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Whole-genome sequencing reveals two prolonged simultaneous outbreaks involving *Pseudomonas aeruginosa* high-risk strains ST111 and ST235 with resistance to quaternary ammonium compounds

A. Rath [a,*], B. Kieninger [a], J. Fritsch [a], A. Caplunik-Pratsch [a], S. Blaas [b], M. Ochmann [b], M. Pfeifer [b,c,d], J. Hartl [a,e], T. Holzmann [a], W. Schneider-Brachert [a]

[a] *Department of Infection Prevention and Infectious Diseases, University Hospital Regensburg, Regensburg, Germany*
[b] *Donaustauf Hospital, Centre for Pneumology, Donaustauf, Germany*
[c] *Department of Internal Medicine II, University Hospital Regensburg, Regensburg, Germany*
[d] *Hospital of the Merciful Brother Regensburg, Regensburg, Germany*
[e] *Hospital of the Merciful Brother "St. Barbara", Schwandorf, Germany*

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SUMMARY

Objective: Water-bearing systems are known as frequent *Pseudomonas aeruginosa* (PA) outbreak sources. However, many older buildings continue to have sanitary facilities in high-risk departments such as the ICU. We present two simultaneous prolonged multi-drug-resistant (MDR) PA outbreaks detected at the ICU of a pulmonology hospital, which were resolved by whole-genome sequencing (WGS).

Methods: Outbreak management and investigations were initiated in August 2019 after detecting two patients with nosocomial VIM-2-positive MDR PA. The investigations involved weekly patient screenings for four months and extensive environmental sampling for 15 months. All patient and environmental isolates were collected and analysed by WGS.

Results: From April to September 2019, we identified 10 patients with nosocomial MDR PA, including five VIM-2-positive strains. VIM-2-positive strains were also detected in nine sink drains, two toilets, and a cleaning bucket. WGS revealed that of 16 VIM-2-positive isolates, 14 were ST111 that carried qacE, or qacEΔ1 genes, whereas 13 isolates clustered (difference of ≤11 alleles by cgMLST). OXA-2 (two toilets), and OXA-2, OXA-74, PER-1 (two patients, three toilets) qacEΔ1-positive ST235 isolates dominated among VIM-2-negative isolates. The remaining seven PA strains were ST17, ST233, ST273, ST309 and ST446. Outbreak containment was achieved by replacing U-bends, and cleaning buckets, and switching from quaternary ammonium compounds (QUATs) to oxygen-releasing disinfectant products.

Conclusion: Comprehension and management of two simultaneous MDR PA outbreaks involving the high-risk strains ST111 and ST235 were facilitated by precise control due to

* Corresponding author. Address: Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany. Tel.: +49 941 944 4897.
E-mail address: anca.rath@ukr.de (A. Rath).

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identification of different outbreak sources per strain, and by the in-silico detection of high-level QUATs resistance in all isolates.

Introduction

Pseudomonas aeruginosa (PA) is an opportunistic Gram-negative rod. Hospitalized patients, especially those in burns units, with immunosuppression or mechanical ventilation, and patients receiving broad-spectrum antimicrobial therapy, are particularly at risk of PA infections [1]. The morbidity and mortality rates among infected patients are associated with the presence of many intrinsic or acquired antimicrobial resistance (AMR) mechanisms in these bacteria, and the spread of multi-drug-resistant (MDR) organisms poses a great threat to the European population [2,3]. While the number of reported clinical PA isolates has been rising, the proportion of MDR isolates among them has decreased across Europe and Germany during the past years. According to EARS-Net, during 2019, the reported proportion of MDR PA isolates (resistant to three of five antimicrobials) reached 12.1% and 6.3% in Europe and Germany, respectively [4].

Infection prevention and control (IPC) of PA is difficult, as it is found ubiquitously in the environment with a preference for wet sources. The bacteria often inhabit wastewater pipes and form a single- or multi-species biofilm [5—7]. While this is widely accepted as normal and unavoidable, water sources in hospitals (e.g., water drains, U-bends, contaminated showers) are a critical source for outbreaks and infections in vulnerable patient populations [1,6,8—10]. Moreover, the strains detected in the wastewater of hospitals differ from strains detected in other water sources [11].

Various research groups have highlighted the importance of addressing these problems to reduce PA colonization and infections [10,12,13]. Numerous PA outbreaks involving water systems were documented worldwide [14]. However, efficient management remains challenging for two reasons: (i) heterogeneous results of outbreak containment measures; (ii) frequent failures to deliver evidence for the suspected transmission sources and the directionality of transmission [15].

