule real-time; PCR, polymerase chain reaction; PDR, pan-drug-resistant; SNP, single nucleotide polymorphism; ST, sequence type; WGS, whole genome sequencing; XDR, extensively-drug-resistant.

Complete genomes: PRJNA631347 and PRJNA631348. Isolate Illumina sequencing reads: PRJNA631491. Metagenomic Illumina sequencing reads: PRJNA631351.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Four supplementary tables, 11 supplementary figures and supplementary video are available with the online version of this article.

DATA SUMMARY

The datasets supporting the conclusions of this article are available in the short read archive (SRA) repository, under the following Bioprojects: the complete genomes for MS14413 (GenBank: CP054302.1) and MS14393 (GenBank: CP054303-CP054305) have been deposited under the Bioprojects PRJNA631347 and PRJNA631348, respectively. All isolate Illumina sequencing reads have been deposited under the Bioproject PRJNA631491. All metagenomic Illumina sequencing reads have been deposited under the Bioproject PRJNA631351.

Impact Statement

Infections with carbapenem-resistant *Acinetobacter baumannii* (CR-Ab) have a high morbidity and mortality in healthcare settings and can be difficult to treat due to limited susceptibility to available antimicrobials. Rigorous surveillance and intervention methods are necessary to combat its spread within hospitals, particularly among patients in critical care. DNA sequencing has become instrumental in the detection and tracking of bacteria in hospitals, but barriers still exist to its routine implementation. Timely reporting and appropriate communication of findings are important features for the success and integration of genomics in healthcare settings. Here we present a thorough investigation of an ongoing CR-Ab outbreak in a tertiary hospital in Brisbane, Australia, between 2016–2018. Continual analysis and timely communication between the genomics and infection control teams allowed for the eradication of both the initial outbreak and recurrent infections, which were traced back to the hospital plumbing. Continued interventions have resulted in significant risk reduction to patients, with no cases in critical care since 2018. Here we provide examples of our genomics reporting scheme (both the original and the adapted version after ongoing feedback) as well as an interactive visualization of the outbreak using the Healthcare-Associated Infections Visualization Tool (HAIviz; https://haiviz.beatsonlab.com/). We believe this work to be of broad interest to both researchers and clinicians alike.

INTRODUCTION

Hospital outbreaks of multidrug-resistant Gram-negative pathogens present great risk to patients and are costly [1, 2]. Whole-genome sequencing (WGS) has been proposed as an effective tool to support infection-control responses to emerging outbreaks within the healthcare environment, but barriers exist to the effective implementation into clinical practice [3].

Acinetobacter baumannii has emerged over recent decades as a major nosocomial pathogen [4]. Its capacity to develop or acquire resistance to multiple antibiotic classes, in addition to intrinsic resistance to desiccation and disinfectants, contributes to persistence of *A. baumannii* in the hospital environment [5, 6]. It has frequently been a cause of nosocomial outbreaks, particularly in the critical care setting [7–9]. *A. baumannii* are often resistant to multiple antibiotic classes and the global incidence of extensively-drug-resistant (XDR) or even pan-drug-resistant (PDR) strains have been increasing [10–12]. Carbapenem-resistant *A. baumannii* (CR-Ab) have been seen at high prevalence in several areas, particularly in the Asian-Pacific region, Latin America and the Mediterranean [13]. Carbapenem resistance in *A. baumannii* usually arises from the acquisition of genes encoding carbapenemases, particularly OXA-type carbapenemases (e.g. OXA-23), and may be associated with high mortality in vulnerable patients [14].

Here we describe a large outbreak of CR-Ab, and other co-infecting MDR Gram-negative pathogens, occurring within an Intensive Care Unit (ICU) and burns facility. Incorporation of WGS in real-time facilitated rapid characterisation of this complex polymicrobial outbreak, provided a detailed understanding of transmission pathways and helped to direct a successful infection control response.

METHODS

Study setting and patient inclusion

Primary isolates were obtained from patients admitted to the Royal Brisbane and Women's Hospital (RBWH), a tertiary referral hospital with 929 beds in South-East Queensland, Australia. The RBWH has a 36 bed ICU providing highly specialist burns care for all of Queensland. The incidence of CR-Ab is low in Australian hospitals [15]. All new CR-Ab strains are routinely stored in the clinical laboratory for future reference. For the outbreak investigation, any patient admitted to the RBWH who cultured CR-Ab from any clinical or screening specimen from May to August 2016 was identified as a case and included in the primary outbreak analysis. Any CR-Ab cases during the outbreak period were also included to determine if plasmid-mediated resistance and dissemination was relevant, with any MDR Gram-negative bacilli (including ESBL-producing *K. pneumoniae*, carbapenem-resistant *S. marcescens* or carbapenem-resistant *P. aeruginosa*) prospectively collected for further genomic analysis. Overall these included 28 CR-Ab, three carbapenem-sensitive *A. baumannii*, ten *K. pneumoniae*, seven *P. aeruginosa*, four *S. marcescens* and three *Enterobacter cloacae* (the *E. cloacae* were isolated in relation to a previous outbreak in the same hospital [16]). Stored CR-Ab isolates from a previous outbreak in 2006 [6], as well as other sporadic cases imported from overseas to the RBWH during 2015/2016 (prior to the outbreak) were included for further analysis. These included 17 historical CR-Ab isolates from earlier in 2016 (n=3), 2015 (n=2) and between 2000–2006 (n=12). *A. baumannii* identified from the outbreak until mid-2018 were also included in the analysis during continued surveillance and infection control monitoring. These included three carbapenem-sensitive

A. baumannii and 19 CR-Ab isolates. A complete list of all isolates is provided in File S2 (available in the online version of this article).

Antimicrobial susceptibility testing

All bacterial isolates were identified by MALDI-TOF (Vitek MS; bioMérieux, France). Antimicrobial susceptibility testing was carried out using Vitek 2 automated AST-N426 card (bioMérieux). For the first eight sequential CR-Ab isolates, additional susceptibility testing was undertaken using Etest to determine MICs for meropenem, imipenem, colistin, tigecycline, fosfomycin, amikacin, sulbactam, doxycyline and ceftolozane/tazobactam, with disc diffusion to determine susceptibility to aztreonam and ceftazidime/avibactam. Additional colistin testing was carried out on suspected colistin-resistant isolates using broth microdilution via the MICRONAUT MIC-Strip Colistin (Merlin Diagnostika GmbH). Carbapenemase activity was assessed by the use of the Carba-NP test (RAPIDEC; bioMérieux) and screened for the presence of common carbapenemases found in Enterobacteriaceae using an in-house multiplex real-time PCR (that targets NDM, IMP-4-like, KPC, VIM and OXA-48-like carbapenemases). Once it became clear that all the outbreak strains had an identical antibiogram, susceptibility testing was confined to the Vitek 2 automated AST-N426 panel with MICs to tigecycline, doxycycline and colistin determined by Etest (as the only susceptible agents).

Bacterial culturing and genomic DNA extraction

All isolates were grown on horse blood agar at 37 °C overnight. For all historical and outbreak isolates collected between May–September of 2016, colonies were scraped from plates and resuspended in 5 ml Luria–Bertani (LB) broth. Then, 1.8 ml of resuspension was used for DNA extraction using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories) as per the manufacturer's instructions. All isolates collected after September 2016 were extracted using the DSP DNA Mini Kit on the QIAsymphony SP (Qiagen).

Isolate whole genome sequencing

Illumina WGS of suspected outbreak patient isolates and historical CR-Ab isolates was performed in four batches of between 10 and 18 samples between June and August 2016 at the Australian Centre for Ecogenomics (ACE), The University of Queensland (see Methods in the Supplementary Material). One CR-Ab isolate (MS14413) and one *K. pneumoniae* isolate (MS14393) were selected for sequencing with Pacific Biosciences (PacBio) Single Molecule Real-Time (SMRT) sequencing on an RSII machine (see Methods in the Supplementary Material). Subsequent Illumina WGS was carried out at Queensland Forensic Scientific Services (QFSS) (see Methods in the Supplementary Material).

Quality control and assembly of WGS data

Illumina raw reads were checked for contamination using Kraken [17] v0.10.5-beta and quality using FastQC v0.11.5 (www.bioinformatics.babraham.ac.uk/projects/). Raw reads were filtered for reads less than 80 bp and quality score less than five using Nesoni clip v0.130 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni). Some reads required further hard trimming with Nesoni clip (10 bp from start, 40 bp from end). Isolates were assembled using SPAdes [18] v3.6.0 at default settings. Contigs less than 10× coverage were removed using a custom script. Assembly metrics were checked for quality using Quast [19] v4.3 (see File S2). Details of the PacBio genome assembly and annotation can be found in Methods in the Supplementary Material (Fig. S1, Table S1).

Genomic analysis and clinical reporting

Between June and August 2016, four reports of detailed bioinformatic analyses were prepared in response to available Illumina data for *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *S. marcescens* and *Enterobacter cloacae* patient isolates. Comparative genome analysis using variant calling, phylogenetic reconstruction, transmission pathway prediction, MLST resistance gene prediction and plasmid characterization used in the clinical reports are given in Methods in the Supplementary Material. For subsequent analyses of the final genome dataset updated or alternative software was used as described below.

Core SNPs were identified using Snippy [20] (v4.3.6) at default settings and trimmed reads against the complete chromosomes for MS14413 (CR-Ab) and MS14393 (*K. pneumoniae*). Parsnp (v1.2) (at default with '-c' flag) was used to visualize phylogenetic relatedness between the outbreak CR-Ab and the historical *A. baumannii* isolates. MLST was performed using mlst [21] v2.6 (https://github.com/tseemann/mlst) against the draft assemblies. Both the Oxford [22] and Pasteur [23] MLST schemes were used for the CR-Ab isolates. Resistance genes were identified using Abricate [24] v0.6 against the ResFinder database [25] (accessed 18 August 2017). Abricate was also used to determine plasmid types using the PlasmidFinder database [26] (accessed 18 August 2017). Comparative analyses were completed using the Artemis Genome browser and the Artemic Comparison Tool (ACT). Figures were constructed using EasyFig [27], BRIG [28] and FigTree [29]. The capsular polysaccharide (K) and lipooligosaccharide outer core (OCL) locus for *A. baumannii* were typed using Kaptive v0.5.1 [30] against the *A. baumannii* databases provided [31] (accessed 16 Dec 2020).

Metagenomic sequencing and analysis

Metagenomic sequencing of environmental samples and analysis was conducted as described previously [16]. Briefly, swab and water samples from the ICU and Burns Unit were collected in July 2018. DNA was extracted using the Qiagen DNeasy Powersoil extraction kit and sequenced at the Australian Centre for Ecogenomics on an Illumina NextSeq500. Metagenomic sequencing data was used to screen for evidence of the current *A. baumannii* outbreak strain, as well as a previously identified *Enterobacter hormaechei* strain responsible for an outbreak in the same ICU in 2015 (described in [16]).

All samples were screened for species using Kraken [17] v1.0 and resistance genes using SRST2 [32] v0.2.0 against the ARG-ANNOT [33] database. Mash [34] v1.1.1 was used at default settings to screen Illumina reads for each samples against our reference CR-Ab sketch (MS14413). Samples that shared ≥90% of hashes were mapped to the reference sequence. Mapped reads were parsed and *de novo* assembled using SPAdes [18] v3.11.1 for MLST analysis using mlst [21] v2.16.2 and nucleotide comparison using ACT [35] and BRIG [28].

Risk reduction assessment

We aimed to estimate the reduced risk of patient colonization following the identification of ST1050 CR-Ab by environmental metagenomic sequencing and the initiation of enhanced decontamination of hospital plumbing. The incidence rate of CR-Ab was measured pre-intervention and post-intervention. The point of intervention was defined as the targeted initiation of routine plumbing maintenance programme within the Burns and Intensive Care units in August 2018. The intervention was expected to generate immediate results with no lag time. The pre-intervention period was defined as May 2016 to August 2018 and post-intervention period as September 2018 to May 2020. All CR-Ab cases recorded in the hospital during these periods were included. Patients admitted to the Burns and Intensive Care units underwent standard clinical swabbing for surveillance and laboratory method for testing did not change over the study period. Statistical analyses were performed on Rv3.5.1.

