Journal of Hospital Infection 150 (2024) 96–104

Available online at www.sciencedirect.com

Journal of Hospital Infection

journal homepage: www.elsevier.com/locate/jhin

Retrospective genome-oriented analysis reveals low transmission rate of multidrug-resistant *Pseudomonas aeruginosa* from contaminated toilets at a bone marrow transplant unit

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ARTICLE INFO

Article history:
Received 11 March 2024
Accepted 28 May 2024
Available online 1 June 2024

Keywords:
Infection control
Genome-oriented infection control
Whole-genome sequencing
Pseudomonas aeruginosa
Outbreak investigation

SUMMARY

Background: Prevention of toilet-to-patient transmission of multidrug-resistant *Pseudomonas aeruginosa* (MDR PA) poses management-related challenges at many bone marrow transplant units (BMTUs).

Aim: To conduct a longitudinal retrospective analysis of the toilet-to-patient transmission rate for MDR PA under existing infection control (IC) measures at a BMTU with persistent MDR PA toilet colonization.

Methods: The local IC bundle comprised: (1) patient education regarding IC; (2) routine patient screening; (3) toilet flushing volume of 9 L; (4) bromination of toilet water tanks, and (5) toilet decontamination using hydrogen peroxide. Toilet water was sampled periodically between 2016 and 2021 (minimum every three months: 26 intervals). Upon MDR PA detection, disinfection and re-sampling were repeated until $\leq$3 cfu/100 mL was reached. Whole-genome sequencing (WGS) was performed retrospectively on all available MDR PA isolates (90 out of 117 positive environmental samples, 10 out of 14 patients, including nine nosocomial).

Findings: WGS of patient isolates identified six sequence types (STs), with ST235/CT1352/FIM-1 and ST309/CT3049/no-carbapenemase being predominant (three isolates each). Environmental sampling consistently identified MDR PA ST235 (65.5% ST235/CT1352/FIM-1), showing low genetic diversity (difference of $\leq$29 alleles by core-genome multi-locus sequence typing (cgMLST)). This indicates that direct toilet-to-patient transmission was infrequent although MDR PA was widespread (detection on 79 occasions, detection in every toilet). Only three MDR PA patient isolates can be attributed to the ST235/CT1352/FIM-1 toilet MRD PA population over six years.

Conclusion: Stringent targeted toilet disinfection can reduce the potential risk for MDR PA acquisition by patients.

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https://doi.org/10.1016/j.jhin.2024.05.015

Introduction

Infection prevention and control (IPC) are critical in healthcare settings, particularly in units caring for patients with high risk for severe infections due to immunosuppression, i.e. bone marrow transplant units (BMTUs) [1]. This makes the challenge of ensuring highest standards of infrastructure essential. Currently, Germany hosts 76 operational BMTUs, undertaking transplantation for more than 3500 patients annually [1].

The acquisition and consequent infections due to *Pseudomonas aeruginosa* (PA) — especially involving multidrug-resistant (MDR) strains — are of particular concern at BMTUs due to high associated mortality [2,3]. The wastewater disposal system (i.e. toilets, drainpipes) has repeatedly been identified as an outbreak source in such settings [4—8]. The long-term persistence of MDR PA in water reservoirs is promoted by the formation of multi-species biofilms, which generate permanent sources for pathogenic bacteria in close proximity to the patients. The bacteria can then be mobilized during toilet flushing, being dispersed as bacteria-carrying aerosols and contaminating surfaces over an area of >1 m around the toilet [9—12]. Given the long hospitalization period of these patients, proper disinfection management of these reservoirs is crucial concerning patient safety [7,13]. Advancements in sanitary technology such as rimless toilets and built-in disinfection systems for water reservoirs have become available and offer potential solutions to these hazards. However, many German BMTUs predate these innovations, which leaves them ill-equipped to address these challenges effectively, whereas refurbishing is expensive and requires extended effort. Consequently, there is still a great need for knowledge on efficient and affordable IPC practices to prevent nosocomial acquisition of MDR PA from toilets.

At the BMTU of our tertiary care hospital in Germany, MDR PA was detected in toilets more than 20 years ago (data not shown). After pursuing unsuccessful attempts to eradicate the bacteria, a simple IPC bundle (ICB) focusing on disinfection (bromination and hydrogen peroxide), education, and surveillance was implemented that — to the authors' best knowledge — has not been described before [14].

This project aimed to evaluate the efficiency of this bundle retrospectively using a genome-oriented approach. Given that since 2016 our institution stores all MDR bacteria detected during routine investigations, we set out to use the unique opportunity to evaluate the possible toilet-to-patient transmission rates for MDR PA during 2016—2021 using whole-genome sequencing (WGS) under the implemented IPC measures.