In recent years, whole-genome sequencing (WGS) has proven very useful in IPC and in managing hospital outbreaks. It currently represents the most accurate tool for analysing isolate relatedness and outbreak dynamics [6,16]. The thereby-generated strain characterization and relatedness analysis enable more accurate identification of affected patients and bacterial fomites than ever before. Halstead *et al.*, for example, concluded from genotyping results that only 5% of affected patients "definitely" and approximately 50% "most likely" acquired their PA from water outlets in their hospital. Moreover, this may enable IPC specialists to achieve an easier understanding of complex transmission pathways [7,17,18]. Additionally, there appears to be a high variability of acquisition rates between different hospitals, which might reflect the different grades of contamination of outlet sources [18].

This issue is not limited to wet environments. PA's ability to enter and leave the persister state depending on environmental conditions promotes the survival of PA on inanimate surfaces for up to 16 months, depending on surface characteristics [19,20]. Thus, efficient disinfection is a central pillar in IPC. However, this is not as trivial as it appears when it comes to PA. Choosing disinfectants, adhering to concentration requirements, and contact time must be prioritized. Above all, disinfectants containing quaternary ammonium compounds (QUAT) have proven problematic for environments with a high PA load [21,22].

In this report, we present two simultaneous prolonged MDR PA outbreaks involving the high-risk PA sequence types (ST) ST111 and ST235 that affected the intensive care unit (ICU) of a pulmonary clinic in South Germany. The old building design, with sanitary facilities in each room, and high-level resistance to QUATs due to carriage of the qacE/qacEΔ1 appear to have been the main outbreak drivers. While the first outbreak was detected based on epidemiological data, the second outbreak, the inefficiency of disinfection, the different attack rate of the outbreak strains, and the difference in outbreak reservoirs and fomites could only be determined using WGS proactively.

Methods

Outbreak definition and management

Upon outbreak suspicion in September 2019, retrospective consultation of medical and laboratory records between January and August 2019 identified further patients who may have been involved in this outbreak. Data of all patients detected with MDR PA during 2019 at the affected ICU were considered for further investigations and decision-making for outbreak management. MDR PA was defined as strains fulfilling the criteria of the German 4MRGN classification (resistance to piperacillin, ceftazidime, cefepime, meropenem, imipenem and ciprofloxacin) and susceptibility to colistin [23]. For these patients, data on age, sex, time and length of hospital stay, days until the first MDR PA detection, clinical significance of the MDR PA strain (infection or colonization), clinical outcome and carbapenemase detection during routine diagnostics were collected. Nosocomial transmission was defined in accordance with the ECDC criteria [24].

At the ICU, screening measures were implemented for all patients immediately, and repeated weekly for four months. Additionally, all patients were screened on admission and before discharge from the ICU during this period. Patient standards of care were adjusted to avoid bacterial transfer between patients and water sources (Table I).

The Ethics Committee of the University Hospital of Regensburg approved the data analysis using the identification number 23-3465-104 from 24.01.2023.

Environmental investigations

The collection of environmental specimens started immediately. Water samples were drawn from every water tap and

Table I
Overview of outbreak management

General interventions	Healthcare worker education in hand hygiene Upgrading cleaning protocols (e.g., 2 × room disinfection after discharge of affected patients) Instruction and training of cleaning personnel Replacement of cleaning buckets and cleaning cart wheels Disinfection of cleaning carts	
	Before the outbreak	After outbreak detection
Screening	Risk adapted	On admission, on discharge, weekly during ICU stay
Patient hygiene	Washable washcloths, tap water, soap	Single-use impregnated washcloths Oral hygiene: packaged sterile water
Surface disinfection	Substance: QUATs Technique: immersion of washable cloths in disinfectant	Substance: oxygen peroxide Technique: packaged impregnated tissues
Cleansing of ventilation and inhalation devices	Tap water	Packaged sterile water
Disposal of fluids	Toilet or sink drain in patient rooms or unclean rooms	Large amounts: unclean room only Small amounts: garbage
Water-bearing systems	None	Use of sanitary facilities was prohibited until after two consecutive rounds of MDR-PA-free test results U-bends were replaced under supervision of an IPC nurse (except unclean room) Periodic disinfection with oxygen peroxide
Storage within room	Around or in the sink	Acquisition of mobile carts

ICU, intensive care unit; IPC, infection prevention and control; MDR PA, multi-drug-resistant *Pseudomonas aeruginosa*; QUAT, quaternary ammonium compound.

toilet, the descaling device, and the central water system disinfection device. To evaluate the outbreak's extent, water specimens were collected upstream and downstream of the ICU water supply line. If MDR PA was cultivated in a source, sampling of the affected source was repeated every four weeks. Once a source tested negative after disinfection, the testing period was lengthened to every three months. The collection intervals were extended to every six months in November 2020, after every tested location yielded at least three consecutive negative results and the outbreak was considered over.

Swabs were collected from each sink drain (SnD), shower drain, cleaning cart, and various surfaces in patient rooms (control panels of the monitoring device, ceiling arm units for ICU equipment, infusion pumps, remote controls, and built-in operator panels of the electric bed in rooms 07, 08, 17, 19, 20 and 22) (Figure 1). Because we identified MDR PA in the respiratory tract of two patients, all bronchoscopes and the bronchoscopy tower were also sampled.