RESULTS

Case study

A 25-year-old patient with extensive burn injuries was retrieved from an overseas healthcare facility. As per infection-control protocols, the patient was placed on contact precautions and provided a single room. Initial nasal and rectal screening swabs were negative for MDR pathogens, including CR-Ab. An extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* was isolated from the patient's respiratory secretions on day 4, and within 24 h a similar organism was isolated from blood cultures. Repeated collection of blood cultures demonstrated a polymicrobial culture with ESBL-producing *K. pneumoniae*, CR-Ab and *Pseudomonas aeruginosa* on day 6, that tested susceptible to all first-line agents. Over the following days, CR-Ab was also isolated from numerous clinical specimens, including a femoral line tip, endotracheal aspirates, rectal swabs, wound swabs and operative specimens collected from debrided tissue. Blood cultures repeatedly grew CR-Ab, (day 15 and 45 of admission), with the emergence of colistin resistance when tested by Etest (MIC 32 μg ml^{-1}) on day 45. *Serratia marcescens* was co-cultured in blood on day 15 and was also grown from respiratory secretions and wounds swabs.

Over the next 5 months in 2016, 18 additional patients within the same ICU area were also found to be colonized or infected with phenotypically similar CR-Ab, *K. pneumoniae*, *S. marcescens* and/or *P. aeruginosa*. This included CR-Ab colonized cases identified in patients discharged from the ICU to the Burns Unit or other surgical wards throughout the hospital, and eventually patients admitted to the Burns Unit. The final CR-Ab case was identified several weeks later in a patient discharged from the Burns Unit and transferred to a hospital in a remote part of Queensland. An outbreak investigation team was constituted as soon as it was suspected that an outbreak of CR-Ab had occurred within the ICU and the use of WGS for strain characterization was initiated.

WGS predicted likely transmission pathways and ruled out non-outbreak cases

Between May to August 2016, a total of 55 isolates were recovered from 22 patients (see File S2). These isolates included *A. baumannii*, *K. pneumoniae*, *S. marcescens, E. cloacae* and *P. aeruginosa*. Species typing and antibiogram analysis alone were insufficient to determine clonal relationships between these isolates. As such, we used WGS to establish the relationship between isolates and predict patient transmission based on SNP accumulation.

We applied WGS in real-time over the course of the outbreak. Four reports aimed at communicating genomic analyses to infection control and other clinical staff at RBWH were delivered during the primary outbreak (22 June, 15 July, 2 August and 29 August). We managed on average a 1 week turnaround time between receiving the isolates and presenting a finalized report, which consisted of (i) a front-page overview of the analysis and key outcomes/interpretations conveyed as short bullet points, (ii) detailed analysis and diagrams on the internal pages, and (iii) method descriptions (see Methods in Supplementary Material). Actual time between receipt of sequencing data and reporting was 8–72 h depending on the complexity of analyses with supplementary interim reports and regular academic-clinical partner meetings necessary to communicate our comparative genomic analyses and help shape the content of the final reports (see File S1 for example reports from 22 June and 29 August, respectively). The Hospital-Acquired Infections Visualization tool (HAIviz) was also developed alongside analysis of this outbreak and was used to interactively display linked cases throughout the hospital wards [File S3 (video)].

The presumed index patient admitted in early May 2016 was identified with ST1050 (Pasteur ST2) CR-Ab, ST515 *K. pneumoniae*, ST979 *P. aeruginosa* and *S. marcescens*. Using WGS, we found that 16 of the 21 patients admitted following the index patient had bacterial infections related to either the ST1050 CR-Ab or the ST515 *K. pneumoniae*. Transmission direction based on the accumulation of SNPs was inferred in patients 10, 11, 13, 14, 15, 16 and 17 (Fig. 1a, as indicated by lines with arrows). CR-Ab isolates from the first nine patients (and patient 12) were identical based on core SNPs, making inference of patient transmission impossible using SNPs alone. However, when combined with SNP information from *K. pneumoniae* isolates, it was possible to

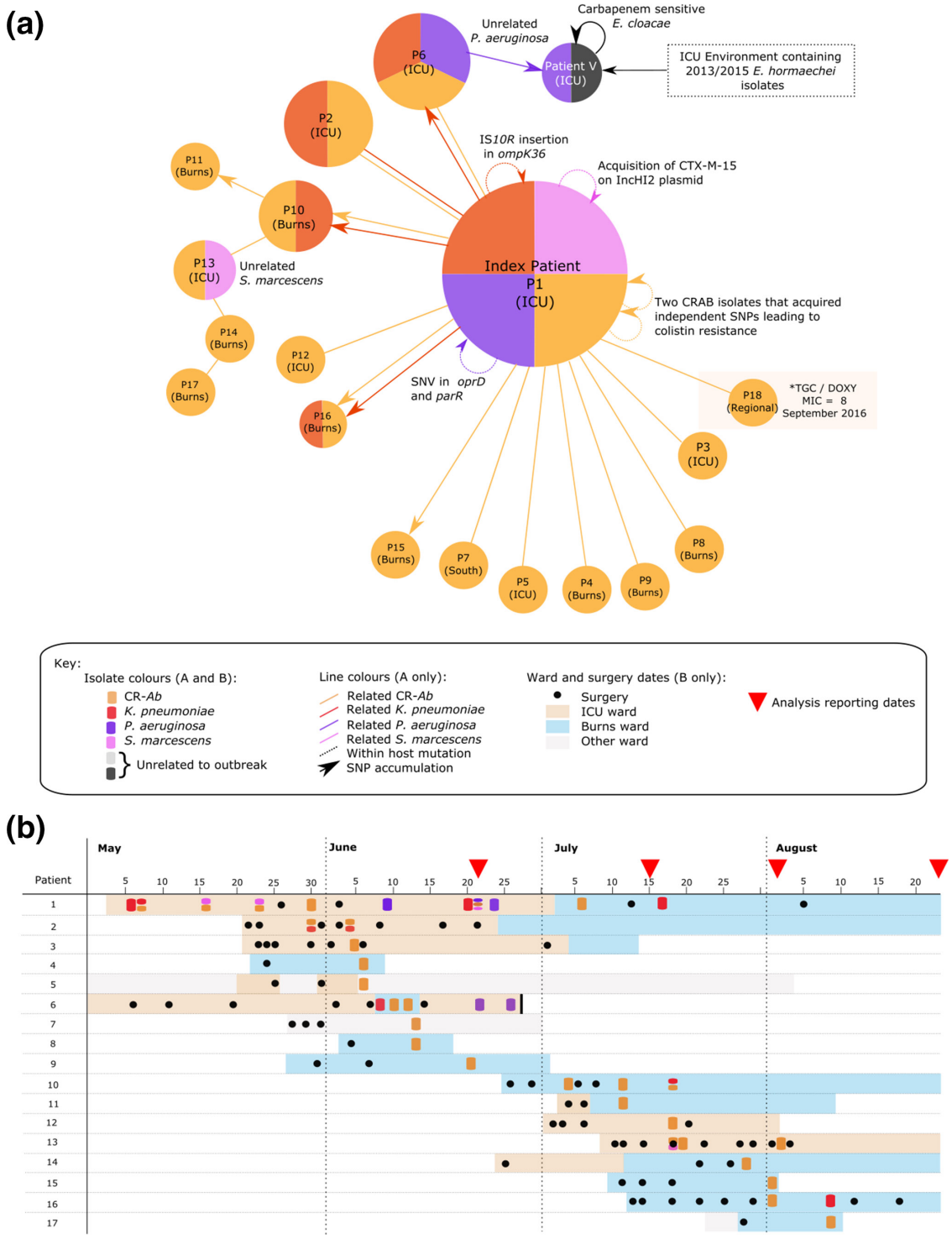

Fig. 1. Patient relationship matrix describing 2016 outbreak of CR-Ab: (a) Each circle represents a patient, where the size of the circle correlates to the number of isolates from that patient. Colours correspond to bacterial species. Straight lines connecting circles represent patients with identical isolates (with the colour of the line indicating the specific species) at the core-genome level (and as such directionality of transmission cannot be inferred). Lines with arrows (also coloured by species) represent predicted direction of transmission based on the accumulation of SNPs between patients' isolates. Circular arrows represent changes in individual patient's isolates, (b) timeline of patient samples, as well as location and surgery dates.

infer co-transmission of *K. pneumoniae* and CR-Ab from the index patient to patient 6 (Fig. 1).

Strains of *S. marcescens* and *P. aeruginosa* specific to the index patient were not found in other patients (see Results in Supplementary Material). Two patients had unrelated *S. marcescens* and *P. aeruginosa* isolates (patients 13 and 6, respectively). Transmission of the unrelated *P. aeruginosa* isolate from patient 6 to another patient in the ICU ward (denoted patient V) was detected. Patient V was also found to have an *Enterobacter cloacae* isolate (later identified as *Enterobacter hormaechei* by WGS) identical to that identified in a 2015 outbreak from the same hospital [16]. This patient also carried an additional carbapenem-sensitive *E. cloacae* (*bla*IMP-4 negative) that was unrelated to the carbapenem-resistant isolate.

Over the course of the outbreak, each species carried by the index patient acquired additional antibiotic resistance mechanisms, via mutations (*pmrB* mutations in CR-Ab, IS*10R* insertions in *ompK36* in *K. pneumoniae, oprD* nonsense mutation in *P. aeruginosa*) or plasmid gain (IncHI2 in *S. marcescens*) (Fig. 1, Results in Supplementary Material and Tables S2 and S3).

An additional CR-Ab was isolated in September 2016 from a patient in a Regional Queensland (QLD) hospital who had previously been admitted to the Brisbane ICU (patient 18, isolate MS14438). Analysis of this isolate found that it was closely related to isolates from the initial outbreak between May to August 2016.

Extensive environmental swabbing throughout the ICU and Burns Unit was conducted on the 16 June 2016, targeting patient bedrooms as well as high-touch areas (e.g. nurse keyboards, trolleys, door handles). However, no bacterial species related to the CR-Ab outbreak were detected in the environment based on traditional culture methods using chromogenic agar.

The outbreak CR-Ab was likely imported into the hospital ICU

In total, 29 CR-Ab isolates related to the ongoing outbreak were collected from 18 patients between May–September 2016. All were found to be ST1050 (Pasteur ST2; global clone [GC] 2) and less than ten SNPs different (Fig. S2). Three carbapenem-sensitive *A. baumannii* isolated at the same time were found to be different sequence types and unrelated to the outbreak. Comparison of the outbreak ST1050 CR-Ab isolates to historical CR-Ab isolates collected between 2000–2016 from the hospital found no close relationship, indicating that the CR-Ab had likely been introduced into the hospital with the index patient (Fig. S3).

All ST1050 CR-Ab isolates related to the index were found to be extensively resistant to carbapenems, β-lactams, cephalosporins, aminoglycosides and quinolones (Table 1). Resistance to colistin appeared in three isolates from the index patient and was mediated by two independent SNP acquisitions in the sensor kinase gene *pmrB* (causing the amino acid changes T235I in MS14413 and its descendant MS14402, and R263C in MS14407). These SNP corresponded to MICs of 8, 16 and 64 μg ml^{-1} for MS14402, MS14413 and MS14407, respectively, by broth microdilution. Antibiotic resistance genes were conserved between all isolates, and included β-lactamases (such as $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-66}}$), streptomycin resistance genes (*strA* and *strB*), and aminoglycoside resistance genes [*aph(3′)-Ic*, *aadA1* and the methylase *armA*]. Finally, a single SNP was found to result in the reversion of a nonsense mutation in a putative type 3 filamentous fimbriae gene (*filB*). This SNP was identified in the majority of CR-Ab isolates taken after the 4 July 2016 and appears to have arisen independently multiple times across the *A. baumannii* lineage (Results in Supplementary Material, Fig. S4).