Methods

Setting

This analysis was conducted retrospectively at a BMTU, where 1898 patients received treatment between 2016 and 2021 with an average stay of 22 days. Of these, 435 patients received allogenic BMT (most frequent diagnosis: 34.7% acute myeloid leukaemia and 13.8% high risk myelodysplastic syndrome), and 352 patients received autologous BMT (most frequent diagnosis: 72.7% multiple myeloma and 16.7% non-Hodgkin lymphoma). The standard for antibiotic prophylaxis included intestinal decolonization with rifaximin for allogenic BMT, whereas ciprofloxacin prophylaxis was administered during neutropenia after autologous BMT. Data on antibiotics consumption was kindly made available from the ADKA-If-DGI project database and is shown in Table III.

The BMTU comprises 16 single-bed rooms, each equipped with a ventilation system, and en-suite bathrooms featuring rimless toilets. Showers were out of service throughout the studied period, and body hygiene was performed with wet wipes. Taps were permanently filtered, and starting in 2018, the drinking water system was treated with chlorine dioxide at a concentration of 0.22 mg/L, whereas patients were instructed to drink only packaged water. Wastewater from each room was either directly drained to the building's main wastewater system (R.1, R.12, R.14, R.17) or linked via a short pipe connecting two rooms to a common pipe draining to the main system (R.2/R.3, R.4/R.5, R.6/R.7, R.8/R.9, R.10/R.11, R.15/R.16). Routine surface disinfection was performed with a glucoprotamine-based product (Incidin™ plus; Ecolab Healthcare, Monheim am Rhein, Germany) and disinfecting ready-to-use wipes containing an oxygen-releasing agent (OxyWipeS; Ecolab Healthcare). All frequent contact surfaces and bathrooms were disinfected at least daily. When MDR organisms (MDRO) were diagnosed, final room disinfection was performed with an oxygen-releasing agent (perform®; Schülke & Mayr GmbH, Norderstedt, Germany).

IPC bundle

Due to the persistent MDR PA (meeting German 4-MRGN criteria) colonization of toilets and shower drains that lasted more than a decade prior to the period analysed here (2016—2021), an infection control bundle (ICB) was already implemented as standard of care before 2016 [15]. This bundle was designed to reduce exposure to MDR PA and appears to be novel in existing literature [14].

The bundle consisted of the following components:

A. *Patients and clinical surveillance*

Routine screenings for MDROs were conducted for all BMTU patients upon admission, every three weeks during treatment, and at discharge using oral and rectal swabs.

B. *Educational elements*

On admission, all patients were instructed to close the lid before flushing the toilet and educated regarding IPC-promoting behaviour, especially hand hygiene. Healthcare workers (HCW) were repeatedly educated (minimum yearly) in

specific IPC (i.e. transmission pathways of MDR PA, protective behaviour including hand hygiene).

C. *Environmental management and surveillance*

Environmental interventions were focused on aggressive toilet disinfection, including continuous bromination (40 g bromine tablets inserted into water tanks every three weeks; Bayrol Deutschland GmbH, Planegg, Germany), and increased flush volume (9 L).

Water samples from all toilets were screened for Gram-negative MDROs at least quarterly. For this purpose, water (250 mL) was collected from toilet vessels using sterile, disposable syringes (Becton Dickinson GmbH, Heidelberg, Germany). The samples were transported directly to the laboratory in sterile plastic bottles containing sodium thiosulphate, 0.05 mol/L (LP Italiana SPA, Milano, Italy). One hundred millilitres of the water samples were filtered through a cellulose nitrate filter membrane (Sartorius stedim, Goettingen, Germany), which was then transferred on to cetrimide agar and cultivated for 48 h at 36 °C with 5% CO_2. If bacterial growth was detected, species identification with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF microflex; Bruker, Bremen, Germany) and antimicrobial resistance testing (AMR) testing using disc diffusion were performed.

Thereafter, detected MDR PA triggered toilet disinfection using an oxygen-releasing agent (perform®; Schülke & Mayr GmbH, Norderstedt, Germany) and subsequent re-sampling. This procedure was repeated as often as needed until contamination was reduced to <4 colony-forming units (cfu) per 100 mL.

Isolate selection for WGS

Since 2016, our institution has built a strain collection of MDRO — including MDR PA — by systematically storing all isolates detected during routine diagnostics and environmental sampling at −80 °C. All available isolates were derived from this collection as described below and thawed for further investigations.