Microbiological investigations

Water samples

Toilet water was collected using a sterile, disposable syringe (Becton Dickinson GmbH, Heidelberg, Germany), whereas tap water was flushed directly into the container. Both specimen types were collected in sterile plastic bottles containing sodium thiosulphate, 0.05 mol/L (LP Italiana SPA, Milano, Italy), and were immediately transported to the laboratory under cooling conditions. Investigations were performed following the German Drinking Water Regulation [25]. Briefly, 100 mL of the sampled water was filtered using a cellulose nitrate filter membrane (Sartorius stedim, Goettingen, Germany), and cultivated on cetrimide agar for 48 h at 36 °C with 5% CO_2. Species identification and AMR testing were performed when the culture yielded growth, as described below.

Swabs

Both patient and environmental swabs were effaced on ChromAgar ESBL (MAST Group Ltd, Reinfeld, Germany). Species identification was performed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MALDI BioTyper, Bruker, Bremen, Germany). AMR was determined using the disc diffusion method, and results were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 9.0). Carbapenemase screening was performed using the β-CARBA Test (Bio-Rad Laboratories Inc., Feldkirchen, Germany). Positive results were confirmed with Xpert Carba-R assay (Cepheid, Krefeld, Germany).

WGS

The bacteria were sub-cultivated on blood agar plates (produced in-house; Merck KGaA, Darmstadt, Germany). DNA was extracted from bacterial lawns cultivated for 24 h, and using the QIAmp DNA Mini kit (#51304; QIAGEn Diagnostics GmbH, Hilden, Germany), following the manufacturer's manual. The DNA concentration and purity were determined using

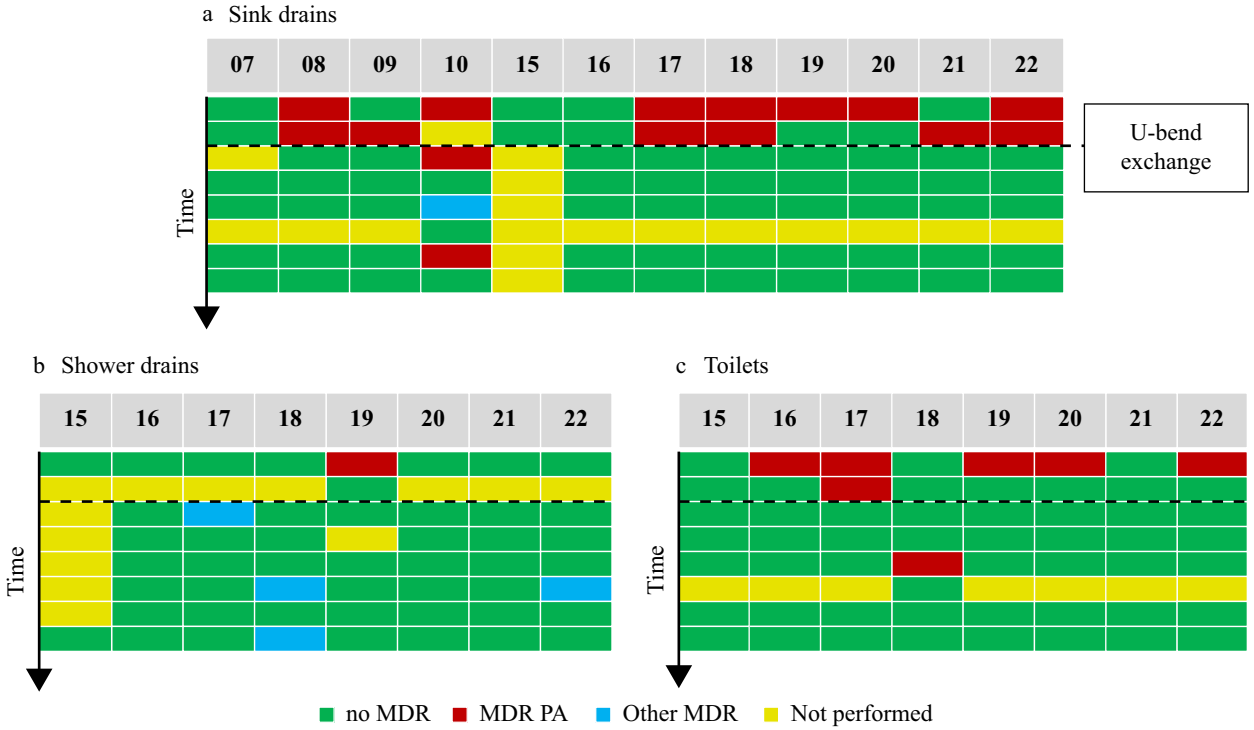

Figure 1. Timeline of environmental investigations. Probes from all sink drains (a), shower drains (b) and toilets (c) were collected at outbreak detection and repeatedly thereafter. (a) Multi-drug-resistant *Pseudomonas aeruginosa* (MDR PA) colonized most sink drains at outbreak detection. After disinfection procedures with an oxygen-releasing agent, most sinks remained positive, and some sink drains that tested negative initially were found to be positive for MDR PA. After U-bend exchange, no MDR PA was detected in sink drains. (b) Only two shower drains were positive for MDR PA. This reservoir was controlled by disinfection procedures. (c) Weekly disinfection measures were very effective in suppressing the growth of MDR PA in toilets.

