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Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks

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ABSTRACT

Increasing isolation rates of resistant bacteria in the last years require identification of potential infection reservoirs in healthcare facilities. Especially the clinical wastewater network represents a potential source of antibiotic resistant bacteria. In this work, the siphons of the sanitary installations from 18 hospital rooms of two German hospitals were examined for antibiotic resistant bacteria and antibiotic residues including siphons of showers and washbasins and toilets in sanitary units of psychosomatic, haemato-oncological, and rehabilitation wards. In addition, in seven rooms of the haemato-oncological ward, the effect of 24 h of stagnation on the antibiotic concentrations and MDR (multi-drug-resistant) bacteria in biofilms was evaluated. Whereas no antibiotic residues were found in the psychosomatic ward, potential selective concentrations of piperacillin, meropenem and ciprofloxacin were detected at a rehabilitation ward and ciprofloxacin and trimethoprim were present at a haemato-oncology ward. Antibiotic resistant bacteria were isolated from the siphons of all wards, however in the psychosomatic ward, only one MDR strain with resistance to piperacillin, third generation cephalosporins and quinolones (3MRGN) was detected. In contrast, the other two wards yielded 11 carbapenemase producing MDR isolates and 15 3MRGN strains. The isolates from the haemato-oncological ward belonged mostly to two specific rare sequence types (ST) (*P. aeruginosa* ST823 and *Enterobacter cloacae* complex ST167). In conclusion, clinical wastewater systems represent a reservoir for multi-drug-resistant bacteria. Consequently, preventive and intervention measures should not start at the wastewater treatment in the treatment plant, but already in the immediate surroundings of the patient, in order to minimize the infection potential.

1. Introduction

The isolation rates of multi-resistant Gram-negative pathogens with resistance to four clinically relevant antibiotic groups (piperacillin, third generation cephalosporines, fluorquinolones and carbapenems) or carbapenemase genes (4MRGN) and pathogens with additional resistance to colistin have increased in recent years (Jeukens et al., 2019; Liu et al., 2016; Müller et al., 2018; Westphal-Settele et al., 2018). Since it is difficult to cure infections with such pathogens effectively, reserve antibiotics are increasingly the last choice for therapy, which results in considerable additional efforts and costs, e.g. through additional hygiene measures. The most common antibiotic resistant, clinically relevant Gram-negative species are extended-spectrum beta-lactamase (ESBL) - and carbapenemase-producing *Enterobacteriaceae* such as *Klebsiella pneumoniae* and *Escherichia coli* as well as non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Infections with these pathogens often affect susceptible patient populations (immunocompromised patients, neonates, etc.) in oncology and intensive care units. Numerous outbreaks in health care facilities could be attributed to highly resistant variants (resistance to carbapenems and colistin) of these pathogens which are able to survive in wet environments, like wastewater systems, for a long time (Rutala and Weber,

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1997). Even colistin resistance, mediated by *mcr*-1 carried on a transferable plasmid has been reported in German wastewaters (Hembach et al., 2017). Furthermore, waterborne bacteria like *P. aeruginosa* and *Klebsiella* strains can adopt a viable but not culturable state. Likewise, their environmental stability is often conferred by the formation of biofilms. In addition, biofilms are an ideal environment for horizontal gene transfer and accumulation of pathogens due to a high bacterial density and species diversity in a confined space (Gattlen et al., 2010; Madsen et al., 2012; Molin and Tolker-Nielsen, 2003; Parkes and Hota, 2018; Schwartz et al., 2003).

Parkes and Hota (2018) state that the overrepresentation of multi-drug-resistant (MDR) organisms in an increasing number of outbreak reports might reflect the wastewater-system related transmission of pathogens that are not commonly included in infection surveillance. Colonization with multi-drug-resistant *Enterobacteriaceae* from a water reservoir may occur by direct and indirect contact, ingestion and aspiration of contaminated water, or inhalation of aerosols (Giani et al., 2015; Weterings et al., 2015). The sewage system in hospitals starts in the siphons of the washbasins, shower siphons, as well as in the toilets. The patients excrete antibiotics by urine and feces. Consequently, the highest antibiotic concentrations in clinical wastewater are measured in the sanitary facilities. An earlier study (Voigt et al., 2019) showed that the concentrations are sometimes higher than the minimal inhibitory concentrations (MICs) proposed by Bengtsson-Palme and Larsson (2016). In addition, it seemed that antibiotics are bound in the matrix of bacterial biofilms and are released again during the stagnation time between two uses of the toilet or washbasin, so that antibiotic residues are nearly always present (Voigt et al., 2019). This might promote the colonization of these facilities with antibiotic resistant bacteria. In order to test this assumption, the study presented here compares the occurrence of MDR-bacteria to the antibiotic concentrations in the water and biofilms of the siphons of washbasins, toilets and shower siphons in vacant patient rooms in three clinical units with widely differing antibiotic administration.

2. Materials and methods

2.1. Sampling sites

Between December 2017 and February 2018, the wastewater of 54 sanitary facilities including washbasin siphons (WS), shower siphons (SS) and water closets (WC) in 18 rooms of three clinical wards (neurological rehabilitation, psychosomatics and oncology) in two different hospitals were examined for occurrence of multi-drug-resistant bacteria.

2.2. Sampling procedure

Sampling was carried out in two phases; 1. status quo and 2. stagnation. The time of sampling ranged from 8:00 a.m. till 4:00 p.m. Sampling of the WC was performed using a 50 ml disposable syringe (Omnifix, Braun) directly in the water phase. An additional inert tube (length: approx. 32 cm) was used for sampling the aqueous phase of WS and SS respectively. The water was bottled, stored at 5 ± 2 °C and analysed within 24 h. Silicone strips (1 cm × 10 cm) were used for growth of the characteristic biofilm of the evaluated facility. They were inserted into the aqueous phase and left there undisturbed for approximately 24 h.