A. *Patients and patient isolates*

All patients diagnosed with MDR PA during 2016—2021 were identified retrospectively using database queries. Sequenced isolates were derived from routine diagnostics (various sample types) (Table I). Additionally, hospitalization data including length of stay, MDR PA status, diagnosis of graft-versus-host disease, and clinical outcome were collected from patient records.

B. *Environmental isolates*

All available isolates from toilet water samples (including isolates from re-sampling) were identified retrospectively from the isolate collection and included in this analysis.

WGS procedure

DNA extraction was performed after sub-cultivation of a single colony per thawed sample and colony morphology on blood agar plates for 24 h (produced in-house; Merck KGaA,

Table I
Characteristics of MDR PA patients

No.	Sex	Age	Sample type	Sample collection	Diagnosis	Detection status	Continuous hospitalization days between BMT and MDR PA detection	Death associated with MDR PA	ST	CT	Carbapenemase
1	M	60	Respiratory	Mar 2017	Pneumonia	Nosocomial	48	Yes	379	3112	None
2	M	70	Screening	Jan 2018	Colonization	Non-nosocomial	–	–	–	–	–
3	M	58	Respiratory	Oct 2018	Pneumonia	Nosocomial	42	Yes	235	1352	blaFIM-1
4	M	63	Blood culture	Jan 2019	Pneumonia	Nosocomial	75	Yes	27	3097	None
5	M	62	Respiratory	Mar 2019	Colonization	Nosocomial	74	Probably	309	3049	None
6	F	49	Urine	Sep 2019	Colonization	Nosocomial	57	–	235	1352	blaFIM-1
7	M	51	Screening	Jul 2019	Colonization	Non-nosocomial	–	–	–	–	–
8	M	59	Screening	Sep 2019	Abscess, abdominal	Nosocomial	No BMT	Probably	309	3049	None
9	F	46	Skin biopsy	Jan 2021	Ekthyma gangraenosum	Nosocomial	232	Unclear	235	1352	blaFIM-1
10	M	63	Urine	Aug 2020	Colonization	Nosocomial	33	–	–	–	–
11	M	55	Screening	Nov 2020	Pneumonia	Non-nosocomial	–	–	207	2606	None
12	M	59	Respiratory	Jan 2021	Pneumonia	Nosocomial	21	Yes	309	3049	None
13	F	56	Respiratory	No data	Unclear	Non-nosocomial	–	–	–	–	–
14	M	74	Urine	May 2021	Urinary tract infection	Nosocomial	No BMT	–	270	3101	None

MDR PA, multidrug-resistant *Pseudomonas aeruginosa*; BMT, bone marrow transplantation; ST, sequence type; CT, complex type.

Table II
Timeline of MDR PA detection, disinfection frequency and strain availability in contaminated toilets

Room	2016					2017				2018				2019					2020				2021			
	Jan	Mar	Jun	Sep	Nov	Mar	Jun	Oct	Dec	Mar	Jun	Sep	Dec	Mar	May	Jul	Sep	Dec	Mar	Jun	Sep	Dec	Mar	Jun	Sep	Dec
1				3••••	1•	1•	1•							1•			1•										
2												1•	1•	3•••			2••		1•						1•	
3	1•				1•	1•								1•					1•						1•	
4						4•••••			1•		1•			3•••			2!!•••	1•	1•		3•••			1•		
5		3••••						1•		1•													1•		4•••••	1•
6						1•											1•						1•		1•	
7								1•		1!••			1•		1•		1•				1•	1•			2•••	
8					1•												1•								1•	
9									1•								1•	1!••	2•••					1•	2•••	1•
10						4•••••										1•	2•••		1•						1•	
11			1•		1•	1•						1•					2•••			3••••			3••••			
12									1•																1•	
14						1•													1•						1•	
15										1•	1•			1•			1•					1•				
16															1•											
17																				3!!••••						

1–4 - number of disinfections until the threshold ≤3 cfu/ml was reached. Sample available for sequencing: • yes; • no.

!! – The disinfection series was interrupted for unknown reasons before the threshold was reached.

Darmstadt, Germany). Bacterial identification was performed with MALDI-ToF (Bruker). Extraction was performed according to the manufacturer's instructions using the QIAmp DNA Mini kit (#51304; Qiagen Diagnostics GmbH, Hilden, Germany). Extraction quality (DNA concentration and purity) was assessed with Qubit (1× dsDNA HS assay kit; Thermo Fisher Scientific, Bremen, Germany), whereas the Illumina Nextera reagents (Illumina GmbH, Berlin, Germany) were used for library preparation. Thereafter, short-read sequencing (acquisition of 2× 150 bp reads) was performed using a mid-output cassette on a NextSeq 550Dx Device (Illumina GmbH, Berlin, Germany).