PacBio sequencing of CR-Ab reveals context of resistance genes and mobile elements

Complete sequencing of a reference CR-Ab isolate (MS14413) from the index patient using long-read sequencing provided a high-quality reference and allowed contextualization of the antibiotic resistance genes (as well as other mobile genetic elements) within the genome. Assembly of the ST1050 CR-Ab reference genome revealed a 4082498 bp chromosome with no plasmids. *StrA, strB* and *sul2* resided within a novel AbGRI1 resistance island most closely related to the *A. baumannii* strain CBA7 (GenBank:NZ_CP020586.1) isolated from Korea in 2017, both of which lacked the *tetA-tetR* genes commonly found in AbGRI1 (Fig. S5). The CR-Ab isolates also carried Tn6279 (also known as AbGRI3-2), which encompassed a large number of resistance genes including *mph(E)* and *msr(E)* (macrolide resistance) and the methylase gene *armA* (gentamicin resistance) (Fig. S6). Resistance to carbapenems in these CR-Ab isolates was likely driven by the presence of three copies of $bla_{\text{OXA-23}}$ residing in separate Tn2006 transposons within the chromosome (two copies proximal to the capsule region, and the third interrupting a diguanylate cyclase gene, which has previously been implicated in biofilm formation [36]). An IS*Aba1* insertion sequence upstream of the chromosomal *ampC* gene was also detected, which has previously been shown to enhance cephalosporin resistance [37]. Additionally, an IS*Aba125* element was identified upstream of the *csu* operon, which is a well-characterized chaperone-usher pili assembly system involved in biofilm formation [38].

Long-read sequencing revealed an OCL1 oligosaccharide outer core and a KL12 capsule (K) locus, which shares 97% nucleotide identity to the capsule region found in the GC1 *A. baumannii* strain D36 (GenBank:NZ_CP012952.1) (Fig. S7). However, the *wzy* gene (a polymerase required for capsular polysaccharide biosynthesis) within the capsule locus was interrupted by an IS*Aba125* insertion sequence in all CR-Ab isolates. Further comparative analysis found a portion of the capsule locus in MS14413 to share 99% nucleotide identity to the capsule from *A. baumannii* strain BAL_097 (GenBank: KX712116), which carries a *wzy* gene at the beginning of the capsule region. This unusual gene placement also appears in MS14413, and likely complements the loss of the internal *wzy*

Table 1. CR-Ab MICs and AB resistance genes: table only shows select representative isolates as all CR-Ab were found to have the same AB resistance gene profile and MIC data. Colours represent mechanism of detection: blue, Etest MIC; Green, Disk diffusion zone diameter; Orange, Vitek2; Grey, Resfinder (accessed August 2017). *A. baumannii* is intrinsically resistant to penicillin and cephalosporins [50]

Strain		MS8413	MS8419	MS8436	MS8442	MS8441
Patient		4	5	6	7	8
Site		Leg wound	ETA	Tissue buttock	Wound Swab	Rectal Swab
Colistin	Colistin	0.125	0.25	0.25	0.125	0.5
Carbapenem	Mero	32	>32	>32	>32	>32
	Imi	>32	>32	N.T	>32	N.T
	Erta	>32	>32	>32	>32	>32
Beta-lactam and Cephalosporins	Sulb	32	32	64	32	64
	MER	R	R	R	R	R
	TIM	R	R	R	R	R
	TAZ	R	R	R	R	R
	CRO	R	R	R	R	R
	CAZ	R	R	R	R	R
	FEP	R	R	R	R	R
	KZ	R	R	R	R	R
	Azt	6 mm R	6 mm R	N.T	6 mm R	N.T
	CTZ/TAZ	>256	>256	128	16	96
	CAZ/AVI	16 mm R	17 mm R	18 mm R	15 mm R	18 mm R
	blaADC-25	+	+	+	+	+
	blaOXA-23	+	+	+	+	+
	blaOXA-66	+	+	+	+	+
Aminoglycosides	Amikacin	>256	>256	>256	>256	>256
	GENT	R	R	R	R	R
	TOB	R	R	R	R	R
	Aph(3′)-Ic-1	+	+	+	+	+
	aadA1	+	+	+	+	+
	armA	+	+	+	+	+
Quinolones	CIP	R	R	R	R	R
	NOR	R	R	R	R	R
Trimmethoprim/ Sulphonamide	TMP	R	R	R	R	R
	SXT	R	R	R	R	R
	Sul1	+	+	+	+	+
	Sul2	+	+	+	+	+
Tigecycline	Tige	2	2	2	2	4
Chloramphenicol	Chloro	6 mm R	6 mm R	N.T	6 mm R	N.T
	catB8	+	+	+	+	+
Fosfomycin	Fosfo	256	512	N.T	128	N.T

Continued

Table 1. Continued

Strain		MS8413	MS8419	MS8436	MS8442	MS8441
Tetracycline	Doxy	4	4	2	2	2
Macrolides	mph(E)_3	+	+	+	+	+
	msr(E)_4	+	+	+	+	+
Streptomycin	strA	+	+	+	+	+
	strB	+	+	+	+	+

N.T, not tested; Mero, Meropenem; Tige, Tigecycline; Sulb, Sulbactam; CTZ/TAZ, Ceftolozane/tazobactam; CAZ/AVI, Ceftazidime/avibactam; Chloro, Chloramphenicol; Fosfo, Fosfomycin; Azt, Aztreonam; Erta, Ertapenem; Doxy, Doxycycline; Imi, Imipenem; KZ, Cephazolin; TMP, Trimethoprim; SXT, Co-trimoxazole; GENT, Gentamicin; TOB, Tobramycin; CRO, Ceftriaxone; CAZ, Ceftazidime; FEP, Cefepime; TAZ, Piperacillin/tazobactam; CIP, Ciprofloxacin; NOR, Norfloxacin; MER, Meropenem; TIM, Ticarcillin/clavulanate.

gene (Fig. 2). The high nucleotide identity at this region also indicates possible recombination.

Overlapping the capsule (K) region in MS14413 is a large 41375 kb tandem duplication, encompassing two copies of Tn*2006* (Fig. 2). Analysis of the other CR-Ab isolates using the Illumina *de novo* assemblies found evidence for this duplication in only one other related colistin-resistant isolates from the index patient (MS14402), suggesting that this duplication arose once and was maintained by a sub-population of CR-Ab within this patient for at least 36 days.

Transmission of *K. pneumoniae* parallel to CR-Ab transmission

Ten ESBL-producing *K. pneumoniae* isolates were collected from five patients during the outbreak and were all found to be ST515. Nine of the ten isolates differed by less than ten core SNPs, indicating direct transmission within the ICU ward (Fig. S8). A single isolate from the index patient (MS14418) was found to have an additional 61 core SNPs, consistent with a hypermutator phenotype. Further investigation of this isolate found an in-frame 9 bp deletion in *mutH*, resulting in the loss of 3 amino acids from this protein (Fig. S9).

All ESBL-positive *K. pneumoniae* isolates had identical antibiotic resistance gene profiles, including the ESBL gene $bla_{\text{CTX-M-15}}$, other β-lactamases (bla_{TEM}, $bla_{\text{OXA-1}}$) and the aminoglycoside resistance gene *aac(6′)Ib-cr* (Fig. S6). Two isolates from the index patient (MS14393 and MS14418) developed resistance to carbapenems, which was likely due to an IS*10R* insertion in the outer membrane porin gene *ompK36* (Fig. S8). Isolate MS14433 (from patient 16) also contained an IS*10R* inserted into *ompK36*, however the insertion was found to be close to the 5′ boundary of the *ompK36* gene and based on *in silico* analysis there was no evidence that it affected the function of the resulting protein. Isolate MS14393 (from the

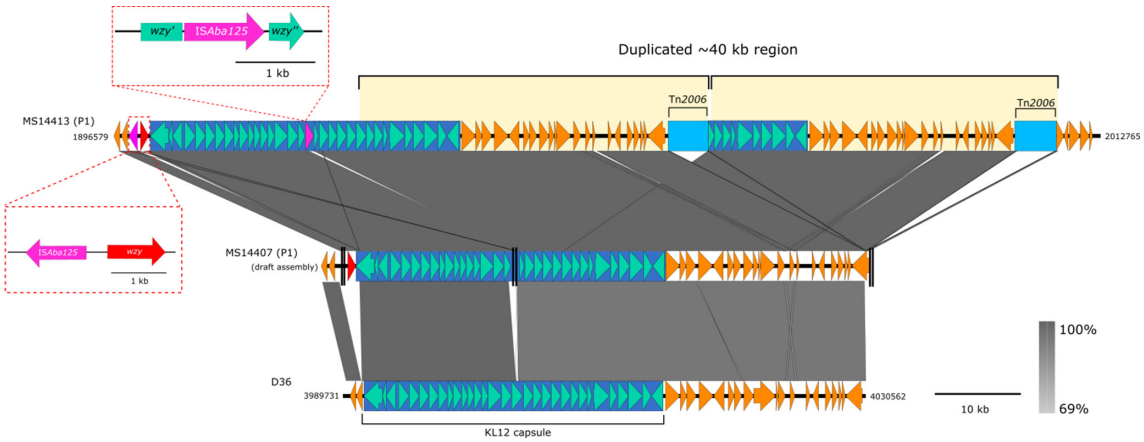

Fig. 2. Large ~40 kb tandem duplication found in MS14413: Duplication of part of the capsule (k) region in the MS14413 complete genome (top line), resulting in three chromosomal copies of Tn*2006* (third copy at alternate locus). This duplication appears to have arisen in some of the index patient isolates, but not other isolates involved in the outbreak (e.g. MS14407 concatenated draft genome, central line; vertical double black lines represent contig break in the draft assembly, presumed to be caused by the same IS as in MS14413). The *wzy* gene in the capsule region was found to be interrupted by an IS*Aba125* element, however a secondary *wzy* gene was identified at the start of the capsule region. Neither the IS*Aba125* insertion or secondary *wzy* gene is found in the KL12 capsule locus of *A. baumannii* strain D36 (bottom line).

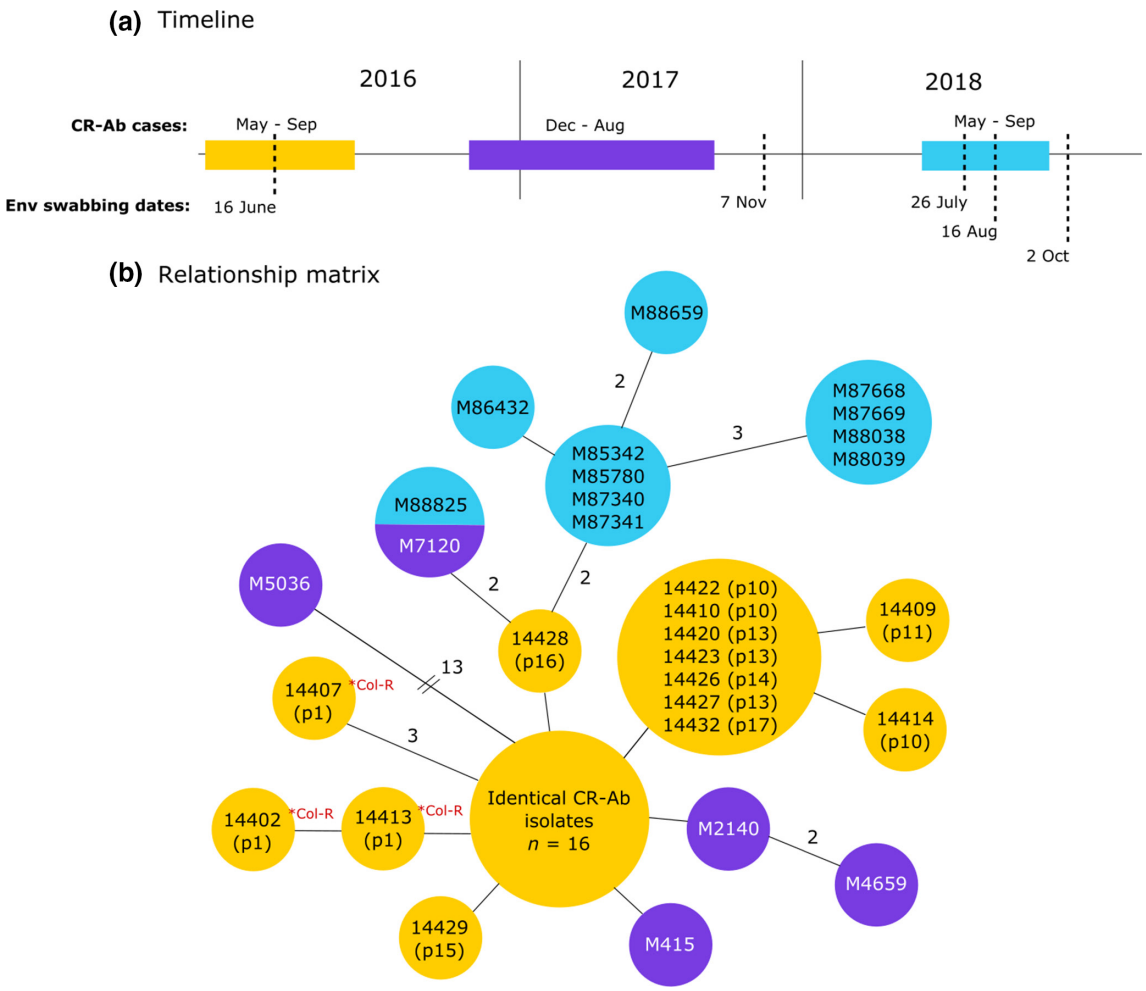

Fig. 3. Ongoing CR-*Ab* surveillance from 2016 to 2018: (a) timeline of CR-Ab cases and dates of environmental swabbing between 2016–2018. (b) Relationship matrix of all CR-Ab isolates related to the initial outbreak. Col-R=predicted colistin resistance via mutation in *pmrB*. Isolates within the same circle are identical at the core genome. Branches represent 1 SNP difference (except where specified). Isolates from the original 2016 outbreak are in yellow. Purple isolates were collected in late 2016–2017. Isolates in blue were collected in 2018. Isolate M88825 was isolated from an Antechamber environment in 2018 and found to be identical at the core SNP level to M7120, isolated in August 2017.