In order to prevent possible falsifications in status quo samples, contact with or mobilization of the biofilm and other incrustations on the walls was strictly avoided during the sampling procedure.

2.2.1. Status quo samples

To determine a base line of contamination in sanitary facilities of clinical wards a “status quo” (SQ) sample was collected. It is characterized as a random grab sample (single sample) taken at a specific time in a vacant patient's room without the knowledge of the last cleaning or use of the washbasin, shower or toilet by the patient or staff. After the sample had been taken, the systems were flushed with approximately 15 l of drinking water that was antibiotic-free and sterilized by filtering through end of pipe filters (PALL, Bad Kreuznach, Germany). Preliminary tests had shown that approx. 15 l of rinsing volume were necessary to eliminate active substances from the water phase (data not shown).

2.2.2. Stagnation samples

After flushing, the sanitary facilities were closed for staff or and patients to ensure the undisturbed growth of the biofilm and diffusion of antibiotics for this study. After one day, the biofilm was disrupted using a syringe connected to an inert tube. 50 ml of the biofilm/water suspension (BFW) was collected, stored or directly analysed.

In addition, samples for chemical analysis of antibiotic concentrations were taken according to Voigt et al. (2019). The chemical analyses of the stagnated water were conducted from water samples taken moments before the disturbance of the biofilm for microbiological analysis.

2.2.3. Sonification of silicone stripes

The silicone stripe was carefully removed before the manipulation of the biofilm and placed into a sterile tube. For the sonification 14 ml sterile 0.9% NaCl was added. The grown biofilm was removed by sonification (ultrasonic bath; Soni) for 5 min at 100% ultrasound intensity using.

2.3. Cultivation, identification and susceptibility testing of antibiotic resistant bacteria

The focus of the cultivation lay on the identification of Gram-negative bacteria including *Enterobacteriales* and non-fermenters (*Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus-baumannii complex*). Samples were also analysed for methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis (VRE),* For detection of Gram-negative bacteria, 2–5 ml of the wastewater were filtered through a sterile nitrocellulose membrane filter (pore size 0.45, Ø 47 mm, MICROFIL® from Millipore, Art. No. MVHAWG 124). After filtration, the membrane was placed on non-selective MacConkey agar plates (Becton Dickinson GmbH, Germany). In addition, 1 ml as well as 100 µl of 10-fold dilutions in sterile 0.9% NaCl were prepared and plated on MacConkey agar (Becton Dickinson GmbH, Germany). Selective chromogenic agar plates [CHROMagar ESBL (MAST Diagnostica GmbH, Reinfeld, Germany, 201470), CHROMagar MRSA (MAST Diagnostica GmbH, Reinfeld, Germany, 201402) and CHROMagar VRE (MAST Diagnostica GmbH, Reinfeld, Germany, 201460)] were inoculated with 100 µl of the samples and incubated at 37 ± 1 °C for 24 h or rather 48 h.

Gram-negative MDR bacteria were selected according to Müller et al. (2018). All colonies detected on CHROMagar ESBL, that were suspected to belong to the *Enterobactericeae*, *Acinetobacter* spp. or *Pseudomonas* spp., were cultivated on 5% sheep blood agar plates (Becton Dickinson GmbH) and incubated at 36 °C for 24 h with 5% CO_2. Pink colonies on CHROMagar MRSA and pink to purple colonies on CHROMagar VRE were also sub-cultured in the same way. Confirmation of the species was conducted using MALDI-TOF MS (bioMérieux, Marcy-l'Étoile, France) with the Myla™ software. Isolates belonging to the *Acinetobacter calcoaceticus-baumannii* (ACB) complex, *P. aeruginosa* and *Enterobacteriaceae* were further processed and tested for multi-drug-resistance.

Antibiotic susceptibility testing was performed using the Micronaut-S MDR MRGN-Screening 3 system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany). The results were interpreted with EUCAST criteria. Bacteria showing resistance against three or four of the four following clinically relevant antibiotics: piperacillin, ciprofloxacin, third generation cephalosporins

(cefotaxime and/or ceftazidime) and carbapenems (meropenem and/or imipenem), were classified as 3MRGN or 4MRGN, respectively. Isolates with phenotypic resistance to carbapenems were further tested for carbapenemase genes while isolates with phenotypic resistance towards colistin were tested for the presence of a *mcr* gene. Colistin (the polypeptide antibiotic polymyxin E) is one of the last resort antibiotics against carbapenemase-producing bacteria. Liu et al. (2016) first reported a transferable colistin resistance encoded by the *mcr*-1 gene in 2016. Carbapenemase producers were also classified as 4MRGN.

2.4. Molecular characterization

For any type of molecular analysis the detected bacteria were cultivated on 5% sheep blood agar (Becton Dickinson GmbH) at 35 °C for 24 h with 5% CO_2.

2.4.1. Detection of resistance genes using real-time PCR

The detection of carbapenemase and *mcr*-1 or *mcr*-2 genes was conducted according to Müller et al. (2018) with a few modifications.

The DNA extraction was performed by resuspending three single colonies in 100 µl nuclease free water and heating to 95 °C for 15 min and centrifugation at 14,000 g for 15 min. 50 µl of the supernatant were carefully removed and used for PCR. In short, determination of the presence of carbapenemase genes and the screening for *mcr-1* and *mcr-2* was carried out using 4Plex PCR including the Biozym 2 × qPCR Mastermix (Biozym, Hessisch Oldendorf, Germany) on a Mic qPCR Cycler (Bio Molecular Systems, Upper Coomera, Australia). The actual reaction mixture contained 0.25 µM of the primers and 0.125 µM of each probe (Table S1), 2 µl of isolated bacterial DNA. The products were amplified using the following protocol: 95 °C for 2 min, followed by 35 cycles with 95 °C for 5 s and 60 °C for 30 s. Strains were counted as positive if they yielded a signal within 30 cycles.