Analysis of WGS data

Isolates were characterized using multi-locus sequence typing (MLST), core-genome (cg) MLST, and in silico AMR (isAMR) [15]. The data were extracted from the draft genomic sequences (FastQ files) and analysed using the SeqSphere+ software (Version 7.1.0; Ridom, Muenster, Germany) including the integrated NCBI AMRFinderPlus tool (v 3.2.3, database version 2019-10-30.1) [16,17]. Quality check was performed with the Aquamis pipeline [18]. iTOL was used for heatmap generation [1].

Results

Patient data

Overall, 14 out of 1898 patients were diagnosed with MDR PA infections (eight cases) or carriage (six cases), of which 10 were nosocomial (Table I). The initial detection occurred 21—232 days post admission (average: 52 days; median: 73 days; Table I). Six of the seven deceased patients were 'likely' or 'definitively' linked to these infections according to the physician's letter (Table I).

WGS of patient isolates

WGS was performed on all obtainable isolates from patients diagnosed with MDR PA, covering 10 of the 14 patients, including nine of 10 isolates from nosocomial cases (Table I). According to MLST and cgMLST, isolates were assigned to six different sequence (ST) and complex types (CT): ST235/CT1352 (three isolates), ST309/CT3049 (three isolates), ST27/CT3049, ST207/CT2606, ST270/CT3101, and ST379/CT3112. isAMR detected the *bla*FIM-1 carbapenemase gene in all ST235/CT1352 isolates, while no other isolates harboured a

Table III
Consumption of selected antibiotics at the bone marrow transplant unit

Substance	2016	2017	2018	2019	2020	2021
Aminopenicillins with β-lactamase inhibitor	1.5	1.2	2.9	2.0	2.1	3.0
Broad-spectrum penicillins	14.3	12.7	15.3	15.0	17.0	21.5
3rd or 4th generation cephalosporins	5.0	3.3	4.6	6.3	5.3	3.3
Carbapenems	26.7	19.4	24.0	26.7	22.5	30.9
Fluoroquinolones	23.8	23.4	31.4	26.2	19.8	18.2
Glycopeptides	25.9	20.2	26.5	31.5	24.9	43.7
Sulfamethoxazole/trimethoprim[a]	12.3	14.6	12.2	13.5	16.2	14.0

Units: defined daily dose per 100 hospitalization days.
[a] Predominantly used for prophylaxis.

carbapenemase gene. Further, the exoU virulence gene was detected in all ST235, ST309, and ST207 isolates, but not in ST27.

Further data analysis of clustering isolates was performed using pairwise comparison by cgMLST. Hereby, three ST235/CT1352 isolates differed only by ≤6 alleles, whereas no epidemiological link was found between these patients (Figure 1). The three ST309/CT3049 isolates differed by ≤4 alleles (Figure 1). Clinical data revealed the simultaneous hospitalization of two patients for one month, whereas the third patient was only admitted 10 months after the second patient left.

Environmental investigations

After conducting 26 sampling rounds (comprising 442 toilet water samples) 79 contamination events were detected (Table II). Per episode, MDR PA was cultivated from one to 10 toilets across the department (average: 3; median: 2), requiring one to four disinfections (average: 1.4; median: 1) to achieve acceptable levels of decontamination. Two toilets were only decontaminated twice, irrespective of the detection of 32 and 28 cfu/100 mL, respectively, after the last disinfection. Every toilet experienced MDR PA growth at least once, with some rooms showing contamination up to nine times (average: 5; median: 5).

Molecular environmental investigations

Out of 117 environmental MDR PA isolates, 90 were available for further study (Table II). Sadly, the preservation of several isolates was lost due to COVID-19-related difficulties. Nevertheless, for 64 out of 79 episodes, isolates from the initial toilet water investigations per interval and 26 isolates from post-disinfection re-sampling were available. All isolates were sequenced, and all were identified as the high-risk clone ST235 with differences of ≤29 alleles by cgMLST in pairwise comparison. cgMLST, however, detected four different complex types: CT1352 (64 isolates including 51 from initial samples), CT3051 (16 isolates including seven from initial samples), CT3054 (three isolates), and CT3055 (seven isolates).

Isolates found in the same toilet vessel during different sampling intervals varied by an average of eight alleles in pairwise comparison (range: 0–25; median: 4; see Supplementary Appendix 1). It is important to emphasize that cultivation from the same toilet was the common denominator for most clusters shown in Figure 1 (column E). Moreover, isolates detected from the same toilet vessel during the same interval (by re-sampling after disinfection) not only had the same ST and CT in 12 out of 17 cases, but also differed by a median of 0 alleles (cgMLST) (Supplementary Appendix 3).