Qubit (1 × dsDNA HS assay kit; Thermo Fisher Scientific, Bremen, Germany).

Library preparation was performed using the Illumina Nextera reagents (Illumina GmbH, Berlin, Germany), following the manufacturer's recommendations. Sequencing was performed on a MiniSeq Device (Illumina GmbH, Berlin, Germany), acquiring 2 × 150bp reads using a high-output cassette.

Analysis of WGS data

FastQ files derived by Illumina sequencing were analysed using SeqSphere+ software (RIDOM, Muenster, Version 7.1.0), and multi-locus sequence typing (MLST) and core-genome (cg) MLST were extracted from the draft genomic sequences. The derived sequence files were analysed by pairwise comparison using the SeqSphere+ integrated MLST (seven alleles) and cgMLST schemes (3867 alleles) [26].

In-silico AMR was determined using the NCBI AMRFinderPlus tool (v 3.2.3, database version 2019-10-30.1) of SeqSphere+, which uses the method published by Feldgarden *et al.* [27]. Only AMR genes with an alignment of 100% and identity of >90% were considered for further analysis.

Results

Setting

The outbreak occurred in the ICU of a 95-bed hospital in southern Germany specializing in pulmonary disease. Due to the hospital's profile, patients admitted to this department predominantly suffer from cystic fibrosis, bronchiectasis and chronic obstructive pulmonary disease.

The department is set in an area repurposed from a normal therapy ward into an ICU. Therefore, each room has a sanitary facility equipped with hospital pattern washbasins (no direct flow into the drain, no overflow holes), a shower and a toilet.

Case finding and outbreak description

The outbreak suspicion was triggered by the identification of two nosocomial MDR PA index cases in August 2019. The PA strains were both detected in respiratory materials, had identical AMR patterns and produced a VIM-2 carbapenemase. Based on this suspicion, the retrospective record analysis for January–August 2019 was initiated, and 29 MDR PA cases (including the initial two cases) were found. Ten of these cases qualified as nosocomial (seven men, three women), and five carried blaVIM-2 (Table II). The earliest isolate (P1) dated back to April 2019, whereas the fourth isolate (P4) was diagnosed in July. In September, VIM-2 carrying MDR PA was diagnosed in one further patient during screening procedures. Two patients died due to an infection with MDR PA (Table II). Non-nosocomial cases without VIM-2, OXA-2, or OXA-74 were not considered outbreak-related and, thus, not sequenced.

As shown in Figure 2a, the patients did not share a common room during their stay, but several overlapping stays at the affected ward were documented. Further, some patients were treated in up to three affected rooms during the outbreak (Figure 2a and b).

Environmental investigations and outbreak management

Environmental investigations

During outbreak investigations, all sink and shower drains, and cleaning devices (including carts and buckets) were swabbed in search of the outbreak's source. A total of 108 swabs from water drains (U-bends) of hand wash basins, 61 swabs from shower drains, and 59 toilet water samples were collected during the 15-month follow-up period across the affected department. The initial evaluation revealed that seven of 25 sink drains, two of 10 shower drains, one of six cleaning buckets, and five of eight toilet water samples were positive for MDR PA (Figure 1). In contrast, cultures outside the ICU did not yield any MDR PA, indicating that the outbreak was limited to this ICU.

Table II
Characteristics of patient isolates of multi-drug-resistant *Pseudomonas aeruginosa* (MDR PA) including relevant clinical information, results of multi-locus sequence typing and antimicrobial resistance genes of patient isolates

Patient	Age (years)	Sex	Entity (infection/ colonization)	Site of first isolation	Outcome	Time from admission to MDR PA acquisition (days)	ST	MBL	*bla* type
P1	62	F	Infection	Urine	Death	27	111	VIM-2	OXA-395
P2	79	M	Infection	Bronchial secretion	Death	78	111	VIM-2	OXA-395
P3	74	F	Colonization	Bronchial secretion	Discharge	2	111	VIM-2	OXA-395
P4	61	M	Colonization	Tracheal secretion	Discharge	20	111	VIM-2	OXA-395
P5	70	M	Colonization	Tracheal secretion	Discharge	7	111	VIM-2	OXA-395
P6	68	F	Colonization	Urine	Discharge	9	235	None	OXA-2, OXA-74 OXA-488
P7[a]	76	M	Colonization	Bronchial secretion	Discharge	Post-discharge	235	None	OXA-2, OXA-74 OXA-488
P8		M	Colonization	Bronchial secretion	Discharge	79	309	None	OXA-50 family
P9	59	M	Colonization	Sputum	Discharge	85	446	None	OXA-848
P10	62	M	Colonization	Tracheal secretion	Discharge	8	17	None	OXA-50

ST, sequence type; MBL, metallo-β-lactamase.
[a] MDR PA detected post-discharge.