index patient) also possessed a nonsense mutation in the antibiotic resistance protein repressor gene *marR*, which could contribute to its overall resistance to antibiotics.

A single *K. pneumoniae* isolate from the index patient (MS14393) was sequenced using PacBio long-read sequencing to generate a high-quality reference genome, consisting of a 5492431 bp chromosome, a 216803 bp IncF plasmid (pMS14393A), and a 125232 bp IncA/C plasmid (pMS14393B). Most of the antibiotic resistance genes resided on the IncA/C plasmid in two main loci (Fig. S6). The larger IncF plasmid did not contain any antibiotic resistance genes, but did harbour several heavy metal resistance operons, including resistance to copper, arsenic and mercury (Fig. S10). Comparison of the short-read assemblies to both plasmids confirmed that all ten *K. pneumoniae* isolates retained both plasmids.

Whole-genome shotgun metagenomics detects CR-Ab in hospital environment

Ongoing surveillance was conducted using WGS following the initial outbreak. Despite continual environmental cleaning and routine swabbing, the outbreak CR-Ab strain persisted through to September 2018 (Fig. 3). Swabs collected from surfaces within the ICU and Burns Unit (e.g. handles, tables, shelves, computer equipment) in 2016 and 2017 were unable to detect CR-Ab in the environment and did not yield enough DNA for direct metagenomic sequencing (data not shown).

Due to 11 new cases of CR-Ab detected between May to September 2018, additional environmental sampling was carried out in the Burns ward environment. Between July to October 2018, areas of presumed high bacterial load (such as floor drains, plumbing, inside burns bath drains etc) were

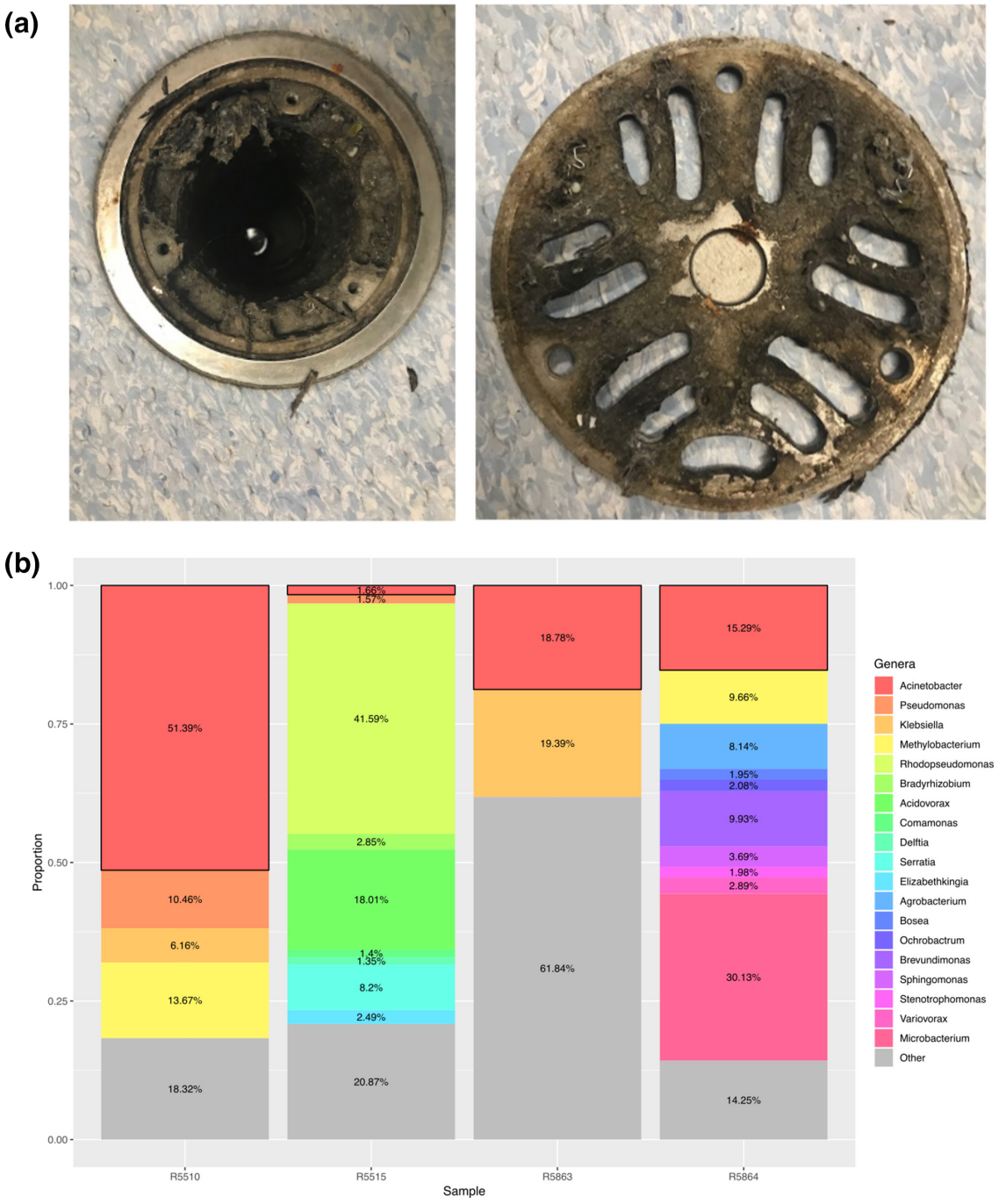

Fig. 4. Burns bath 3 floor trap and metagenomic read abundance profiles: (a) an example of the biomass uncovered under the floor trap in a Burns Unit bathroom. Areas of high biomass (such as this one) were targeted for environmental screening. (b) Each column shows the relative abundance of paired-end reads for each environmental sample that were classified at a bacterial genus level by comparing against a database of bacterial genomes from RefSeq. Only bacterial genera with a relative abundance >0.5% are shown as distinct. Genera with an abundance of <0.5% are grouped together as 'Other' (grey). Boxes outlined in black represent abundance of '*Acinetobacter*'.

targeted for environmental sampling (Fig. 4). All samples were subjected to culture using traditional methods (on chromogenic media) and direct DNA extraction and shotgun metagenomic sequencing. Of 50 environmental samples, two were culture positive for CR-Ab (R5666 and R5864), while four were positive based on analysis of the metagenomic sequencing data (R5515, R5510, R5863 and R5864) (Table S4, Fig. 4).

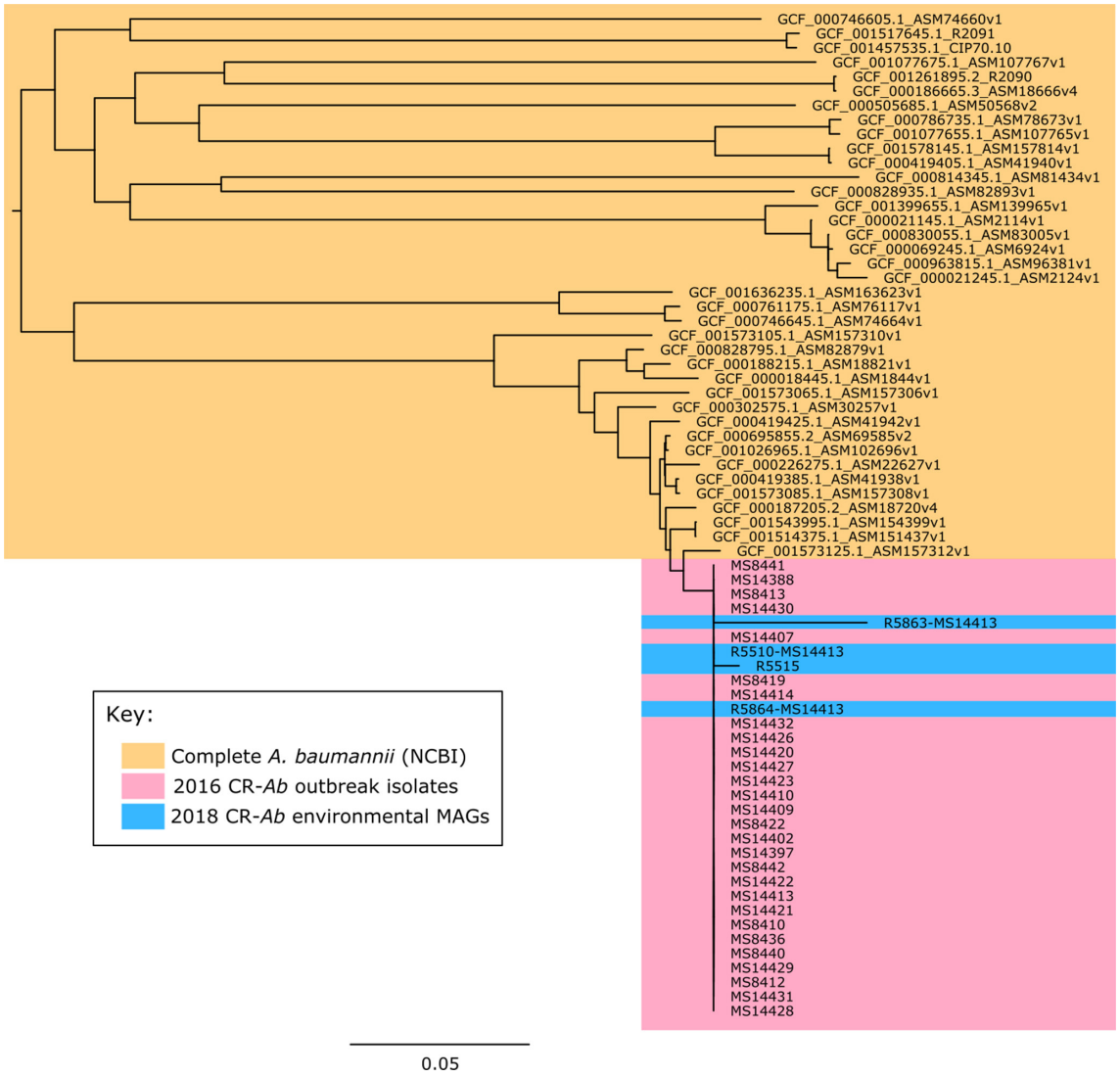

Fig. 5. Clustering of MAGs with outbreak strains: Mid-point rooted core genome SNP phylogenetic tree contextualizing the metagenome assembled genomes (MAGs) with *de novo* assemblies of the outbreak strains and publicly available complete *A. baumannii* genomes (yellow) showing clustering of the MAGs (blue) within the outbreak clade (pink).