The greatest variability was detected when comparing isolates collected across the department on the same day. Per interval, the pairwise comparison revealed differences of 13 alleles on average (range: 0–28; median: 13; see Supplementary Appendix 2). This suggests that mitigation from one room to another was a less probable cause for MDR PA growth in toilet vessels than persistence in the vessel.

The isAMR analysis found two different carbapenemases to be present in all but one environmental isolate: FIM-1 (66 isolates) and VIM-2 (22 isolates). One additional isolate carried both carbapenemases. However, the distribution of the carbapenemases was not fully congruent with the cgMLSTyping. Although all ST235/CT3055 isolates and 59 out of 64 ST235/CT1352 isolates were positive for FIM-1, three of the latter carried blaVIM-2 and one isolate carried both FIM-1 and VIM-2. Further, all isolates typed as ST235/CT3051 and ST235/CT3054 carried blaVIM-2.

Analysis of possible MDR PA transmissions

WGS results demonstrate that MDR PA toilet-to-patient transmissions were infrequent throughout the entire analysed period (six years). The strain ST235/CT1352/FIM-1 was only detected in three of 10 sequenced patient isolates (patients 3, 6, and 9; Table I and Figure 1), with no evidence of simultaneous hospital treatment, suggesting unlikely patient-to-patient transmission. The overall low genetic diversity among isolates over time (difference in alleles by cgMLST: ≤29 between ST235 isolates, and ≤20 between CT1352 isolates) reinforces the likelihood that strain acquisition from toilets is the most likely scenario.

Further, three patients (patients 3, 8, and 12; Table I and Figure 1) had closely related strains of the type ST309/CT3049/no-carbapenemase. The hospitalization data revealed that the first two patients who acquired the strain had stayed in the BMTU simultaneously for one month (Figure 1). Patient-to-patient transmission is, thus, plausible. The third patient, however, was admitted 10 months after the second patient had been discharged. This strain was not detected in toilet water.

Discussion

Despite permanent implementation of the described ICB, MDR PA was repeatedly detected at the BMTU in toilets

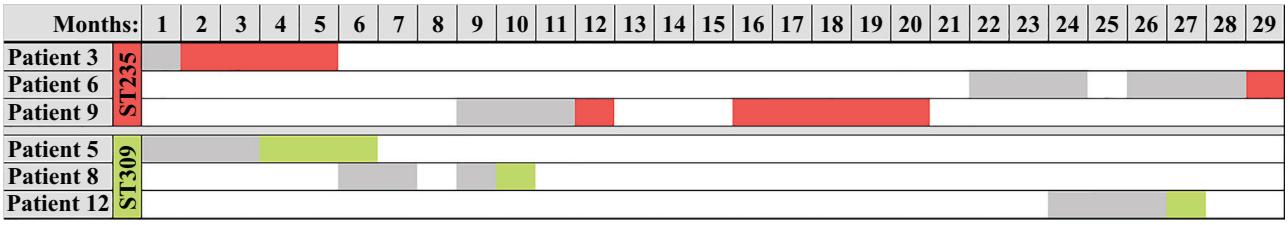

Figure 1. Timeline of multidrug-resistant *Pseudomonas aeruginosa* (MDR PA) sequence type (ST)235 and ST309 detection in patients. The three patients with MDR PA ST235 isolates had no overlapping hospitalization periods. By contrast, two of the patients with ST309 were hospitalized simultaneously for a month. Patient numbers correspond to those listed in Table I. The chronological representation starts with the admission of the first patient in each cluster, and not on the same calendar date. Grey areas mark the hospitalization before MDR PA detection, whereas coloured strips denote hospitalization after detection of the pathogen.

(Table II) and in patients (Table I) throughout the retrospectively evaluated six-year period. Throughout this period 14 MDR PA cases — including 10 nosocomial cases (median of 52.5 days post transplant) — were diagnosed. Seven patients died, with six deaths 'directly' or 'likely' linked to the MDR PA infection according to the physician's discharge letter (Table I). The incidence and mortality were calculated at 0.47 per 100 patients at risk and 50% of patients with MDR PA, respectively. Thus, the intended WGS-based analysis proved highly relevant for a thorough evaluation of the transmission probability.

WGS of 10 MDR PA patient isolates revealed six different STs and CTs including two clusters of three patients each: ST309/CT3049/no-carbapenemase and ST235/CT1352/FIM-1. Although pairwise comparison showed high clonality within the clusters (Figure 2), hospitalization data linked only two of the ST309/CT3049/no-carbapenemase patients (Figure 1). Thus, WGS was performed on all available environmental MDR PA isolates aiming to identify other possible MDR PA transmission pathways.