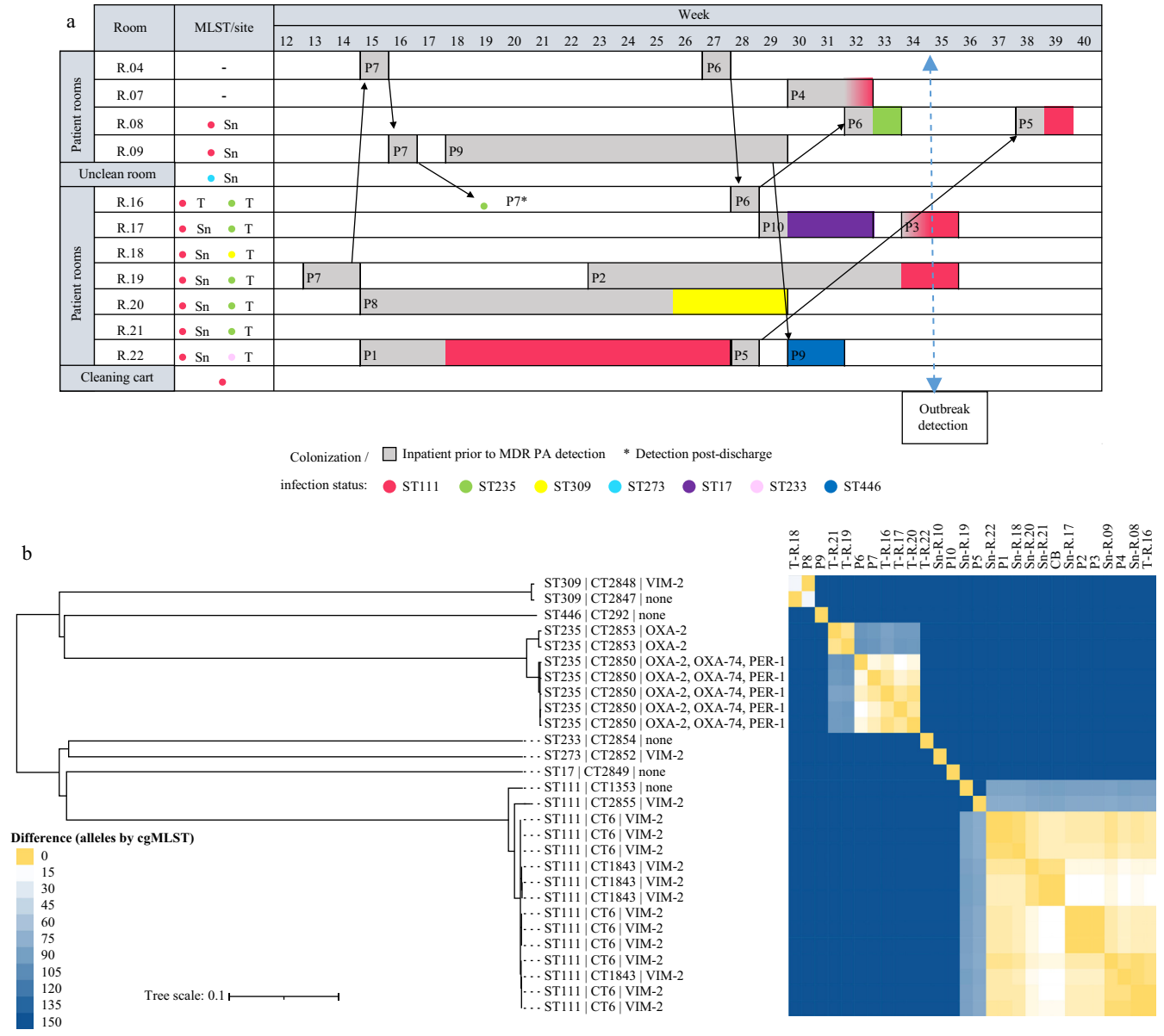

Figure 2. Distribution in space and time of patient isolates and environmental isolates according to the results of whole-genome sequencing (WGS), multi-locus sequence typing (MLST) and core-genome MLST (cgMLST). All patient isolates (P1—P10) and environmental isolates (T, toilet; Sn, sink drain; CB, cleaning bucket; R.04—R.22, room number 04—22) were sequenced and analysed by MLST (colour) and cgMLST (spanning distance). (a) The sequencing data was matched with accommodation data and positive sites per room. Arrows mark patient movement. (b) MLST identified seven sequence types (STs), of which three had more than one isolate, and two had sub-clusters (yellow and white areas) with difference of ≤11 alleles by cgMLST, and even identical isolates (yellow squares) in pairwise comparison by cgMLST. Sub-cluster 1 consists of ST111 isolates (13 isolates: CT6 and CT1843), followed by ST235 with Sub-Clusters 2 (five isolates: CT2850) and 3 (two isolates: CT2853). MDR PA, Multi-drug-resistant *Pseudomonas aeruginosa*.