An ST1050 CR-Ab was cultured using traditional methods from the environmental sample R5666, taken from a crack in a toilet seat being used by a patient colonized with the ST1050 CR-Ab. The depth of sequencing obtained from the same environmental sample, however, was not sensitive enough to be able to confidently detect the presence of the CR-Ab in the metagenomic data. The second positive ST1050 CR-Ab culture came from an environmental sample taken from an Antechamber room connected to patient rooms that had previously been colonized with ST1050 CR-Ab (R5864). Parallel metagenomic sequencing was also able to detect this same ST1050 CR-Ab in the environmental sample (Fig. 5).

Additionally, three other samples were found to have ST1050 CR-Ab based on metagenomic sequencing, despite being culture negative using traditional methods (Fig. 5, Table S4). Samples R5515 (burns bath 2 floor trap water sample) and R5510 (burns bath 2 bath drain hole [interior]) were both positive for ST1050 CR-Ab. Both samples were taken at the same time from proximal locations, and patients colonized with ST1050 CR-Ab were using the burns bath in question. Samples R5863 was also positive for ST1050 CR-Ab, and was taken from the room previously occupied by a patient known to be colonized with ST1050 CR-Ab.

Plumbing maintenance programme implemented in response to genomic investigation

Shotgun metagenomic detection of the outbreak strain in the hospital plumbing provided the evidence base for implementation of a sustainable infection prevention strategy. Consequently, a routine plumbing maintenance programme

was instituted. Every month, pipes were soaked for 30 min in sodium hydroxide, with additional soaking and scrubbing of drain plates. Since the implementation of these measures, no further cases of CR-Ab have been detected in the Burns Unit or Intensive Care Unit (ICU) following 28 September 2018. Periodic environmental surveillance of CR-Ab in drains and plumbing in the Burns unit has been ongoing as of May 2020.

Significant reduction of risk following interventions

A total of 32 CR-Ab cases were recorded over 28 months in the pre-intervention period, compared to four CR-Ab cases over 21 months in the post-intervention period. All cases identified at pre-intervention period were admitted to the Burns or Intensive Care Units during their hospitalization. Conversely, three out of the four CR-Ab cases detected in the post-intervention period had no obvious epidemiological link to exposure in the Burns and Intensive Care Units. The fourth case attended an external wound clinic, which was also attended by RBWH patients, although no specific patient or environmental link was proven. The last CR-Ab case detected in the Burns and Intensive Care unit was in September 2018, and there was no new case detected in the hospital after May 2019 (Fig. S11). The incidence rate post-intervention was two CR-Ab cases per year, significantly reduced from the pre-intervention incidence rate of 13 CR-Ab case per year (P<0.001). The post-intervention CR-Ab incidence rate was reduced by 17 % compared to the pre-intervention period (incidence rate reduction=0.17, 95 % CI: 0.06–0.47).

DISCUSSION

CR-Ab are an increasingly dire threat to global public health. Their proficiency at surviving for long periods of time in environments whilst under antibiotic pressure is largely due to the positive selection of both intrinsic and acquired resistance and survival mechanisms. As such, they present a significant problem in healthcare settings, which typically have high antibiotic use as well as a large cohort of vulnerable patients. Understanding the mechanisms behind their resistance and transmission, as well as their possible environmental reservoirs, is key to combating further colonization and infection in hospital settings. Here we present a comprehensive analysis of an outbreak of CR-Ab using isolate and environmental metagenomic sequencing to fully elucidate transmission, determine new cases rapidly and detect possible environmental reservoirs within the hospital.

Genomics is being rapidly established in clinical settings, particularly in response to outbreaks [39, 40]. This is due not only to the higher discriminatory power that WGS provides, but also the complete picture that WGS captures by yielding the entire genome. The current cost and turnaround time for sequencing and analysis also make this type of investigation more feasible in nosocomial settings. In this study, initial sequencing of the outbreak CR-Ab isolates (and associated bacterial species) confirmed an already suspected outbreak, and so despite providing more insight into possible transmission routes, it did not greatly affect the infection control response. However, genomics superseded traditional methods when it came to (i) contextualizing outbreak isolates with previous CR-Ab strains from the hospital (to determine the likely source), and (ii) contextualizing new CR-Ab isolates as they appeared after the initial outbreak to determine whether there was an ongoing problem in the hospital. While having a slightly faster turnaround time, traditional methods alone would not have been able to confidently assess either of these scenarios. Regular meetings and reporting of the genomic results provided the hospital with actionable information and greater insight into the ongoing outbreak. These cross-disciplinary discussions facilitated the communication of complex genomic data into the clinical setting, providing guiding principles for subsequent WGS reporting of multidrug resistant bacterial pathogens at this hospital, and prompting the development of an interactive online visualization for communicating genomic epidemiology data (see HAIviz; File S3).

In addition to providing evidence for related isolates, WGS was also a valuable tool for discerning unrelated isolates, in many cases preventing ward or operating theatre closures and mitigating the associated financial costs to the hospital [41, 42]. It is plausible that with continued, ongoing sequencing of clinically significant bacteria in high-risk environments (e.g. ICU and Burns Unit) the risk of outbreaks could be reduced if evidence of transmission was detected early. During this study, we were able to detect transmission of an *E. hormaechei* unrelated to the outbreak at hand, but linked to a *bla*IMP-4 carbapenemase-producing Enterobacteriaceae (CPE) outbreak from the same hospital the year prior [16]. We were also able to identify transmission of an unrelated meropenem-resistant *P. aeruginosa* isolate, highlighting how WGS can detect transmission well before it becomes known to staff. Routine WGS can also lead to a reduction in the costs associated with responding to an established outbreak. A study of a similar outbreak in Brisbane determined the cost per patient related to the outbreak to be six-times higher than unrelated patients [43]. However, the feasibility (i.e. access to sequencing facilities and analysis) of routinely sequencing multidrug-resistant organisms is not yet achievable for many hospitals, particularly in low-resource settings. Despite this, recent collaborative efforts in the Philippines [44] have demonstrated how retrospective sequencing and capacity building for prospective sequencing can be achieved. WGS is also becoming increasingly cheap and portable (e.g. Oxford Nanopore Technology), and when coupled with more accessible cloud-based infrastructure, routine sequencing in these settings has greater potential.

Determining relatedness and transmission using genomics has historically relied on the number of core SNP differences between isolates [45–47]. However, this approach has several flaws, including a general lack of consensus on SNP cutoffs and what number defines a related isolate within a particular species, as well as the fact that it largely ignores other genomic differences, such as large insertions, inversion and rearrangements. It also does not account for hypermutators, which we observed in the case of the *K. pneumoniae* isolate MS14418.

More recent methods have explored the use of transmission probabilities by taking into account isolation time and species mutation rate [48], but these methods appear more suited to outbreaks spanning large timeframes. Most studies to date that have used SNP distances have used them retrospectively and under research conditions, thereby avoiding the necessity to conform to standardized metrics and allow case-by-case judgments to be made on isolates. Moving forward, translating this approach into standardized clinical settings will likely present several hurdles. In our study, with the exception of the hypermutator strain MS14418 there was no ambiguity using SNP distances to determine relatedness due to the observed low mutation rate. However, because of this, many isolates were unable to be discriminated, with several identical at the core-genome level. We were surprised that the initial polymicrobial nature of this outbreak enabled deduction of transmission routes by examining SNP differences between their respective companion *K. pneumoniae* isolates, which appeared to have coinfected with the CR-Ab. However, all of these transmissions were from the index patient and were already recognized by the clinical team. In contrast, the spread of CR-Ab between the ICU and Burns Units in July could be traced to transmission of CR-Ab carrying a discriminatory SNP from the index patient to patient 10 in the Burns Unit with subsequent transmission of CR-Ab to patient 11, 14 and 17 in the Burns Unit and patient 13 in the ICU (Fig. 1). Further work into routinely automating the identification of both SNPs and pan-genome markers (such as gain/loss of regions or movement of mobile elements) could assist in further characterizing this outbreak and others.

Metagenomic sequencing of the environment was able to identify several areas positive for ST1050 CR-Ab. In one case, metagenomic sequencing analysis and traditional culture methods were concordant and both identified the ST1050 CR-Ab. In all other cases, either traditional culture or metagenomic sequencing was able to recover the ST1050 CR-Ab, highlighting the advantage of using both methods during an outbreak. In addition to the ST1050 CR-Ab, we were also able to use the metagenomic sequencing data to search for a previously identified *E. hormaechei* strain, which caused a small outbreak in 2015 in the same ICU [16] and was found again during this study. This highlights the wider utility of metagenomic sequencing to search for not only a single strain of interest, but potentially several, while also gaining greater insight into the microbial communities within the hospital plumbing.

While metagenomic sequencing was able to recover more positive results than the traditional methods, it has several limitations, including the necessity for high bacterial loads (such that there is sufficient starting DNA to sequence) and the increased costs (in our study, we observed that at least 5 Gigabase pairs of sequencing data is required to get a basic amount of depth and sensitivity when looking for specific strains, roughly equivalent to 5x the required sequencing for a single isolate). In future, initial PCR from the environmental DNA targeting a known marker in the outbreak strain could help narrow the candidates for complete metagenomic sequencing. Further work is required to refine these methods and determine an accurate guideline, particularly as it relates to sequencing depth and sensitivity.

All of the positive sequencing and culture results from the environmental sampling were from areas presently or previously being used by patients colonized with the ST1050 CR-Ab. As such, we cannot be sure that the identified ST1050 CR-Ab was present in these environments prior to colonization, or if it was shed from the patient. Subsequent environmental sampling was carried out after each round of cleaning, and no CR-Ab was detected afterwards. It is most likely that the CR-Ab detected in the environmental reservoirs were shed from the patients, however this result does indicate the ease of transmission of this organism from colonized patients to fomites within the hospital, where they then might transmit to other areas or to hospital staff [49]. Burns baths are a particular risk to patients, as denuded skin is easily colonized and presents a high risk for subsequent infection. 'Splash-back' from sinks and/or drains where MDR bacteria, such as CR-Ab, reside could present a hypothetical route for reinfection and ongoing transmission.

CONCLUSION

By using WGS to assist in a large outbreak of CR-Ab (and other MDR Gram-negative bacilli) we show how genomics can be used to improve rapid respond measures and outbreak management, as well as provide in-depth characterization of the outbreak strains to establish a historical database that can be used to guide responses to future outbreaks. We also show how direct sequencing of environmental samples was able to detect evidence of the outbreak strain leading to key changes in infection control policy.

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Author contributions

L.W.R., P.N.A.H. and S.A.B. designed study. P.N.A.H., G.R.N., N.G., J.M. and J.L, coordinated patient inclusion and isolate collection from the hospital. L.W.R. and T.H. collected environmental samples. T.H. and K.H. coordinated infection control response. L.W.R., B.M.F. and W.L. performed experiments. L.W.R., S.A.B., B.M.F. and P.N.A.H. prepared clinical reports. All authors contributed to the interpretation of results. S.A.B., P.N.A.H., M.A.S. and D.P. supervised aspects of the project and provided essential expert analysis. L.W.R., P.N.A.H., S.A.B., W.L. and T.H. wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

P.N.A.H. has received research grants from MSD and Shionogi Ltd, outside of the submitted work, and speaker's fees from Pfizer paid to The University of Queensland. D.L.P. reports receiving grants and personal fees from Shionogi and Merck Sharp and Dohme and personal fees from Pfizer, Achaogen, AstraZeneca, Leo Pharmaceuticals, Bayer, GlaxoSmithKline, Cubist, Venatorx, and Accelerate. J.L. has received personal fees from Pfizer and MSD and grants from MSD paid to The University of Queensland. The other authors have no conflicts of interest to declare.

Ethical statement

Ethics approval was provided by the RBWH HREC as a low-risk study with waiver of consent (HREC/16/QRBW/581).

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Outlook

FW: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

From Teresa Inkster [redacted]
Date Fri 24/01/2025 15:08
To teresa inkster [redacted]

OFFICIAL-SENSITIVE

OFFICIAL-SENSITIVE

From: Teresa Inkster
Sent: 21 January 2025 15:30
To: Bagrade, Linda [redacted]; Kelly, Allana [redacted]
Cc: Devine, Sandra [redacted]; Leighanne Bruce [redacted]; NSS ARHAIinfectioncontrol [redacted]
Subject: RE: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

Hi Linda, thanks for your responses, I have forwarded to relevant colleagues in NHS Assure.