2.4.2. Molecular typing of resistant bacterial isolates

For molecular typing, three single colonies of the isolated strains were resuspended in 80 µl of ultra-pure water and heated for 5 min at 95 °C. After centrifugation at 14.000*g* for 5 min 40 µl of the supernatant was removed and directly used for typing according to previously described standard procedures or stored at −20 °C until analysis. Typing of *E. cloacae* complex (Miyoshi-Akiyama et al., 2013) was performed as multi-locus sequence typing (MLST), while typing of *P. aeruginosa* (Basset and Blanc, 2014; Curran et al., 2004) was conducted as double-locus sequence typing (DLST). Purification of PCR products was performed using the GeneJET Gel Extraction Kit (Fisher Scientific GmbH, Schwerte, Germany). The samples were sent to GATC Biotech AG (Konstanz, Germany) for sequencing. Determination of the sequence types was performed using the specific multi-locus sequence typing (MLST) and DLST websites for the species (https://pubmlst.org/paeruginosa; https://pubmlst.org/bigsdb?db=pubmlst_ecloacae_seqdef; http://dlst.org/Paeruginosa/). Conversion of DLST types into MLST types was realized by MLST typing of one isolate.

2.5. Analysis of antibiotic concentrations

The concentration of antibiotic residues was analysed by High Performance Liquid Chromatography (HPLC) with an Agilent LC-System 1290 II (Waldbronn, Germany) coupled to a QTRAP® 6500 + mass spectrometer from AB Sciex GmbH (Darmstadt, Germany), as described in Voigt et al. (2019). The separation of target analytes was performed with a Nucleoshell RP18Plus® column 2 mm × 100 mm, 2.7 µm (Macherey & Nagel, Düren, Germany) after dilution (1:1 with a water:acetonitrile (95:5, v/v) mixture with (0.8 g/l Na_2EDTA) and filtration (H-PTFE filter, 0.45 µm pore size of Macherey & Nagel (Düren, Germany). The sample injection volume was 20 µL. The chromatography was performed with a binary gradient (eluent A: water:acetonitrile (98 + 2, v/v) and eluent B: methanol:acetonitrile (80 + 20, v/v)). The target molecules were dispersed by electrospray ionization in positive mode (5500 V). Scheduled multiple reaction monitoring was used for detection to obtain a sufficient number of datapoints per analyte. The observed concentrations were compared with the no effect concentrations (PNECs) and the lowest MICs of the susceptible population of this species as described by Bengtsson-Palme and Larsson (2016). The analytical standards were purchased from Sigma Aldrich (Taufkirchen, Germany), Cayman Chemicals (Ann Arbor, United States), Toronto Research Chemicals Canada (Toronto, Canada) and USP Reference Standard (Basel, Switzerland). LC columns and micropore filters used were obtained from Macherey and Nagel (Düren, Germany).

3. Results

Determination of possible hot spots in clinical sanitary facilities and correlation between the presence of antibiotic resistant bacteria and antibiotic concentrations.

In total, 18 patient rooms or 54 facilities (wastewater samples 18 SS, 18 WC, 18 WS) were examined for the occurrence of multi-drug-resistant bacteria and concentrations of antimicrobial agents.

None of the samples contained MRSA and/or VRE bacteria. Residues of third generation cephalosporins or imipenem as well as extensively resistant bacteria with susceptibility to only one or two antibiotic classes were not detected in any sample. The MICs of trimethoprim/sulfamethoxazole ("co-trimoxazole") were tested in combination. A possible correlation between presence of MDR bacteria and antibiotic residues was evaluated for the different sampling procedures and types of wards.

3.1. Psychosomatic ward

The psychosomatic ward (18 sanitary facilities of six rooms) was tested as a single control sample because of the low antibiotic therapy rates with carbapenems and colistin compared to the rehabilitation and haemato-oncological wards (Fig. 1). Only one MDR strain, a 3MRGN *Citrobacter freundii* was detected. Other isolated bacteria belonged to the species *P. aeruginosa* (11), *Acinetobacter* spp. (5) and sensitive *Enterobacteriales* (*E. coli* and *C. freundii*). Seven strains were classified as phenotypic ESBL producers. Furthermore three strains were characterized as colistin resistant, with one of those being a phenotypical ESBL producer as well, but none harboring *mcr*-1 or *mcr*-2 genes.

3.2. Rehabilitation ward

At the rehabilitation ward, 15 sanitary facilities in five rooms were sampled. In this ward, patients are treated frequently with carbapenems and vancomycin. However, no VRE was detected. In total 13 multi-drug-resistant Gram-negative bacteria were isolated; two isolates were classified as 4MRGN, seven isolates as 3MRGN, four isolates as ESBL (Fig. 2). Furthermore, there were five colistin resistant strains, two of which were also found to be 3MRGN bacteria but none tested positive for the presence of *mcr*-1 or *mcr*-2. The 4MRGN were found in the toilet and in the siphon of a washbasin respectively. The majority (4/7) of the 3MRGN were detected in the shower siphons. Two 3MRGN bacteria with additional resistance against colistin were found in a washbasin siphon and a toilet, respectively. All in all, the predominant species were *P. aeruginosa* (18), *E. cloacae* complex (4) and *C. freundii* (3). The two most resistant isolates were 4MRGN *P. aeruginosa* and *K. pneumoniae* producing VIM and KPC, respectively. The 3MRGN bacteria were identified as *E. cloacae* complex, *C. freundii* and *P. aeruginosa*. Further *E. cloacae* complex, *P. aeruginosa* and *A. calcoaceticus-baumannii* complex isolates were found to be only ESBL producers. Chemical analyses are presented in Fig. 2 (B).