Cultures from other sites in the rooms of affected patients, from the bronchoscopy equipment, from the descaling device, from the water disinfection system and the cleaning machine did not show any growth of MDR PA.

IPC interventions

QUAT-containing disinfectants were immediately replaced with oxygen-peroxide-containing products. After extensive disinfection procedures, six sink drains (including two that initially tested negative) and one toilet were still positive, whereas shower drains were free of MDR PA (Figure 1). With the replacement of all U-bends (except for the unclean room due to the high risk of recontamination during the disposal of body fluids and greywater), no more MDR PA was cultivated from sink drains in patient rooms. The permanent switched to and use of oxygen peroxide in the unclean room was successful for six months until blaVIM-positive *Enterobacterales* (*Klebsiella spp.*, *Citrobacter freundii)* were detected in this drain, followed by a blaVIM-positive PA isolate one month later. The ST of the PA isolate did not match the outbreak isolates (data not shown).

Six of 14 initial toilet water samples were positive for MDR PA, but only two were contaminated by blaVIM-2-positive MDR

PA strains. Further, in one toilet water sample (R.16, Figure 2), we found both a blaVIM-2-positive and a blaVIM-2-negative strain. Four of the rooms with contaminated toilets were inhabited by affected patients at some point during 2019. Despite periodic disinfection, the toilet in room 07 remained positive for MDR PA until December 2019 (Figure 1). One initially MDR-PA-positive toilet remained negative for seven months and then tested positive again in April 2020.

Sequencing results

All available MDR PA from environmental sources (18 isolates) and one isolate per patient (10 isolates) were collected and sequenced by WGS within one week of detection. Although one isolate was detected post-discharge (P7), it was considered nosocomial and was thus also included. Moreover, the isolate had AMR mechanisms similar to non-VIM-2-producing isolate P6 according to routine diagnostics.

MLST

MLSTyping based on WGS data revealed seven different strain types (ST): ST111, ST235, ST309, ST233, ST273, ST446 and ST17, of which two accounted for 78.6% of all isolates. Fifteen isolates stemming from patients (P) 1 to 5, and nine environmental sites (predominantly SnD) were assigned to ST111 (see Figure 2). The second-largest group is represented by ST235 and includes seven isolates: P6, P7, and five T isolates. Among the other isolates, two (P8 and T-R.18) belonged to ST309, of which only T-R.18 was blaVIM-2 positive. The remaining four were identified as singletons (including blaVIM-2-positive ST273 isolate in the unclean room).

One additional strain was detected in December 2019 in a toilet that had been free of MDR PA for seven months. However, the strain type found in this toilet did not match any of the two outbreak strains according to MLST, but that of the patient inhabiting the room at that moment (data not shown).

cgMLST

Using cgMLST, we detected low clonality among isolates of the same ST ($\leq$150 alleles in pairwise comparison by cgMLST, as opposed to $>$3000 between STs). Within the same ST, isolates differed by a median of six (ST111, range 0—150 alleles) and seven (ST235, range 0—99 alleles) alleles, respectively, in pairwise comparison by cgMLST. After pairwise comparison by cgMLST of all isolates, the necessity for a narrower definition arose. Both within ST111 and ST235 we identified groups of isolates which differed by $\leq$11 as opposed to $\geq$78 alleles from the other isolates within the same ST. Three sub-clusters were identified thereby (Figure 2b): Sub-Cluster 1 included 13 of 15 ST111 (CT6 and CT1843) isolates, whereas ST235 comprises Sub-Cluster 2 (five isolates including P6 and P7 — CT2850) and Sub-Cluster 3 (two isolates — CT2853). If the definition of the cluster was narrowed even further to $\leq$3 alleles as it has been suggested for enterococci, all isolates within Cluster 1 still formed smaller clusters of up to three isolates and 18 matching pairs were found, whereas only two ST235 isolates still met clustering criteria [28].

The line list represented in Figure 2a depicts the patient movement around the department and the distribution of MDR PA strains across the department. Pairwise comparison according to cgMLST results showed that only P2 and P3 carried isolates with no allelic difference (by cgMLST) to isolates detected in the SnD of rooms they inhabited. P2 and P5—7 stayed in rooms contaminated with isolates of the same ST, whereas P6 only differed by seven alleles from the isolate found in the toilet. In the rooms of P6 and P7, however, additional strains of different ST were found. Finally, while MDR PA of a different ST was found in the room of P8, P9, and P10, no MDR PA was detected in the rooms of P4. Moreover, among the 18 pairs within ST111 which differ by 0—3 alleles according to cgMLST, five patients match isolates detected in other rooms including one patient who matched two such isolates. The criteria were also met twice for neighbouring rooms.