With regards to historical issues we are referring to previous cases of Cupriavidus in RHC which have featured in the Scottish Hospitals Inquiry.

Re point 4a , it would not be appropriate for me to make assumptions based on work I undertook over 5 years ago. Can you clarify what is meant by 'low levels' , and whether we can take this to mean < 10 cfu/100ml

Kr
Teresa

From: Bagrade, Linda [redacted]
Sent: 15 January 2025 18:28
To: Teresa Inkster [redacted]; Kelly, Allana [redacted]
Cc: Devine, Sandra [redacted]; Leighanne Bruce [redacted]
Subject: RE: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

OFFICIAL-SENSITIVE

Hi Teresa,

Please see below our response to the questions from colleagues in ARHAI and NHS Assure engineering team. Please do not consider this as an update to the closed incident as many of these questions are not relevant to the incident we investigated and reported and many of the questions have been answered already in previous communications.

1. *With respect to the locations where Cupriavidus samples have been identified, have NHS GG&C undertaken a review to establish whether these locations have returned positive samples previously?*
   Yes, we have a very rigorous routine system for sampling and review of results in place and I have clarified the findings in my previous e-mail (see below).

2. *With respect to the noted testing strategy can NHS GG&C advise if any additional testing for Cupriavadus has been undertaken in Ward 2A? From the assessment notes provided it would appear to have led to additional testing in Ward 4D and dialysis only.*
   Ward 2A in RHC is routinely tested for GNB so no additional testing is required.

3. *Can NHSGGC clarify the decision not to undertake typing given the historical issues with Cupriavidus*

Could colleagues in ARHAI and NHS Assure engineering team clarify what exactly is meant by "historic issues" and why would these issues lead to a typing of isolates reviewed in this particular situation?

4. NHS GG&C have identified that "an outlet that has been repeatedly low level positive for Cupriavidus sp. Repeat samples from September onwards have been clear for this outlet.". Can NHS GG&C confirm:

a. What they deem to constitute "low levels" and against what benchmark is this being compared?
The cut-off level is 10CFU/100ml as was agreed in NHS GGC at the time when you were Lead ICD and the ICD for RHC so I understand you will be more aware of the reasons why this number was chosen and what was the evidence base.

b. Whether they have considered typing cultures from these positive samples with the newly identified positive results.
No, it is not required at this point.

c. Whether any additional investigation/testing of the water/drainage systems connected to the previous identified positive sample were undertaken?
We have already reported all the actions we have considered and put in place. All positive results from routine testing are addressed immediately in real time according to the existing SOPs.

d. Were any remedial works undertaken e.g. replacement of outlets/sanitaryware/etc?
No, this is not required in response to this particular situation. The outlets with positive results are managed in real time according to the existing SOPs.

4. NHSGGC have rated risk of transmission as minor .Can the reason for this be clarified given that investigations are ongoing?
I have already responded to this question, please see previous email below. HIAAT assessment was done at the PAG meeting by the group of specialists in their particular field and after the review of information the decision was to rate it as minor risk. Could you please explain why do you disagree with this decision?

5. Similarly, NHSGGC have rated public anxiety as minor. Can the reason for this be clarified given the historical issues and ongoing Scottish hospitals inquiry?
Could you please clarify what is meant by "historic issues" and how is ongoing Scottish Hospitals Inquiry relevant to this particular situation?

6. Can NHSGGC confirm whether clinician duty of candour has been undertaken ?
I already answered this question, please see the previous e-mail below.

Hope this answers your questions.

Kind regards,

Linda

OFFICIAL-SENSITIVE

From: Teresa Inkster
Sent: 03 December 2024 16:56
To: NSS ARHAIinfectioncontrol [redacted]; Bagrade, Linda [redacted]; Kelly, Allana [redacted]
Cc: Devine, Sandra [redacted]; Leighanne Bruce [redacted]
Subject: RE: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

OFFICIAL-SENSITIVE

Hi Linda, I appreciate you are busy but would you be able to give us an indication re timescale for the questions below.
Thanks
Kr
Teresa

From: NSS ARHAIinfectioncontrol
Sent: 26 November 2024 13:39
To: Bagrade, Linda [redacted]; NSS ARHAIinfectioncontrol [redacted]; Kelly, Allana [redacted]
Cc: Devine, Sandra [redacted]; Leighanne Bruce [redacted]; Teresa Inkster [redacted]
Subject: RE: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

Dear Linda,

Thank you for your responses. I have some further queries below from ARHAI and NHS Assure engineering colleagues and would be grateful if you clarify these points;

1. With respect to the locations where *Cupriavidus* samples have been identified, have NHS GG&C undertaken a review to establish whether these locations have returned positive samples previously?

2. With respect to the noted testing strategy can NHS GG&C advise if any additional testing for *Cupriavadus* has been undertaken in Ward 2A? From the assessment notes provided it would appear to have led to additional testing in Ward 4D and dialysis only.

3. Can NHSGGC clarify the decision not to undertake typing given the historical issues with *Cupriavidus*

4. NHS GG&C have identified that "an outlet that has been repeatedly low level positive for *Cupriavidus* sp. Repeat samples from September onwards have been clear for this outlet.". Can NHS GG&C confirm:

    a. What they deem to constitute "low levels" and against what benchmark is this being compared?
    b. Whether they have considered typing cultures from these positive samples with the newly identified positive results.
    c. Whether any additional investigation/testing of the water/drainage systems connected to the previous identified positive sample were undertaken?
    d. Were any remedial works undertaken e.g. replacement of outlets/sanitaryware/etc?

5. NHSGGC have rated risk of transmission as minor .Can the reason for this be clarified given that investigations are ongoing?

6. Similarly, NHSGGC have rated public anxiety as minor. Can the reason for this be clarified given the historical issues and ongoing Scottish hospitals inquiry?

7. Can NHSGGC confirm whether clinician duty of candour has been undertaken ?

Kind regards
Teresa

Dr Teresa Inkster
Consultant Microbiologist/Infection Control Doctor
ARHAI Scotland
NHS Scotland Assure
NHS National Services Scotland
Tel: [redacted]

From: Bagrade, Linda [redacted]
Sent: 20 November 2024 16:26
To: NSS ARHAIinfectioncontrol [redacted]; Kelly, Allana [redacted]
Cc: sandara.devine [redacted]; Teresa Inkster [redacted]; Jennifer Barrett [redacted]
Subject: Re: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

Dear Leighanne,

Please find below (in purple) the additional information you requested:

We understand why this has been submitted as a single incident whilst you investigate any correlation between patient cases however, as the isolates have been identified in different populations, we are actively considering whether 2 separate ORTs may be required. We will be back in touch if there are any resultant actions for either organisation to enable that.

Thank you but our reasons for having a PAG was the occurrence of the two cases in a short period of time. The investigation is focused on why this may have occurred so it would seem logical that this is reported as a single incident.

We note the investigations to date, the 4 x working hypotheses provided and the current / intended control measures for both the RCH and QEUH buildings as per your ORT submission, but we would be grateful if you could clarify some additional points for us please?

- *The HIIAT assessment recorded does not appear to reflect the current risk of transmission given that the source remains unknown. Could you please provide some further detail that will help us understand why risk of transmission and also public anxiety are being assessed as minor?*

We have provided information via ORT, and we do not have any reason to doubt the appropriateness of our assessment. HIIAT assessment is a dynamic tool and will be re-assessed should the situation change.

These are two separate areas of the hospital, so we do not believe that there is an ongoing risk of transmission. There is no reason to believe that public anxiety is more than minor. We would, however, be very interested to know your reasons for questioning this assessment.

This is, as you know, an independent process where discussion have taken place, and a collective decision has been made.

- We've noted that a meeting was held on Friday the 15th of November, could you confirm if this was a PAG or an IMT and are any further meetings planned?

  This was a PAG and no further meetings are planned at the moment.

- Can you advise what the board have undertaken in terms of Duty of Candour for both patient cases?

  DoC is not applicable at this stage.

- Could you confirm if typing of the isolates has been requested?

  Not at this stage.

- Can you advise if you have identified any specific intravenous solutions or drug products during further investigation supporting hypothesis number IV?

  No, this is still an early stage of data gathering.

- Does / has patient case 2 attended level 2 (dialysis) at the QEUH for recent / established haemodialysis sessions since line their line was inserted on the 8th October?

  Yes.

- Can you advise if there are / have been any water controls in place including POUFs on level 2 of the QEUH (which pre-date the identification of the adult case)? and if not, have such measures been considered?

  No, there is no evidence at the moment to consider this area as the source of infection.

- As part of current monitoring, can you advise if chlorine dioxide concentrations have been adequate at outlets in the associated areas being reported?

  Cl dioxide levels are checked regularly throughout the campus and consistently are achieving the expected levels.

- Has Cupriavidus been identified in any water samples obtained from the QEUH or RHC sites (as part of routine testing or other testing) within the past 6 months?

For the routine water testing programme in total this year we have tested 32 642 water samples in QEUH and RHC and 2500 samples have been specifically tested for GNB which will include *Cupriavidus* sp. Of those 2500 samples, *Cupriavidus* sp. have been isolated in 2 samples from non-clinical areas within wards 2A/B in RHC (one in February, one in September), both are different outlets and subsequent repeat samples have been clear. All outlets in 2A/B are fitted with PoUF.

Testing designed specifically for an area under reconstruction has identified an outlet that has been repeatedly low level positive for *Cupriavidus* sp. Repeat samples from September onwards have been clear for this outlet.

- Could you please advise if there have there been any IC or clinical audits undertaken of the line care which is being provided within the renal unit at the QEUH, ward 4D QEUH and / or Wd 2A / 2B RHC?

There is a regular multidisciplinary assurance review process in place for RHC ward 2A which includes line reviews. No reasons for concern identified.

Audit for QEUH ward 4D is planned in near future.

- Did case one attend RHC as an outpatient at any point between their hospital admissions ?

  Yes

Hope this answers your questions,

A53385584

Dr Linda Bagrade

Lead Infection Prevention and Control Doctor

Consultant Medical Microbiologist

NHS GGC

[redacted]

From: NSS ARHAIinfectioncontrol [redacted]
Sent: 19 November 2024 11:52
To: Bagrade, Linda [redacted]; Kelly, Allana [redacted]
Cc: sandara.devine [redacted]; NSS ARHAIinfectioncontrol [redacted];
Teresa Inkster [redacted]; Jennifer Barrett [redacted]
Subject: ARHAI Scotland request for further information regarding HIIAT2024-GGC-South-369

Dear colleagues

Thank you for submitting the ORT to ARHAI Scotland following the recognition of the above Cupriavidus pauculus incident.

We understand why this has been submitted as a single incident whilst you investigate any correlation between patient cases however, as the isolates have been identified in different populations, we are actively considering whether 2 separate ORTs may be required. We will be back in touch if there are any resultant actions for either organisation to enable that.

We note the investigations to date, the 4 x working hypotheses provided and the current / intended control measures for both the RCH and QEUH buildings as per your ORT submission, but we would be grateful if you could clarify some additional points for us please?

- The HIIAT assessment recorded does not appear to reflect the current risk of transmission given that the source remains unknown. Could you please provide some further detail that will help us understand why risk of transmission and also public anxiety are being assessed as minor?
- We've noted that a meeting was held on Friday the 15th of November, could you confirm if this was a PAG or an IMT and are any further meetings planned?
- Can you advise what the board have undertaken in terms of Duty of Candour for both patient cases?
- Could you confirm if typing of the isolates has been requested?
- Can you advise if you have identified any specific intravenous solutions or drug products during further investigation supporting hypothesis number IV?
- Does / has patient case 2 attended level 2 (dialysis) at the QEUH for recent / established haemodialysis sessions since line their line was inserted on the 8th October?
- Can you advise if there are / have been any water controls in place including POUFs on level 2 of the QEUH (which pre-date the identification of the adult case)? and if not, have such measures been considered?
- As part of current monitoring, can you advise if chlorine dioxide concentrations have been adequate at outlets in the associated areas being reported?
- Has Cupriavidus been identified in any water samples obtained from the QEUH or RHC sites (as part of routine testing or other testing) within the past 6 months?
- Could you please advise if there have there been any IC or clinical audits undertaken of the line care which is being provided within the renal unit at the QEUH, ward 4D QEUH and / or Wd 2A / 2B RHC?
- Did case one attend RHC as an outpatient at any point between their hospital admissions ?