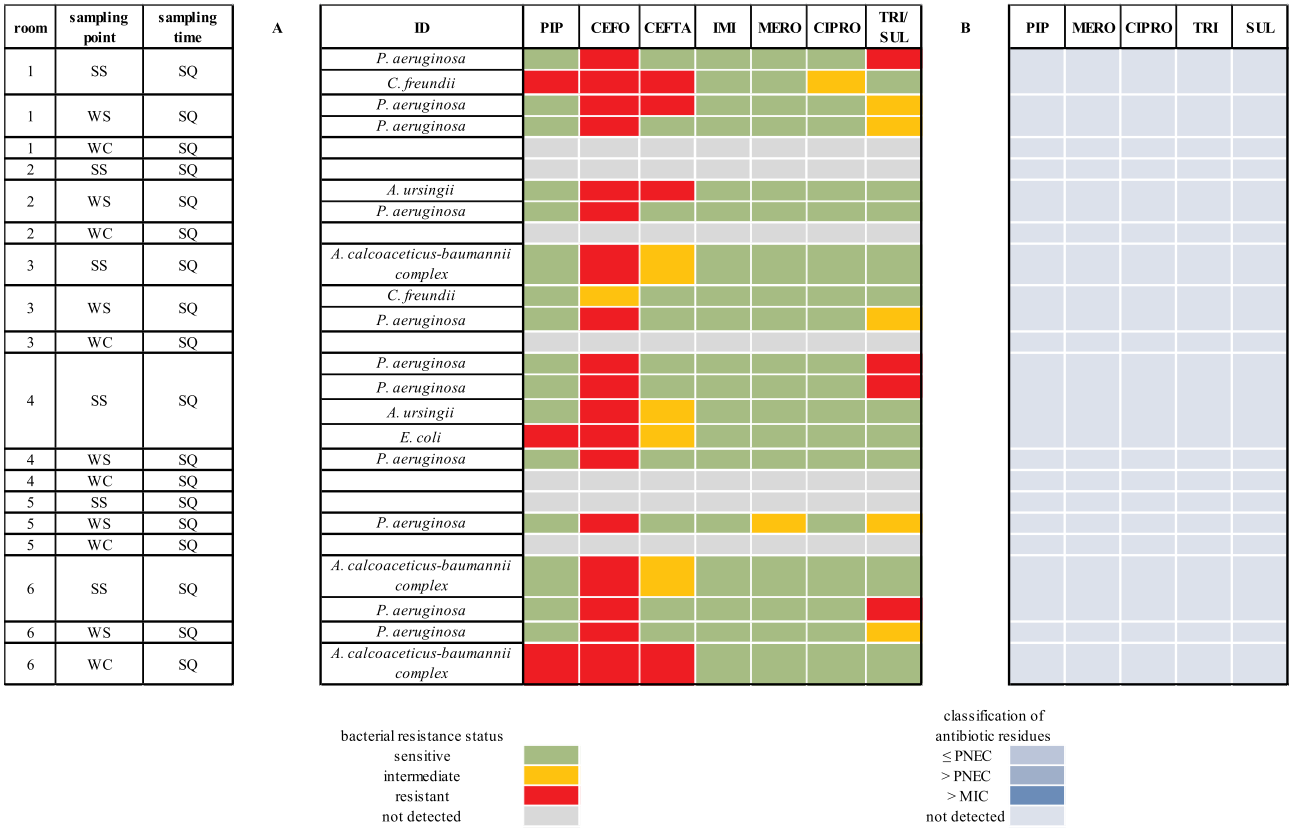

Fig. 1. Antibiotic resistance patterns of the isolated bacteria (A) and the detected antibiotic residues (B) in the sanitary facilities in six different rooms of a psychosomatic ward. To put the antimicrobial substances into relation, the Predicted No Effect Concentration (PNEC) and Minimum Inhibitory Concentration (MIC) values from Bengtsson-Palme and Larsson (2016) were used. The concentrations of residual antibiotics were further subdivided to distinguish between different concentration levels: ≤ PNEC (light blue); ≥ PNEC (blue); > MIC (dark blue). These are then compared to the antibiotic resistance of the bacteria. For the random samples (SQ) no correlation between A and B was detected: (A). The bacteria displayed diverse antibiotic resistance patterns (susceptible = green; intermediate = orange; resistant = red). (B) The chemical analyses of the water samples showed no evidence of any antibiotics (not detected = grey). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
PIP: piperacillin; CEFO: cefotaxime; CEFTA: ceftazidime; IMI: imipenem, MERO: meropenem; CIPRO: ciprofloxacin; TRI/SUL: trimethoprim/sulfamethoxazole; TRI: trimethoprim; SUL: sulfamethoxazole.

3.3. Haemato-oncology ward

The testing of 21 sanitary facilities in seven patient rooms of the haemato-oncology ward at the beginning and the end of a stagnation time of one day revealed nine 4MRGN strains, ten colistin resistant 3MRGN isolates and six isolates characterized as phenotypical ESBL, not harbouring *mcr*-1 or *mcr*-2 (Fig. 3). Most of these 25 bacterial isolates originated from the shower siphons (three 4MRGN, nine 3MRGN and two ESBL), seven from the toilets (six 4MRGN and one ESBL) and four from washbasin siphons (one 3MRGN and three ESBL). The colistin resistant bacteria were mostly found in the shower siphons (9/10). The predominant species were *E. cloacae* complex (12/25) and *P. aeruginosa* (8/25). All colistin resistant bacteria belonged to the *E. cloacae* complex. One room did not yield any resistant isolates. Chemical analyses are presented in Fig. 3 (B).