WGS and MLST found only the high-risk strain ST235 in all toilet water samples [19]. According to isAMR, 75.6% were positive for the FIM-1 carbapenemase, 25.6% carried the more common VIM-2 carbapenemase, one isolate carried both carbapenemases and one isolate had no carbapenemase. Although ST235 is found worldwide, FIM-1 remains a rarity since it was first described in Italy in 2013 [7,20,21]. According to reports from the German National Reference Centre, FIM-1 was only detected in 10 isolates sent for investigations during the past five years [22—26]. Three of these cases are described in this paper. The nosocomial detection, recurrent detection in toilets, and long periods of hospitalization, thus, strongly suggest that acquisition is linked to toilet exposure. However, the 'dark digit' level cannot be estimated due to the lack of obligation to report PA that fulfils the German 4-MRGN criteria.

Furthermore, even though cgMLST showed a slightly higher diversity among environmental isolates, ST235/CT1352/FIM-1 (51 initial isolates: 78.5%) remained the most common strain in toilets, followed by ST235/CT3051/VIM-2 (seven initial isolates: 10.7%) (Figure 2). Remarkably, a maximum difference of 29 alleles by cgMLST was detected between ST235 isolates collected throughout the described period, whereas isolates of the sub-type ST235/CT1352 differed by $\leq$20 alleles (Figure 2). This indicates a highly stable population at our BMTU over the six-year period. Thus, toilet-related MDR PA incidence and mortality were recalculated to 0.16 per 100 patients at risk and 7.1% of patients with MDP PA, respectively.

Our results strongly indicate that although the frequency of MDR PA detection in toilets was high during the analysed period, ongoing disinfection procedures and surveillance succeeded in a low transmission rate of only 0.16 per 100 patients at risk. But what made our standard of care work? The management of water sources is difficult for many reasons, including incomplete removal of bacteria from the toilet vessel during flushing which facilitates biofilm formation, and difficulties in mechanical removal of residue during cleaning procedures due to the unfavourable design of appliances [8,27]. A similar toilet-related and WGS-supported outbreak report from Germany was associated with a 3.6% lethality and could be ended after the installation of rimless toilet vessels [28]. Successful usage of this particular design has become common in outbreak management bundles in BMTUs due to easier cleaning [14]. Thus, this had been implemented at our BMTU early on, being in place throughout the studied period. Due to insufficient availability of data before 2016, it is unknown when these toilets were installed. Kossow *et al.*, however, even report that attributable mortality fell to 0% after toilet replacement [28]. Nonetheless, while it appears that they are less frequently contaminated with MDR PA (6.1% vs 18.9%), they did not remain free of MDR PA, indicating that permanent decontamination may be necessary even when using modern technologies [28]. This corresponds well with our experience.

Flushing the toilet is a high-risk situation at BMTUs due to mobilized and dispersed infectious particles [27,29,30]. Managing this hazard, thus, seemed crucial and it was addressed twofold: stopping droplets from leaving the vessel by closing the lid, and reduction of spreadable bacteria in the vessel through disinfection. Although the first approach seems intuitive, closing the lid when flushing is neither very common — a Chinese study found that only 56.1% of participants habitually followed this recommendation — nor fully efficient in preventing dispersal [31,32]. Moreover, lids are not necessarily mounted on every toilet. Thus, patient education was combined with permanent disinfection.

Although chlorination is known to be highly efficient and is most commonly used for the management of water sources in BMTUs, in our setting it proved insufficient (data not shown) [8,14,33]. Consequently, bromination was introduced due to its reported higher efficiency compared to chlorine [34]. Nevertheless, MDR PA was detected during our surveillance over six years in a minimum of one toilet per sampling event, and at least once in every toilet. Overall, 79 out of 442 water samples grew MDR PA. Presumably, MDR PA persistence happened due to biofilm-enhanced resistance to biocides and impaired efficiency of bromine under protein exposure [34—36]. Disinfection using hydrogen peroxide upon MDR PA detection, however, proved more efficient. Although up to four disinfections were necessary to achieve $\leq$3 cfu/100 mL in four cases, 57 toilets were free of MDR PA after the first disinfection (Table II). However, similar to the report of Kossow *et al.*, complete resolution of the contamination could not be achieved at our BMTU [28]. Thus, these procedures had to be maintained throughout the study period.