We aim to inform the HAI policy unit of this incident today and would greatly appreciate any information you can provide in advance of this.

Kind regards

Miss Leighanne Bruce
Senior Nurse Infection control
ARHAI Scotland
NHS Scotland Assure
NHS National Services Scotland
Tel: [redacted]

We are NHS National Services Scotland. We offer a wide range of services and together we provide national solutions to improve the health and wellbeing of the people of Scotland. Find out more in our [NSS Strategic Framework 2024-2026](#).

Cover your mouth and nose when sneezing, coughing, wiping and blowing your nose.

Use a disposable tissue, then wash your hands with soap and water.

NHS SCOTLAND

Outlook

FW: SMVN ¦ Cryptococcal data request from ARHAI Scotland

From Teresa Inkster [redacted]
Date Thu 19/06/2025 18:52
To [redacted]

From: Bal, Abhijit [redacted]
Sent: 02 December 2024 09:02
To: Teresa Inkster [redacted]
Cc: Shona Cairns [redacted]; NSS ARHAIdatateam [redacted]; Mackenzie, Fiona M [redacted]; Laura Imrie [redacted]
Subject: Re: SMVN ¦ Cryptococcal data request from ARHAI Scotland

Thanks Teresa. The document you have kindly provided makes it clear that sharing of personal data should be justifiable and proportionate (paragraph 6) and on a need to know basis with only the minimum necessary being shared (paragraph 21). It is the personal responsibility of those obtaining or disclosing the data to ensure confidentiality (paragraph 23).

The email below provides no detail as to what is the purpose of this request and so no conclusion can be drawn as to what is the justification and how much data can be considered proportionate. CHI number (an obvious patient identifier), health board, ward, specialty, and department, can provide a significant amount of information about a patient's identity, their geographical location, and their diagnosis. Under GDPR, providing disproportionate amount of personal data without justification might be a serious breach of confidentiality.

Please discuss it internally within ARHAI and inform the Scottish Government of my concerns as you have suggested.

We should be able to provide anonymous and de-duplicated data within the suggested time frame. I have asked the laboratory IT team to generate the data.

Regards,

Abs

Abhijit M Bal
MBBS, MD, DNB, MNAMS, FRCP, FRCPath, FISAC, FRAS, Dip Med Mycol
Consultant, Head of Service, and Infection Control Doctor
Department of Microbiology
Queen Elizabeth University Hospital, Glasgow
Honorary Clinical Associate Professor, University of Glasgow

From: Teresa Inkster [redacted]
Sent: 28 November 2024 16:12
To: Bal, Abhijit [redacted]
Cc: Shona Cairns [redacted]; NSS ARHAIdatateam [redacted];

Mackenzie, Fiona M [redacted]; Laura Imrie [redacted]
Subject: RE: SMVN ¦ Cryptococcal data request from ARHAI Scotland

Hi Abs, I am aware that you have raised concerns previously in relation to this issue and Dr Anna Lamont had provided the information attached. If despite this information you still have concerns we will escalate internally within ARHAI and inform SG. In the meantime as there is some time pressure we are happy to accept anonymised patient data from NHSGGC

Kr

Teresa

From: Bal, Abhijit [redacted]
Sent: 28 November 2024 14:50
To: Mackenzie, Fiona M [redacted]
Cc: Shona Cairns [redacted]; Teresa Inkster [redacted]; NSS ARHAIdatateam [redacted]
Subject: Re: SMVN ¦ Cryptococcal data request from ARHAI Scotland

Hi Fiona,

I think if patient identifiable information is to be provided, it needs Caldicott approval. Have ARHAI obtained Caldicott approval? Please can you ask around SMVN what other health boards think.

Thanks,

Abs

--

Abhijit M Bal

MBBS, MD, DNB, MNAMS, FRCP, FRCPath, FISAC, FRAS, Dip Med Mycol
Consultant, Head of Service, and Infection Control Doctor

Department of Microbiology
Queen Elizabeth University Hospital, Glasgow
Honorary Clinical Associate Professor, University of Glasgow

From: Mackenzie, Fiona M [redacted]
Sent: 28 November 2024 12:23
To: Mackenzie, Fiona M [redacted]
Cc: Shona Cairns [redacted]; Teresa Inkster [redacted]; NSS ARHAIdatateam [redacted]
Subject: SMVN ¦ Cryptococcal data request from ARHAI Scotland

OFFICIAL-SENSITIVE

Dear SMVN Lab-based Steering Group Members,

Please see the request below for Health Boards to send data on all Cryptococcal clinical isolates since January 2020, to ARHAI Scotland.

Best wishes

Fiona

OFFICIAL-SENSITIVE

From: Teresa Inkster [redacted]
Sent: 27 November 2024 14:47
To: Mackenzie, Fiona M [redacted]; nss smvn [redacted]
Cc: NSS ARHAIdatateam [redacted]; Shona Cairns [redacted]
Subject: Cryptococcal data request

OFFICIAL-SENSITIVE

Dear Fiona,

ARHAI have been asked by Scottish Government colleagues to obtain data in relation to Cryptococcal infections in Scottish health boards.

Due to the limitations of ECOSS we would be grateful if labs could send us data on all Cryptococcal clinical isolates (all species) including positive Cryptococcal antigen results from January 2020 to present day. We do not require the data to be de-duplicated.

We have attached a template to assist with this and would be grateful if labs could get back to us by 5pm on Friday 6th Dec . Completed forms should be sent to ; [redacted]

Kr

Teresa

Dr Teresa Inkster

Consultant Microbiologist/Infection Control Doctor

ARHAI Scotland

NHS Scotland Assure

NHS National Services Scotland

Tel: [redacted]

Outlook

FW: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

From Teresa Inkster [redacted]
Date Thu 19/06/2025 18:30
To teresa inkster [redacted]

OFFICIAL-SENSITIVE

OFFICIAL-SENSITIVE

From: Teresa Inkster
Sent: 20 March 2025 10:52
To: Laura Imrie [redacted]
Subject: RE: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

Comments as discussed

Whilst Coag neg Staphs are of low virulence in some patient populations they are a significant cause of sepsis in paediatric ICU patients particularly cardiac surgery patients who may have prosthetic material , lines and ECMO support. (as has been reported here)

From the information provided there are epidemiological links in time/place/ person and WGS results which confirm an outbreak. This strain has variable MICs to Vancomycin and is resistant to several other agents , reducing the options for treatment

The fact the strain has been circulating for at least one year might point to a staff carrier, has this been considered as a hypothesis. (Could reference previous CNS outbreak linked to staff carrier in this patient group and requirement for decolonisation)

Kr
Teresa

From: Teresa Inkster
Sent: 20 March 2025 09:44
To: Laura Imrie [redacted]
Subject: RE: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

Hi Laura, can we discuss email trail below when you have time
Thanks
Teresa

From: Anna Munro [redacted]
Sent: 20 March 2025 08:31
To: Teresa Inkster [redacted]
Subject: FW: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

OFFICIAL-SENSITIVE

Hi Teresa

A53385584

See response below. I'm not sure there would be anything else we would go back on in this case as it is on their radar as something to investigate if clustering noted? The isolate mentioned from the year before didn't specify if it was from a BSI, so that's probably why it wasn't stored?

Happy to discuss.

Kind regards

Anna Munro
Nurse Manager Infection Prevention and Control
ARHAI Scotland
NHSScotland Assure
NHS National Services Scotland
Tel: [redacted] | [redacted] | [redacted]

OFFICIAL-SENSITIVE

From: NSS ARHAIinfectioncontrol [redacted]
Sent: 19 March 2025 16:56
To: Anna Munro [redacted]
Subject: FW: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

OFFICIAL-SENSITIVE

OFFICIAL-SENSITIVE

From: Bagrade, Linda [redacted]
Sent: 19 March 2025 16:55
To: NSS ARHAIinfectioncontrol [redacted]
Subject: RE: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

OFFICIAL-SENSITIVE

Dear Anna,

No, the isolate is not available for typing. And of course, we have done appropriate investigations with look back.

We don't know how widespread this type of *Staphylococcus epidermidis* is and how long it has been circulating in the population. Colleagues from microbiology lab have sent just a few isolates that clustered for whole genome sequencing but we have to keep in mind there have been around 37 *S.epidermidis* isolates in PICU in the last 2 years with enough clinical significance to be identified to species level with sensitivity testing performed.

We also need to note that this microorganism belongs to the group of Coagulase negative Staphylococcus (CoNS) which is one of the most common skin commensals with limited clinical significance. Very few CoNS get identified to species level and very few of these get antimicrobial sensitivity tested. Therefore we are currently operating with a very limited information and should avoid making generalised conclusions. We will keep monitoring this situation.

Kind regards,

Linda

OFFICIAL-SENSITIVE

From: NSS ARHAIinfectioncontrol
Sent: 19 March 2025 10:14
To: Bagrade, Linda [redacted]
Cc: NSS ARHAIinfectioncontrol [redacted]
Subject: Query re HIIAT2025-GGC-Paediatrics-103 - Ward 1D, S. epidermidis

OFFICIAL-SENSITIVE

Dear Dr Bagrade

Thank you for adding the information regarding the WGS for this incident. We note that you have identified that the outbreak strain was present approximately a year ago. Can we ask you have done any lookback for case ascertainment and if the isolate is available for typing to confirm it is the same?

Thank you for your time responding to these queries.

Kind regards

Anna Munro
Nurse Manager Infection Prevention and Control
ARHAI Scotland
NHSScotland Assure
NHS National Services Scotland
Tel: [redacted] | [redacted]

OFFICIAL-SENSITIVE

please (i) contact the sender by email reply; (ii) delete the email from your system; .
and (iii) do not copy the email or disclose its contents to anyone.

SBAR: Environmental testing

November 2024

Teresa Inkster
Shona Cairns

Version history

Version	Date	Summary of changes

Approvals

Version	Date Approved	Group / Individual

Contents

1 Situation

Following a significant water incident at the Queen Elizabeth University Hospital (QEUH) in Glasgow in 2018, the subsequent Case Note Review report identified an inconsistent approach to environmental testing as an area requiring improvement. Several years on, inconsistencies persist, alongside ongoing challenges related to sampling protocols and environmental testing in Scottish hospitals. These issues have been a focus of the Scottish Hospitals Public Inquiry, with evidence provided by numerous witnesses to explore these concerns in depth.

A paper produced in March 2020 by the then Centre of Excellence (CoE) states in its executive summary that ‘a lack of expert environmental microbiological capacity was identified and we recommend that the CoE ensures the growth, development and retention of this expertise in Scotland’. They suggest that the possibility of using Scottish Government laboratories should be explored, making use of government funded laboratories to fulfil environmental testing requirements.

Subsequently a recommendation from the gap analysis of Public Health Microbiological services in Scotland (August 2023) has the following recommendation for NHS Assure ‘Sampling methodology and infrastructure within the NHS environmental investigations require development. ‘

There is currently a varying laboratory approach in Scotland with regards routine water testing with a combination of local and external labs undertaking this. This applies to both routine testing of hospital water systems and specialist systems such as endoscopy rinse waters.

Furthermore, Scotland currently lacks defined sampling strategies and capacity to undertake reactive water testing and typing or whole genome sequencing in response to waterborne incidents or outbreaks. This means that the full epidemiological picture is lacking, and incidents may not be investigated fully, another finding of the Case Note Review.

Not all boards have expertise in water testing and in some there is a reluctance to test and particularly for organisms other than Pseudomonas and Legionella, that do not have UKAS accreditation.

Similar challenges exist for both environmental surface swabbing and air sampling

2 Background

The water incident in the QEUH, Glasgow in 2018 highlighted the important role of the microbiology laboratory in undertaking environmental testing and supporting such incidents or outbreaks.