To evaluate the influence of 24 h stagnation time on the antibiotic concentrations in the wastewater, samples were taken twice in four rooms of the oncology ward (day 1 and day 2). In addition, the biofilms in the sanitary units were sampled by disruption in biofilm/water suspensions for antibiotic resistant bacteria. The results showed that compared to the water the yield of resistant bacteria was higher, when the biofilm had been disrupted. In addition former results that had indicated that antibiotics are released from the biofilm after flushing with 15 l of water were confirmed (Voigt et al., 2019).

In total seven isolates, originating from five different rooms of the haemato-oncological ward, that had been characterized as VIM producing *P. aeruginosa* were typed using DLST. All bacteria had been isolated either from the biofilm/water suspension or the sonificated silicone stripe and originated from the toilets (6/7) or shower siphons (1/7). The results showed that all investigated strains belonged to the double locus sequence type 64–105 and MLST typing showed that this DLST allele combination belongs to ST823. This strain had been encountered earlier in this hospital and the susceptibility profiles of these clones are compared in Table S2.

Furthermore, all ten 3MRGN isolates of the *E. cloacae* complex from three different rooms of the haemato-oncology ward were typed via MLST. Bacterial isolates originated either from the showers (9/10) or the washbasin siphon (1/10). The sequences of all seven alleles showed that all isolates could be assigned to the sequence type ST167.

3.3.1. Intervention approaches

During sampling of the oncological ward, the biofilms in the shower siphons were macroscopically visible. Interestingly two rooms were cleaned before or during sample collection (rooms two and six). In room two, the sanitary facilities were cleaned in the evening before obtaining the first sample (SQ) using antiseptics and a surface cleaning procedure, however without disturbing the biofilm deep in the siphon. In these water samples no resistant bacteria and only low concentrations of antibiotics could be observed. However, after 24 h viable MDR bacteria could still be isolated from the biofilm. In room six the SQ sample was taken before a routine cleaning and did not show a contamination with MDR bacteria. After cleaning and a subsequent