Finally, some interesting aspects found during pairwise comparison of toilet isolates by cgMLST combined with the architectural features of the BMTU should be highlighted. Detection in the same vessel was the most frequent common denominator in identical (by cgMLST) or clustering isolates (Figure 2, column E). However, in accordance with previous reports, the biofilm population is not monoclonal but, as can be seen, for example, in R.4, rather a combination of ST235 subtypes which differ by up to 25 alleles (cgMLST) in time (Figure 2) [37]. This suggests both MDR PA persistence over time and a low mutation rate. Thus, the success of toilet vessel replacement seen in France appears even more plausible [38].

The architecture of the sewerage system could also be relevant for understanding the ST235/CT3051/VIM-2 cluster (cut-off value of $\leq$3 alleles by cgMLST) in R.10 and R.11. Six isolates detected in the two rooms with a common drainpipe system stand out compared with the rest of the department. However, the association with the isolate from R.4 remains unclear. Similar implications of architectural features are also suspected within clusters from R.2/R.3, R.4/R.5, and R.8/R.9 (Figure 2). However, further analysis is impaired by the overall

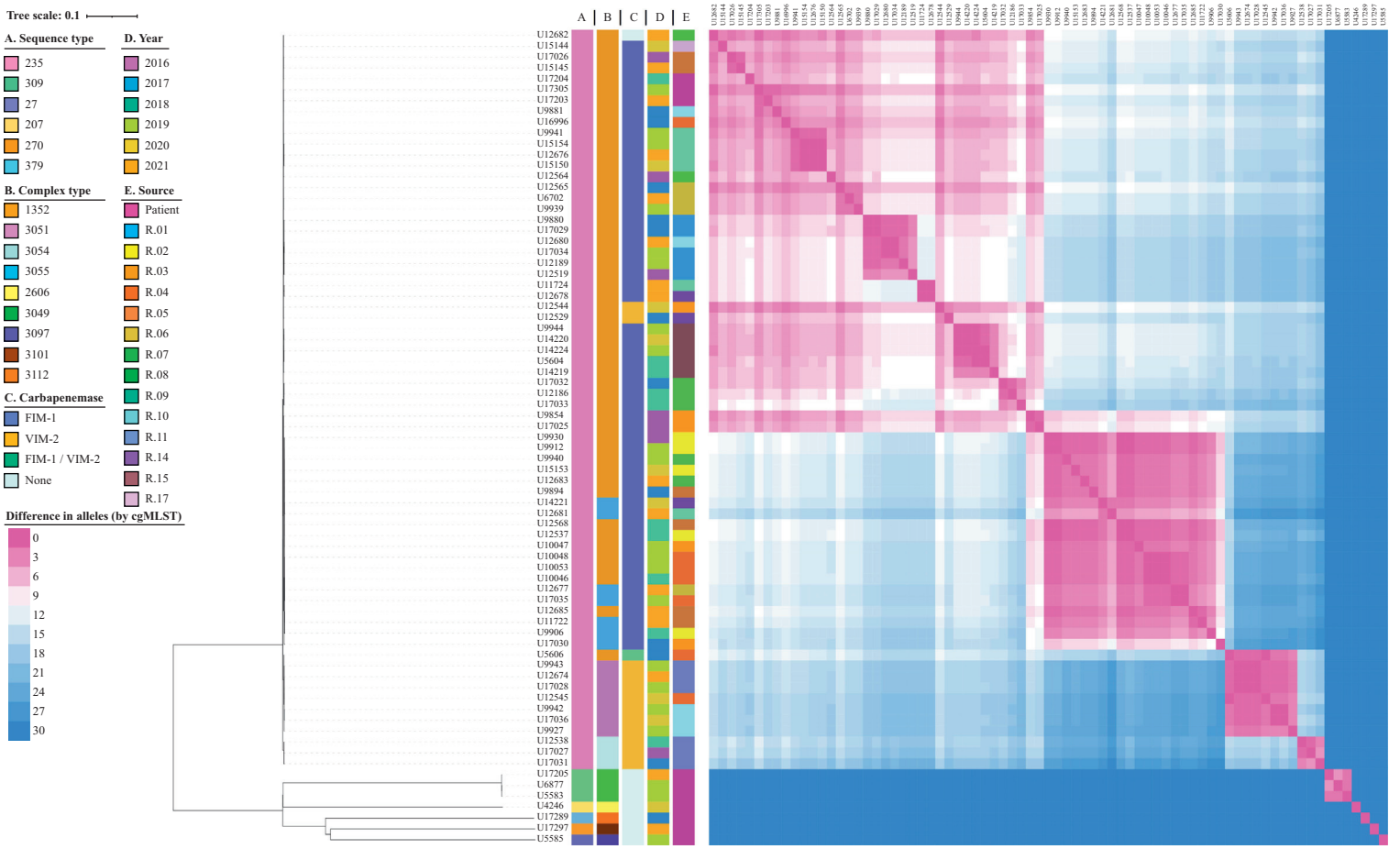

Figure 2. Distribution and genetic relatedness of multidrug-resistant *Pseudomonas aeruginosa* (MDR PA) strains according to whole-genome sequencing. Three MDR PA patient isolates and all isolates from toilet water were attributed to sequence type (ST)235, which suggests acquisition from toilets at the bone marrow transplant unit. Despite low genetic diversity among isolates from the same toilet over time, it remains unclear from which specific room patients contracted the isolates. This uncertainty arises from pairwise comparison of all isolates by core-genome multi-locus sequence typing, revealing a maximum of 29 allelic differences throughout time. The remaining patient isolates, however, were typed to five other STs whereas three ST309 isolates clustered closely. As neither of the five STs were detected in toilet vessels, it is assumed that the latter seven isolates were acquired from sources other than the toilets.

low diversity across the department (difference in alleles by cgMLST, all ST235: ≤29, and ST235/CT1352: ≤20). Moreover, since the department was in use since 1998 with a history of MDR PA toilet colonization that started before 2016, our ability to retrace and determine the dissemination vehicles of MDR PA (i.e. possible fomites or erroneous behaviour of HCW or cleaning staff) was impaired. Therefore, we consider that more genome-oriented research is needed to enable tailored IPC in the future (i.e. replacement of affected elements versus drainpipe design versus modifying staff behaviour).