In-silico AMR

The in-silico AMR detection confirmed the presence of blaVIM-2 in isolates assigned to three different sequence types: ST111 (14 isolates), ST309 (one isolate), and ST273 (one isolate). Six of seven ST235 isolates were carbapenemase negative according to WGS, whereas one isolate carried a class A carbapenemase, blaGES. Additionally, all ST235 strains were positive for blaOXA-2, and five for blaOXA-74 and blaPER-1. Further genes that induce resistance to beta-lactam antibiotics were detected and are depicted in Table II (patients) and Table III (environmental).

All patient and environmental isolates were also found to carry the qacEΔ1 (21 isolates) or the qacE (one isolate) gene, which are responsible for enhanced resistance to QUATs.

Discussion

PA is known to be a leading cause of nosocomial outbreaks, which are linked to water sources and insufficient cleaning of medical devices [9]. We described two parallel outbreaks of MDR PA strains with in-silico high-level resistance to QUATs, which were detected in August 2019 in the ICU of a pneumology clinic in southern Bavaria. The outbreak scenario was solved using a WGS-based investigation approach.

Our analyses revealed 10 nosocomial MDR PA detections during 2019, but only five of these isolates produced VIM-2. VIM-2 is the most common carbapenemase in PA in Germany, and can be detected in different PA strains [29]. As no obvious link was detected between these patients, the outbreak still had to be confirmed. In recent years, WGS has proven to be a useful tool in outbreak management. Its benefit lies in the detailed insight into clonality of outbreak isolates that it provides, which can help detect outbreak sources and transmission events more accurately. Thus, isolate relatedness was evaluated by WGS, and data analysis using MLST, cgMLST, and in-silico AMR detection was performed. Thereby, both the variable VIM-2 detection and a polyclonal outbreak were confirmed. According to MLST, P1-5 isolates were assigned to ST111, P6 and P7 to ST235, while the rest were singletons of different ST. ST111 and ST235 are found worldwide, and they are considered 'high-risk' clones due to frequent association with hospital outbreaks [30]. Further analysis identified three sub-clusters of ST111 and ST235 with an allelic difference of $\leq$11 alleles in pairwise comparison by cgMLST. Not only is such low diversity plausible to occur during an outbreak, but it strengthens the assumption that these findings do not merely reflect typical sewage flora [11]. Second, the general population structure of PA is rather network-like, not monoclonal [17]. Although this makes source attribution and tracing of PA more difficult, it

Table III
Antimicrobial resistance genes of the environmental multi-drug-resistant *Pseudomonas aeruginosa* isolates

ID	ST	MBL	bla	Biocide
Sn-R.08	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn.R.09	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
T-R.16	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn-R.17	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn-R.18	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn-R.19	ST111	VIM-2	$bla_{OXA-395}$	qacE
Sn-R.20	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn-R.21	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
Sn-R.22	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
CB	ST111	VIM-2	$bla_{OXA-395}$	qacEΔ1
T-R.16	ST235	none	bla_{OXA-2}, $bla_{OXA-488}$, bla_{OXA-74}, bla_{PER-1}	qacEΔ1
T-R.17	ST235	none	bla_{OXA-2}, $bla_{OXA-488}$, bla_{OXA-74}, bla_{PER-1}	qacEΔ1
T-R.20	ST235	none	bla_{OXA-2}, $bla_{OXA-488}$, bla_{OXA-74}, bla_{PER-1}	qacEΔ1
T-R.21	ST235	none	bla_{OXA-2}, $bla_{OXA-488}$	qacEΔ1
T-R.19	ST235	GES	bla_{OXA-2}, $bla_{OXA-488}$, $bla_{class\ D}$	qacEΔ1
T-R.22	ST233	none	$bla_{OXA-486}$	none
T-R.18	ST308	VIM-2	bla_{OXA-50}	qacEΔ1

could represent an even better explanation for the genesis of these sub-clusters.

As a common outbreak source was suspected, but still unknown, environmental probing was initiated immediately. The results showed gross environmental contamination with MDR PA with a clear dominance of VIM-2-producing strains in SnD (including eight ST111 isolates) and blaVIM-2-negative (including five ST235 isolates) strains in toilets, respectively (Figure 2 and Table III).

Even though the directionality of bacterial transfer remains unclear in most cases of this outbreak, we suspect that the two strains might have been dispersed across the ICU through different pathways. This is supported by the dominance of ST111 in SnD, and ST235 in the toilet. Although the initial outbreak source could not be determined for ST111, its spread might have been facilitated by a contaminated cleaning bucket. For ST235, conversely, both patients had stayed in rooms tested positive for this particular strain at some point (Figure 2a). However, the evaluation of transfer directionality is impaired by the fact that environmental sampling was carried out only after the strains were detected in both patients.