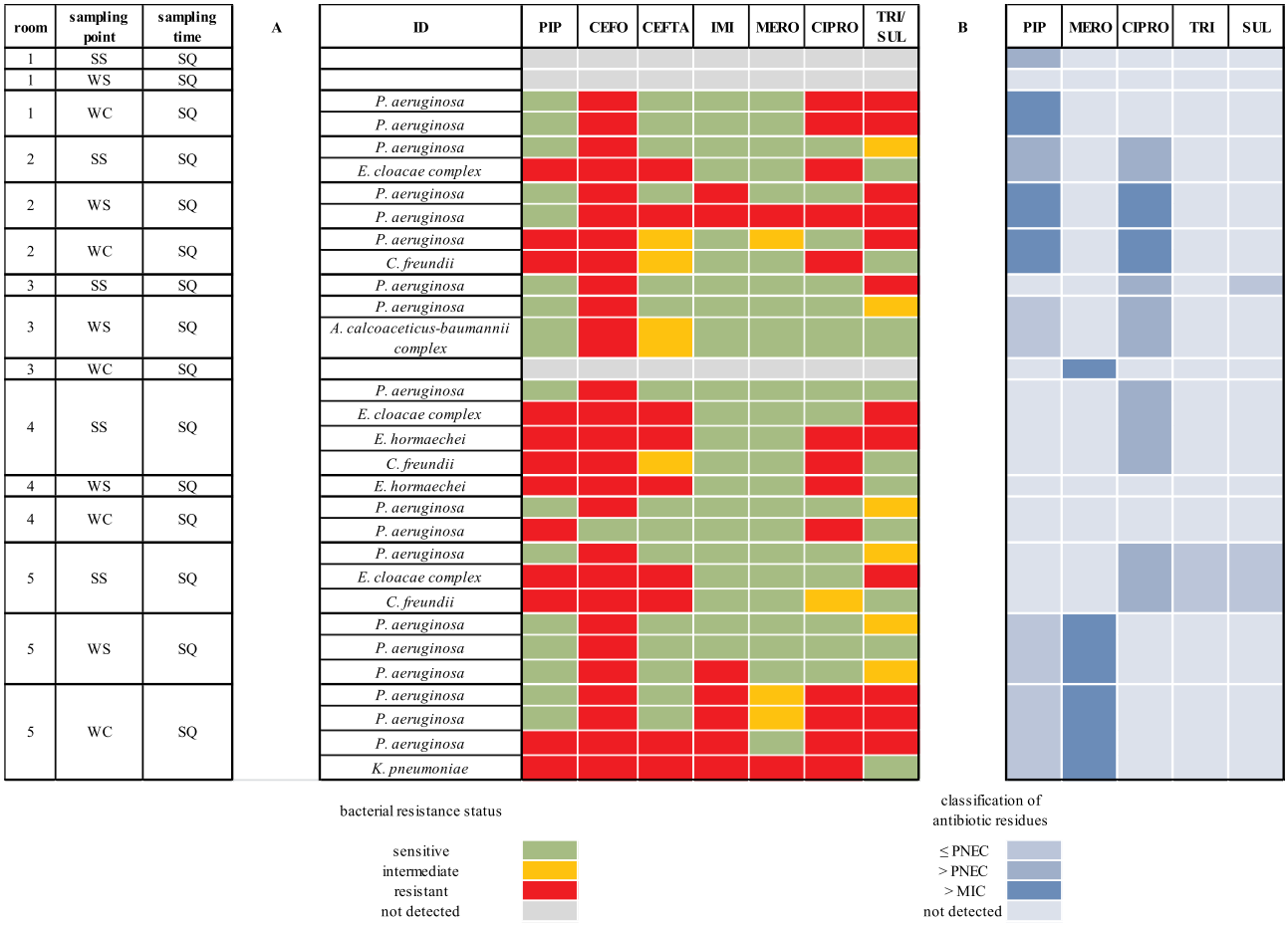

Fig. 2. Antibiotic resistance patterns of the isolated bacteria (A) and the detected antibiotic residues (B) in the sanitary facilities in six different rooms of a rehabilitation ward. To put the antimicrobial substances into relation, the Predicted No Effect Concentration (PNEC) and Minimum Inhibitory Concentration (MIC) values from Bengtsson-Palme and Larsson (2016) were used. The concentrations of residual antibiotics were further subdivided to distinguish between different concentration levels: ≤ PNEC (light blue); ≥ PNEC (blue); > MIC (dark blue). These are then compared to the antibiotic resistance of the bacteria. The bacteria displayed diverse antibiotic resistance patterns (susceptible = green; intermediate = orange; resistant = red). Chemical analysis showed that antimicrobial substances could be detected in almost all of the samples with some concentrations reaching or exceeding the lowest MIC as postulated by Bengtsson-Palme and Larsson (2016). Trimethoprim (TRI) and sulfamethoxazole (SUL) were identified in two rooms but only below the postulated PNEC. Piperacillin (PIP) and ciprofloxacin (CIPRO) were found in a broad range of concentrations and meropenem (MERO) was only detected in high concentrations (dark blue). In room 3, high amounts of meropenem were found in the toilet but no resistant bacteria were observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
CEFO: cefotaxime; CEFTA: ceftazidime; IMI: imipenem; TRI/SUL: trimethoprim/sulfamethoxazole.

stagnation, the disruption of the biofilm revealed one phenotypical ESBL *E. cloacae* complex in the siphon of the toilet.

4. Discussion

The hospital wastewater system and water-related devices in the patients' environment may serve as a reservoir of waterborne pathogens in healthcare settings (Munoz-Price et al., 2010, 2013). They are prone to colonization with these bacteria, in part, because wastewater temperatures are suitable for bacterial growth. In addition, the complex structures of hospital wastewater systems often lead to stagnation, corrosion, and biofilm formation (Tzouvelekis et al., 2012). Kanamori et al. (2016) identified multiple studies describing (waste)water-associated outbreaks in healthcare facilities, emphasizing that multi-drug-resistant organisms are often linked to contaminated washbasins. The most common waterborne pathogens causing these infections are Gram-negative bacteria including *Pseudomonas* spp., *Enterobacter* spp., *Serratia* spp., *Stenotrophomonas* spp. and *Klebsiella* spp. (Kanamori et al., 2016).

Typing of the carbapenemase-producing *P. aeruginosa* strains isolated from the haemato-oncology ward showed that all isolates belonged to ST823 and produced the VIM carbapenemase. 4MRGN strains of the same sequence type had already been isolated 6 years ago (Table S2) and had been found repeatedly in corresponding samples of the connected wastewater system of this building in 2016 and 2017 (Müller et al., 2018). A search in PubMLST showed that seven strains of this MLST type were isolated in India and Malaysia and there is one further publication describing this sequence type in the Arabian Emirates and Qatar (Zowawi et al., 2018). A comparison of the allele combinations also showed that ST823 is not a member of any wide-spread pandemic clonal complex (e.g. CC111, CC175, CC235, CC308 or CC357). Therefore, it can be assumed that, unlike the high-risk clones, ST823 *P. aeruginosa* is not a wide-spread clone in Germany and that there might be a stable colonization with this strain in the wastewater system of this hospital building. The isolated 3MRGN E. *cloacae* complex strains belonged to sequence type ST167. This sequence type was also present throughout the downstream wastewater system (unpublished results). So far, to our knowledge, there is also no publication concerning this ST type and the allele composition indicates that it is also not a member of any well-known *E. cloacae* clonal complex (e. g. CC114, CC234, CC74 (Izdebski et al., 2015)).

Fig. 3. Antibiotic resistance patterns of the isolated bacteria (left) and the detected antibiotic residues (right) in the sanitary facilities in six different rooms of a haemato-oncology ward. To put the antimicrobial substances into relation, the Predicted No Effect Concentration (PNEC) and Minimum Inhibitory Concentration (MIC) values from Bengtsson-Palme and Larsson (2016) were used. The concentrations of residual antibiotics were further subdivided to distinguish between different concentration levels: ≤ PNEC (light blue); ≥ PNEC (blue); > MIC (dark blue). These are then compared to the antibiotic resistance of the bacteria. The bacteria displayed diverse antibiotic resistance patterns (susceptible = green; intermediate = orange; resistant = red). Samples show the antibiotic concentrations and antibiotic resistant bacteria in the water on day 1 (SQ) and on day 2 (BFW). Bacterial samples from the biofilms (BFW) were acquired by manipulation of the biofilms on day 2 after the water sample for chemical analysis had been taken (SQ). In almost all of the sampling sites trimethoprim (TRI) and sulfamethoxazole (SUL), which are dispensed in a combination called “co-trimoxazole” (TRI/SUL) could be found in different concentrations ≤ PNEC. In the rehabilitation ward, the presence of bacteria with resistance against co-trimoxazole seemed to be independent of the concentration of those antimicrobial agents. Ciprofloxacin was also found in most of the water samples and varied in its concentration. Ciprofloxacin (CIPRO) and, in one room, trimethoprim were the only antibiotics with concentrations higher than the MICs postulated by Bengtsson-Palme and Larsson (2016). Carbapenems were not detected in any of those samples although carbapenem resistant strains with carbapenemases were isolated from some of the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
PIP: piperacillin; CEFO: cefotaxime; CEFTA: ceftazidime; IMI: imipenem, MERO: meropenem.

The antibiotic concentrations in the wastewater of the sanitary installations of some rooms of the rehabilitation and the haemato-oncology wards were above the PNECs and MICs defined by Bengtsson-Palme and Larsson (2016). However, a correlation between the high concentrations of specific antibiotics and the resistance phenotypes of the colonizing bacteria was not obvious. For example, in room 5 of the rehabilitation ward, the patient inhabiting the room before sampling had been dosed with meropenem and high concentrations of this substance were found in the washbasin and the toilet, but only three out of seven isolated bacteria showed resistance towards meropenem. This may be explained by the fact that non-growing cells in biofilms show a lower susceptibility to betalactams, which kill only growing cells. Thus, the bacteria may survive the exposure to antibiotics in biofilms, however would not be able to multiply if the antibiotics are present.