Limitations of our study include sequencing of only one isolate per sample, which may lead to underestimation of strain diversity, and subsequently to overestimated IPC efficiency. However, no recommendations are currently available regarding the number of colonies to be sequenced. Second, though it offers high precision, short-read sequencing does not allow comparison of whole genomes and may lead to an overestimation of strain relatedness. Third, the definition of complex types by cgMLST suggests that multiple genotypes contaminate the toilets. However, the allelic difference between isolates of different CTs within ST235 was <30, which possibly suggests a common ancestor for all ST235 isolates. Fourth, the bromine intervals for the water tanks were determined empirically during the early phase of the bundle implementation (before 2016). The bromine was not measured during the study period. Thus, we cannot exclude that underdosing could occur, or that different usage frequencies of toilets could use up the bromine faster and promote the MDR PA detection rate in rooms with high occupancy. However, whether higher doses of bromine would have been more efficient could not be determined due to legal dosing limitations [39]. Fifth, patients had variable lengths of stay and thus a different time at risk, which could have influenced the acquisition risk. Finally, no data were available on compliance with hand hygiene and lid closing, or with bromination intervals. However, the highest number of positive results were found on three occasions: September 2019, March 2020, and September 2021. Inquiries have revealed that bromination had been accidentally skipped at least once before each of these sampling events (oral communication). We considered this a strong indicator of proper compliance with bromination at other times, and of ongoing necessity for disinfection.

In conclusion, our genome-oriented IPC approach emphasizes the necessity of permanent IPC procedures including toilet disinfection in combating toilet-related nosocomial MDR PA transmissions in BMTU. Despite frequent environmental contaminations, however, only three of 1898 patients at risk were definitively affected by strains found in toilets over six years, highlighting the sustained success of our strategy. The WGS-oriented analysis provided the currently highest possible resolution and demonstrated the persistence of MDR PA ST235 with low genetic diversity (overall ≤29 alleles in pairwise comparison by cgMLST; ≤20 alleles within a CT) [40]. This low level of genetic diversity seen in our cohort over six years points to a need for defining what a feasible threshold for clear genome-oriented outbreak identification could be in the future.

Acknowledgements

We extend our gratitude to the infection control nurses, particularly T. Kocyba and J. Hartl, for their diligent sampling efforts and bundle implementation supervision. Special thanks to P. Beer, R. Fechter, L.-M. Fischer, U. Klemens, T. Prenzel, and C. Schießl for their exceptional technical support in our molecular epidemiology and outbreak laboratory, and to F. Wimmer for his role in bromination processes. Lastly, we appreciate all patients and BMTU staff for embracing and executing the infection control bundle. We also thank M. Steib-Bauert for extracting the antibiotic consumption data from the ADKA-If-SGI project.

Ethics statement
This project was approved by the Ethics Committee of the University Hospital of Regensburg within the application 23-3465-104 from 24.01.2023.

Conflict of interest statement
None declared.

Funding sources
No funding was received for conducting this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2024.05.015.

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