The assumptions above, however, have to be assessed critically and the limitations of the laboratory methods must be considered. First, only one colony per site and colony morphology were examined by WGS. Secondly, colony selection, isolation site, and culture conditions may influence the quantified allelic difference. Thirdly, the relatedness level by MLST and cgMLST could be overestimated. Although they are established as a reliable method for strain comparison during outbreak management, it should be mentioned that its main limitation is the use of only the defined 3867 alleles, and not the whole genome [26]. This might falsely generate the impression of too low or too high genetic diversity. Nevertheless, genome plasticity and missing data regarding cut-off values for whole-genome comparison make the use of only certain, highly conserved genome segments the most feasible tool for strain comparison on a daily basis. Finally, low transmission incidence and prolonged events are not unusual in outbreaks involving PA, which can benefit the accumulation of SNP [1].

Furthermore, we suspect that the routinely used QUAT disinfectants promoted the outbreak. The patients treated at this facility are prone to PA infection due to chronic lung disease. Thus, the import of PA is particularly high and makes the choice of disinfectants of utter importance. QUATs, however, are known to be less effective against Gram-negative bacteria. This hypothesis is sustained by the identification of the qacE and qacEΔ1 genes in all outbreak isolates (Table III) [31,32]. Moreover, the switch from QUATs to an oxygen-releasing disinfectant, and finally, after the outbreak ended, to a disinfectant based on glucoprotamine, immediately terminated the spread of MDR PA. After September 2019, MDR PA was only detected in the unclean room, and in one toilet. The toilet isolate, however, matched the isolate found in the patient residing there, but not the outbreak isolates (data not shown). Thus, the directionality of this coincidence is unclear. The isolate in the unclean room was also not closely related to the outbreak isolates. Thus, it appears more likely that it was inoculated during disposal of the grey water from the department. Moreover, as the U-bend was not exchanged here, it could have already inhabited the sink drain biofilm throughout the study period.

Finally, water sources are well known to promote PA outbreaks in hospitals [8]. The affected ICU was retrofitted from a normal ward and thus did not meet recommendations to avoid water sources in departments treating vulnerable patients (e.g., ICU). U-bends in particular have been shown to be problematic in various outbreak reports due to device geometry and difficulties in eliminating biofilm, whereas the elimination of MDR PA from the water-carrying systems is frustrating and rarely successful [8]. Therefore, replacing these components is recommended to end an outbreak. Garvey *et al.* recorded a 50% reduction in clinical isolates of PA after replacing contaminated tap outlets [9]. The risk for recontamination of sinks is high in the clinical setting due to their misuse for disposal of greywater or body fluids, and placement of patient care materials adjacent to sinks [35]. As these appear to be the most likely transmission route(s) during this event, major elements in outbreak management were behavioural measures and healthcare worker education such as

limitation of greywater disposal exclusively into a designated sink drain in the unclean room, and education on hand hygiene (Table I). Further, mobile carts were purchased for storage to avoid the transfer of bacteria by fomites deposited near water sources.

In conclusion, IPC strategies should make use of modern diagnostics. For these outbreaks, proactive WGS implementation was indispensable in outbreak management. Its high resolution not only confirmed the outbreak suspicion involving the VIM-2-positive ST111 strain, but also detected an unsuspected simultaneous outbreak involving blaVIM-2-negative ST235 MDR PA. Outbreak confirmation was necessary in both cases as the presence of VIM-2 is neither limited to ST111, nor is it proof of isolate relatedness [29]. Secondly, as the ST235 isolates did not stand out through morphological or AMR criteria, the relatedness between these isolates, and therefore the outbreak involving two patients and five toilets, would have been overlooked without WGS. Moreover, although all ST235 isolates were shown to carry rarely described beta-lactamases suspected to cause carbapenem resistance (OXA-2 and OXA-74), these are not detected during routine diagnostics [36]. The identification of this strain is even more dramatic, as they all carried a type II secretion system, exoU, which is associated with poor clinical outcomes [33,34].

Even though the transmission pathways could not be elucidated completely, by using the genetic data, we could narrow the number of involved patients and environmental sites and establish a tailored monitoring system. Moreover, WGS revealed that different environmental sites were affected by different strains with different attack rates. This realization enabled these sources and potential fomites to be addressed quickly, and thus hastened outbreak termination.

Finally, the high number of potential outbreak sources strengthens the current policy of avoiding 'one suits all' strategies in IPC. In high-risk environments, such as ICUs, water sources in patient rooms should be avoided, and disinfectant choice should be driven by expected bacterial spectrum (e.g., avoid QUATs in pulmonary clinics due insufficient efficiency against Gram-negative bacteria).

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