Even after exchanging the whole water volume of the siphons for 15 l of sterile drinking water, the antibiotic concentrations increased again during 24 h of stagnation but reached lower concentrations than before. This confirmed earlier results and indicates that the antibiotics are probably absorbed by the biofilms and are released again after flushing (Voigt et al., 2019). This effect was especially observed for ciprofloxacin and co-trimoxazole (Fig. 3) which was found almost "ubiquitously" in the haemato-oncology ward. Ciprofloxacin and co-trimoxazole are administered prophylactically to each oncological patient with a neutropaenia below < 500 cells per microliter (for daily doses see Table S3) and both antibiotics of this combination were present concentrations that surpass the PNECs or even MICs of susceptible strains in the sanitary installations of several rooms (Fig. 3) (Bengtsson-Palme and Larsson, 2016). As only single random samples were taken, the concentrations measured in this study represent only a snapshot and the longterm concentration profiles may vary. However, two facts have to be considered, (i) the fluctuations in antibiotic concentrations seemed to be buffered by the biofilm and (ii) the stable presence of the ST823 *P. aeruginosa* and ST167 *E. cloacae* complex strains in the sanitary units of the haemato-oncological ward. The stable presence of resistant strains and the high antibiotic concentrations indicate that the long-term medication with co-trimoxazole and ciprofloxacin might exert a selective effect towards the resistance development directly in the siphons and result in stabilizing a long-term colonization with resistant strains, Interestingly, the selective effect of fluoroquinolones has also been held responsible for the world-wide dissemination of the ST235 *P. aeruginosa* clone (Treepong et al., 2018).

After rinsing of the siphons and the toilets with 15 l of sterile water, the antibiotics were detected again in some water samples after 24 h of stagnation. In contrast, the latest recommendations and sustainability efforts of the European Green Public Procurement (EU GPP) criteria covering procurement actions for toilet flushing systems limit the amount of water to be used for one flush to a maximum of 6 l and aim at flush volumes below 3 l (Genty, 2018). Such low flush volumes might stabilize selective concentrations of antibiotics in the wastewater system even better than the 15 l used in this study and possible health risks arising from this guideline should be considered in hospital hygiene. Therefore, flush volumes of more than 15 l of water should at least be tested for risk areas with highly vulnerable patients (e.g. haemato-oncology) in order to observe the effect on residual antibiotics and bacterial colonization, as long as alternative approaches, such as self-sterilizing siphons, are not available If this approach is confined to the risk areas, the additional consumption of water will not be high.

Biofilms contain concentrated numbers of bacteria and a higher amount of resistant bacteria was isolated after manipulating the biofilm. Particularly in the oncological ward, the biofilms in the shower siphons were macroscopically visible. Therefore, basic mechanical cleaning of all parts of the siphons that are accessible should be introduced into the cleaning practice. The fact that viable bacteria were isolated from the biofilm even after routine cleaning, shows that biofilm associated bacteria are impervious to antiseptics. An optimized shower siphon design, which allows easy cleaning or even replacement of an integrated shower drain fitting, would be desirable and in the end such measures could reduce the dissemination of antibiotic-resistant bacteria into the wastewater and back to the patient.

5. Conclusions

These results substantiate that hospital wastewater systems with elevated antibiotic pressure are major reservoirs of multi-drug-resistant bacteria. Furthermore, in contradiction to common belief the results underline that the wastewater system begins in the siphons and that siphons of sanitary facilities have to be taken into account when searching for sources of antibiotic residues and MDR bacteria. Therefore, the question arises whether the direct patient sanitary environment (siphons, drains, etc.) offers optimal opportunities for preventive approaches in terms of risk minimization and interruption of transmission chains with regard to possible feedback to humans along the wastewater path.

To reduce biofilm formation, siphons should be flushed and cleaned daily, particularly in high risk areas harbouring immunocompromised patients. So far, self-disinfecting siphons only are available to be installed under washbasins. Other effective disinfection methods are not yet commercially available. Thus, design of the sanitary equipment and technical solutions reducing the microbial load of siphons might in future play a key role in preventing transmission from contaminated wastewater systems to patients and into the environment. The protection of water and wastewater networks from contamination with pathogens and antibiotic resistance genes is of paramount importance for public health protection.

Conflicts of interest

The authors declare no conflict of interest.

This study complies with the ethical guidelines of the Declaration of Helsinki by the "World Medical Association" from 1964. The ethics committee of the Medical Faculty of the University of Bonn was involved and approved the procedures and the publication of the results (reference no. 120/16).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.03.006.

References

Basset, P., Blanc, D.S., 2014. Fast and simple epidemiological typing of Pseudomonas aeruginosa using the double-locus sequence typing (DLST) method. Eur. J. Clin. Microbiol. Infect. Dis. 33, 927–932.

Bengtsson-Palme, J., Larsson, D.G., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149.

Curran, B., Jonas, D., Grundmann, H., Pitt, T., Dowson, C.G., 2004. Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa. J. Clin. Microbiol. 42, 5644–5649.

Gattlen, J., Amberg, C., Zinn, M., Mauclaire, L., 2010. Biofilms isolated from washing machines from three continents and their tolerance to a standard detergent. Biofouling 26, 873–882.

Genty, A., 2018. European Green Public Procurement Criteria for Flushing Toilets and Urinals. Joint Research Centre, European Commission´s in-house science service.