During such incidents there is a requirement for the microbiology lab to react in a timely fashion and capacity is required to process large sample volumes in a short space of time. The incident also highlighted the increasing number of waterborne pathogens and the need for laboratories to have expertise in identifying these in addition to the acceptance that UKAS accreditation for each different organism tested is not required.

More recent incidents in Scottish hospitals have further highlighted the lack of resource in Scotland to undertake the large volumes of water testing required during a suspected outbreak linked to the hospital water supply. In particular, there are challenges where more unusual organisms are implicated such as Stenotrophomonas and Acinetobacter, where UKAS accreditation does not exist.

Aside from outbreaks there is a requirement for routine water testing in hospitals including that of specialist equipment such as rinse water endoscope washer disinfector, sterilizer, heater cooler units, hydropools and renal waters.

A recent Aspergillus outbreak in one health board has highlighted the lack of capacity for reactive air sampling and for expertise in processing these samples. Significant delays have been incurred by relying on an external company to undertake these samples. There is reduced capacity from the Bristol mycology reference lab to undertake identification of isolates, which the external lab is unable to do.

3 Assessment

1. Resource, expertise, and capacity to respond to environmental incidents in Scotland is often lacking. It is a challenge to process large numbers of water samples in a timely fashion when the work is unanticipated.

2. Many boards are reluctant to undertake testing on water samples on organisms other than Pseudomonas aeruginosa and legionella, citing lack of UKAS accreditation for each organism and the inability to interpret results. Private labs have significant resource for filtering water samples but not for organism identification and often are dependent on sending to NHS labs to undertake further identification.

3. Priority for using laboratory automated methods is for clinical isolates, so there can be some delay in getting identifications on water samples. Results can take up to several weeks which impacts response time relating to any significant findings.

4. Reference laboratories currently do not have the planned capacity for typing or whole genome sequencing of large numbers of water samples and the recommended multiple colony picks. (up to 20-30 colonies per plate). There is lack of capacity to undertake this work in real time and it is often retrospective which again impacts the commencement of mitigating measures in response to any significant findings.

5. Whilst there is now expertise in the NHS Greater Glasgow and Clyde (NHSGGC) water lab there is currently limited capacity to support other health boards who don't have experience in water testing in response to incidents. NHSGGC have already had to provide support to other health boards. ARHAI have recently published Chapter 4 of the NIPCM which means awareness of the risks from water continues to grow and these requests have become more frequent.

6. In contrast to the rest of the UK, Scotland has not always been routinely testing for Pseudomonas in water. The current water literature review and subsequent proposal for Gram negative guidance may challenge boards in

terms of capacity and expertise. This is challenging for boards in terms of relevant laboratory expertise and capacity.

7. There are risks with sending water samples to external labs. These include sample retention or storage issues and the lack of ability to send isolates for further analysis and typing and compare them with patient isolates should outbreaks occur. Some external labs do not have MALDI-TOF which NHS labs utilise for rapid organism identification. This can result in external labs sending isolates to NHS diagnostic labs for identification further adding to a time delay.

8. Similar challenges exist with regards to surface environmental testing and air sampling, with few labs equipped to undertake these.

9. Research on development of more rapid testing methods such as PCR for waterborne organisms is needed. As an example, current culture times for NTMs are up to 6 weeks and development of PCR would have significant advantages in both routine and outbreak situations. Having a dedicated lab in Scotland would enable such research to be undertaken.

4 Recommendations

A national or once for Scotland approach to provision of environmental testing would support NHS boards in this priority area where gaps exist. ARHAI should collaborate with colleagues in PHS to progress this work

Consideration should be given to the following options for increasing lab resource.

1) Increasing resource and capacity at the NHSGGC GRI lab to make this a national reference laboratory for environmental testing and typing or WGS.
2) Creating four regional environmental laboratories (potentially utilising the existing public analyst labs)
3) Options 1 and 2 combined, with more complex testing and typing going to NHSGGC, for example rare organisms, requirement for typing including whole genome sequencing.

4) Option 2 with complex testing and typing going to UKHSA.

Scottish Hospitals Inquiry

NHS National Services Scotland (NSS) Review of 'GGC Expert (HAD) Report - Chapter 8 - Dr Samir Agrawal's Calculations re Invasive Aspergillosis'

Prepared by: Shona Cairns, BSc (Hons), PGDip, MSc, ARHAI Scotland, NSS

Date: 20 June 2025

Contents

Executive Summary

1. This review is based on the additional information made available to NSS regarding the calculations undertaken by Dr Agrawal in preparation of Chapter 8 of the HAD report (**GGC Expert (HAD) Report - Chapter 8 - Dr Samir Agrawal's Calculations re Invasive Aspergillosis[1] and Copy of Cases Over Time Protected (updated)[2])**. This document was made available on 10th June 2025. The review was constrained by the limited time available and does not involve a comprehensive re-analysis of the data provided by NHSGGC or Dr Agrawal. However, with the information available it has been possible to determine that there remains a lack of detail on the calculations undertaken by Dr Agrawal and there are, what appears to be, several inconsistencies between the case definitions provided in the report and the final datasets. This demonstrates a lack of transparency on the case definitions used and may impact on the final interpretation of the analysis.

[1] Bundle 44, Volume 2, Page 107.
[2] Bundle 44, Volume 2, Page 109-119.

Introduction and Background

2. This review has been prepared to further assist the Inquiry in its considerations of the NHS Greater Glasgow and Clyde (NHSGGC) commissioned 'Expert Report for the Scottish Hospitals Inquiry on the evidence of risk of infection from the water and ventilation systems at the Queen Elizabeth University Hospital and Royal Hospital for Children, Glasgow' (**Bundle 44, Volume 1, Page 5 (**NHS GGC – Expert Report by Prof Peter Hawkey, Dr Samir Agrawal and Dr Lydia Drumright – The evidence of risk of infection from water and ventilation systems at QEUH and RHC – 24 July 2024 **("HAD Report")**)). The HAD report was admitted into evidence following a Procedural Hearing on 11 March 2025. At this time, NSS offered offer to provide an epidemiological commentary on the HAD report.

3. NSS provided a commentary on Chapters 7 and 8 of the HAD report on 28th May 2025 (**Bundle 44, Volume 2, Page 685**). As noted in Paragraph 2.4.4 of the NSS commentary, the details of the calculations undertaken and data interrogated by Dr Agrawal in his production of Chapter 8 were not available to NSS at time of review. A document describing the calculations (**Bundle 44, Volume 2, Page 107**) and the data used (**Bundle 44, Volume 2, Page 109-119**) were made available for review on 10th June 2025.

4. There remains a lack of detail on the calculations undertaken by Dr Agrawal. Dr Agrawal did not provide any additional methodological information in the newly provided calculation document and it instead refers to sections of the HAD report. The issues noted Paragraphs 4.2 and 4.3 of the NSS commentary (**Bundle 44, Volume 2, Page 702**) remain outstanding. The lack of additional information provided has made the assessment of the calculations undertaken by Dr Agrawal challenging.

Review of Paediatric Aspergillus Data

5. In order to support the review of the limited information provided by Dr Agrawal, it was necessary to undertake a rudimentary analysis of the paediatric

Aspergillus dataset provided by NHSGGC (**COMPLETE ASPERGILLUS POSITIVES, ADULTS 2013-2023 & PAEDS 2005 - 2022**)[3].

6. Dr Agrawal describes the de-duplication in his calculations document and in the HAD report as: "*Where the same patient had multiple positive tests for the same admission episode, this was classified as one case*" (**Bundle 44, Volume 1, Page 123, Section 8.1**). There is no further information provided regarding the application of positive results to patient admission data to identify cases of infection. In order to apply this case definition, Dr Agrawal would need to have linked the Aspergillus microbiology data to the admission data. There is no evidence in the dataset provided by Dr Agrawal that these datasets were linked to identify new cases of infection during each admission. Instead the cases appear to have been identified using the Aspergillus microbiology dataset only. Furthermore, the analysis of the NHSGGC Aspergillus dataset (**COMPLETE ASPERGILLUS POSITIVES, ADULTS 2013-2023 & PAEDS 2005 - 2022**)[4] suggests that the identification of cases was not linked to admissions. This lack of clarity between the case definition and the data presented makes interpretation of the final case numbers challenging.

7. As noted in Paragraph 4.2 of the NSS commentary (**Bundle 44, Volume 2, Page 702**), the case definition provided by Dr Agrawal does not include a parameter to indicate the time that should elapse between positive samples before a subsequent episode is considered a new case. There are paediatric patients with multiple samples that have been included as a single case and a patient with multiple samples that has been included as two cases. The calculations document ((**Bundle 44, Volume 2, Page 107**) and dataset (**Bundle 44, Volume 2, Page 109-119**) provided by Dr Agrawal do not provide further clarification on this issue. Consistent epidemiological case definitions are essential in such an analysis and should be transparent to readers of the report. This further lack of clarity on how cases have been defined make robust interpretation of the report challenging.

[3] This document contains patient identifiable data therefore is not included in a Hearing Bundle. See Inventory of Documents received from CLO/NHS GGC (Bundle 44, Volume 1, Page 224).
[4] Ibid.

8. The laboratory tests described by Dr Agrawal for case identification includes positive polymerase chain reaction (PCR) testing for *Aspergillus* spp. The information provided by Dr Agrawal does not describe any further exclusion criteria in relation to *Aspergillus* spp. PCR testing results. A note in the dataset indicated that a case has been excluded due to the high cycle threshold (CT) value despite having a positive *Aspergillus* spp. result (detected). A high PCR CT value indicates a low concentration of genetic material. This additional exclusion criterion is not noted in the methods provided in either the HAD report (**Bundle 44, Volume 1, Page 5**) or reiterated in the calculations document. The application of additional exclusion criteria that are not defined in the methods affects the interpretation of the case numbers presented in the report.

9. The methods provided in the HAD report (Section 8.1, page 123) state "*BDG is the only mycological criterion, this is not indicative of an invasive aspergillosis as it is a pan-fungal marker*" and for this reason patients with a beta-D-glucan result only are not included in the case numbers. Dr Agrawal's description of the case definition indicated these were applied "*regardless of host and imaging factors*" suggesting that no clinical review of imaging was undertaken. However, Dr Agrawal's notes in the datafile suggest that a clinical review of imaging was undertaken to exclude the cases. This is not in line with the case definitions provided in the HAD report and calculations document. In this instance, six cases in 2022 were reviewed and excluded based on both beta-D-glucan results and clinical imaging. This does not affect the case numbers as the cases would have been excluded based on beta-D-glucan result only. However, the review of clinical imaging is another deviation from the case definition as described in the report.

10. A specific issue was noted in relation to a patient with an "equivocal" *Aspergillus* spp. PCR result in the dataset (**Bundle 44, Volume 2, Page 109-119**). Whilst exclusion of "equivocal" PCR results is acceptable, the excluded patient also had a positive *Aspergillus fumigatus* PCR result so was excluded in error (presumably due to the accompanying "equivocal result"). This has resulted in one fewer case in the case numbers in 2014.

Review of Adult Aspergillus Data

11. Due to time constraints, the review of the adult Aspergillus data has not been as comprehensive as the paediatric review. The raw NHSGGC adult data file has not been analysed to support this review, instead the datafile provided by Dr Agrawal was reviewed. However, the points identified from the review of the paediatric datafiles are also relevant to the adult data. Namely:

    a. There is no evidence or description of how the adult Aspergillus data was linked to admission data to apply the case definition provided in the report.

    b. No description of an episode definition has been provided. The adult dataset includes patients with more than one episode that appear to have been included based on the judgement of Dr Agrawal.

    c. Adult patients with beta-D-glucan results only should have also been excluded according to the case definition provided in the report. The author appears to have undertaken clinical review of imaging to assess whether these cases should be included/excluded.

    d. Dr Agrawal also appears to have considered PCR CT values in determining whether cases should be included. This criterion is not included in the case definitions described in the report.

SCOTTISH HOSPITALS INQUIRY

Bundle of documents for Oral hearings commencing from 19 August 2025 in relation to the Queen Elizabeth University Hospital and the Royal Hospital for Children, Glasgow

Bundle 44 Volume 3 - Substantive Core Participants' Direction 5 Responses to GGC Expert (HAD) Report & Supplementary Report/Comments on Chapter 8