Giani, T., Arena, F., Vaggelli, G., Conte, V., Chiarelli, A., Henrici De Angelis, L., Fornaini, R., Grazzini, M., Niccolini, F., Pecile, P., Rossolini, G.M., 2015. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing Klebsiella pneumoniae traced to clonal expansion of an mgrB deletion mutant. J. Clin. Microbiol. 53, 3341–3344.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8, 1282.

Izdebski, R., Baraniak, A., Herda, M., Fiett, J., Bonten, M.J., Carmeli, Y., Goossens, H., Hryniewicz, W., Brun-Buisson, C., Gniadkowski, M., MOSAR WP2, WP3, WP5 study groups, 2015. MLST reveals potentially high-risk international clones of Enterobacter cloacae. J. Antimicrob. Chemother. 70, 48–56.

Jeukens, J., Freschi, L., Kukavica-Ibrulj, I., Emond-Rheault, J.G., Tucker, N.P., Levesque, R.C., 2019. Genomics of antibiotic-resistance prediction in Pseudomonas aeruginosa. Ann. N. Y. Acad. Sci. 1435, 5–17.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome. Open. Res. 3, 124.

Kanamori, H., Weber, D.J., Rutala, W.A., 2016. Healthcare outbreaks associated with a water reservoir and infection prevention strategies. Clin. Infect. Dis. 62, 1423–1435.

Liu, Y.Y., Wang, Y., Walsh, T.R., Yi, L.X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.H., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect. Dis. 16, 161–168.

Madsen, J.S., Burmølle, M., Hansen, L.H., Sørensen, S.J., 2012. The interconnection between biofilm formation and horizontal gene transfer. FEMS Immunol. Med. Microbiol. 65, 183–195.

Miyoshi-Akiyama, T., Hayakawa, K., Ohmagari, N., Shimojima, M., Kirikae, T., 2013. Multilocus sequence typing (MLST) for characterization of Enterobacter cloacae. PLoS One 8, e66358.

Molin, S., Tolker-Nielsen, T., 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr. Opin. Biotechnol. 14, 255–261.

Munoz-Price, L.S., Hayden, M.K., Lolans, K., Won, S., Calvert, K., Lin, M., Stemer, A., Weinstein, R.A., 2010. Successful control of an outbreak of Klebsiella pneumoniae carbapenemase-producing K. pneumoniae at a long-term acute care hospital. Infect. Control Hosp. Epidemiol. 31, 341–347.

Munoz-Price, L.S., Poirel, L., Bonomo, R.A., Schwaber, M.J., Daikos, G.L., Cormican, M., Cornaglia, G., Garau, J., Gniadkowski, M., Hayden, M.K., Kumarasamy, K., Livermore, D.M., Maya, J.J., Nordmann, P., Patel, J.B., Paterson, D.L., Pitout, J., Villegas, M.V., Wang, H., Woodford, N., Quinn, J.P., 2013. Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. Lancet Infect. Dis. 13, 785–796.

Müller, H., Sib, E., Gajdiss, M., Klanke, U., Lenz-Plet, F., Barabasch, V., Albert, C., Schallenberg, A., Timm, C., Zacharias, N., Schmithausen, R.M., Engelhart, S., Exner, M., Parcina, M., Schreiber, C., Bierbaum, G., 2018. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. FEMS Microbiol. Ecol. 94.

Parkes, L.O., Hota, S.S., 2018. Sink-related outbreaks and mitigation strategies in healthcare facilities. Curr. Infect. Dis. Rep. 20, 42.

Rutala, W.A., Weber, D.J., 1997. Water as a reservoir of nosocomial pathogens. Infect. Control Hosp. Epidemiol. 18, 609–616.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43, 325–335.

Treepong, P., Kos, V.N., Guyeux, C., Blanc, D.S., Bertrand, X., Valot, B., Hocquet, D., 2018. Global emergence of the widespread Pseudomonas aeruginosa ST235 clone. Clin. Microbiol. Infect. 24, 258–266.

Tzouvelekis, L.S., Markogiannakis, A., Psichogiou, M., Tassios, P.T., Daikos, G.L., 2012. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. Clin. Microbiol. Rev. 25, 682–707.

Voigt, A.M., Faerber, H.A., Wilbring, G., Skutlarek, D., Felder, C., Mahn, R., Wolf, D., Brossart, P., Hornung, T., Engelhart, S., Exner, M., Schmithausen, R.M., 2019. The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: a neglected source of antibiotic residues. Int. J. Hyg Environ. Health. https://doi.org/10.1016/j.ijheh.2018.12.013.

Westphal-Settele, K., Konradi, S., Balzer, F., Schönfeld, J., Schmithausen, R., 2018. [The environment as a reservoir for antimicrobial resistance : a growing problem for public health?]. Bundesgesundheitsblatt - Gesundheitsforsch. - Gesundheitsschutz 61, 533–542.

Weterings, V., Zhou, K., Rossen, J.W., van Stenis, D., Thewessen, E., Kluytmans, J., Veenemans, J., 2015. An outbreak of colistin-resistant Klebsiella pneumoniae carbapenemase-producing Klebsiella pneumoniae in The Netherlands (July to December 2013), with inter-institutional spread. Eur. J. Clin. Microbiol. Infect. Dis. 34, 1647–1655.

Zowawi, H.M., Syrmis, M.W., Kidd, T.J., Balkhy, H.H., Walsh, T.R., Al Johani, S.M., Al Jindan, R.Y., Alfaresi, M., Ibrahim, E., Al-Jardani, A., Al Salman, J., Dashti, A.A., Sidjabat, H.E., Baz, O., Trembizki, E., Whiley, D.M., Paterson, D.L., 2018. Identification of carbapenem-resistant Pseudomonas aeruginosa in selected hospitals of the Gulf Cooperation Council States: dominance of high-risk clones in the region. J. Med. Microbiol. 67, 846–853.