International Journal of Antimicrobial Agents 50 (2017) 436–440

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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

Wastewater is a reservoir for clinically relevant carbapenemase- and 16s rRNA methylase-producing Enterobacteriaceae

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ARTICLE INFO

Article history:
Received 2 December 2016
Accepted 12 April 2017

Keywords:
Carbapenems
Aminoglycosides
Resistance
Wastewater

ABSTRACT

The aim of this study was to evaluate wastewater for carbapenemase-producing Enterobacteriaceae (CPE) and 16S rRNA methylase-producing Gram-negative bacteria (MPB) and to assess their occurrence following wastewater treatment. Wastewater samples were collected between June 2015 and March 2016 in the sewage network of the city of Basel (Switzerland) from sites located before and after influx of wastewater from the hospital into the sewage network. Samples were also obtained from the influent and effluent of the receiving wastewater treatment plant. Samples were screened for CPE and MPB using selective media. *Escherichia coli* and *Klebsiella pneumoniae* were typed by multilocus sequence typing (MLST). Carbapenemase and 16S rRNA methylase genes were identified by PCR and sequencing. Resistance profiles were determined by the disk diffusion test and Etest. The occurrence of CPE and MPB was increased downstream of hospital wastewater influx. Of 49 CPE isolates, 9 belonged to OXA-48-producing *E. coli* clone D:ST38, 7 were OXA-48-producing *Citrobacter freundii*, and 6 were KPC-2- or OXA-48-producing *K. pneumoniae* belonging to clonal complex 258. NDM (NDM-1, -5 and -9) and VIM (VIM-1) producers were detected sporadically. MPB included ArmA- and RmtB-producing *E. coli* and *Citrobacter* spp. Isolates corresponding to strains from wastewater were detected in the effluent of the treatment plant. Conclusively, CPE and MPB, predominantly OXA-48-producing Enterobacteriaceae, are readily detected in wastewater, survive wastewater treatment and are released into the aquatic environment. OXA-48-producers may represent an emerging threat to public health and environmental integrity.

1. Introduction

Dissemination of carbapenemase-producing Enterobacteriaceae (CPE) is a major concern for healthcare providers worldwide. Carbapenemases are β-lactamases, usually plasmid-encoded, that hydrolyse almost all β-lactam antibiotics. Most CPE are also resistant to multiple other classes of antimicrobials and therefore treatment options for infections are limited. Among the few remaining antimicrobials, tigecycline, fosfomycin, colistin and aminoglycoside antibiotics (mainly gentamicin) are the currently considered chemotherapeutic options for the treatment of severe infections caused by multidrug-resistant (MDR) CPE [1]. However, high-level resistance to aminoglycosides owing to the production of plasmid-encoded 16S rRNA methylase is emerging in Enterobacteriaceae and may be associated with the production of carbapenemases [2]. Clinically relevant carbapenemases include the class A KPC, the class B metallo-β-lactamases VIM, IMP and NDM, and the class D OXA-48-type enzymes [3]. In the hospital setting in Switzerland, CPE are isolated predominantly from patients exposed via previous contact with healthcare services in CPE-endemic countries such as India, Cyprus, Greece and Italy, whereby the most important carbapenemase-producing species continues to be *Klebsiella pneumoniae* [4,5]. However, the last years have seen the emergence of other carbapenemase-producing species such as *Escherichia coli*, *Enterobacter* spp. and *Citrobacter* spp. The development of resistance to carbapenems among *E. coli* is of particular concern because of its potential to disseminate carbapenemases to the community and the environment generally, a situation analogous to the global spread of extended-spectrum β-lactamases (ESBLs), especially of *E. coli* sequence type (ST) 131 harbouring $bla_{\text{CTX-M-15}}$, during the last decade [4]. One of the most pressing challenges is to determine reservoirs and transmission pathways of CPE and other MDR bacteria in order to reduce the risk to public health. Wastewater has repeatedly been described to reflect the current status of antibiotic-resistant bacteria in the population [6,7]. Hospital wastewater (HWW) is reported to serve as an important reservoir of CPE [8], however the correlation between clinical isolates of CPE and CPE

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http://dx.doi.org/10.1016/j.ijantimicag.2017.04.017
0924-8579/

isolated from HWW remains unclear [9,10]. The aim of this study was to evaluate the presence of CPE and 16S rRNA methylase-producing bacteria (MPB) in wastewater. Samples were collected over a 9-month period from different sites within the sewage disposal network of the city of Basel, Switzerland. The sites under scrutiny corresponded to municipal wastewater (MWW), HWW, treatment plant influent (TPI) and treatment plant effluent (TPE). Isolates were examined for (i) species identity, phylogenetic group or multilocus sequence type (MLST), (ii) carbapenemase or 16S rRNA methylase genes, ESBL genes and the plasmid-mediated colistin resistance gene *mcr-1* and (iii) antimicrobial susceptibility profile.

2. Materials and methods

2.1. Study setting and sampling

This study targeted multiple sites within the sewage disposal network of the city of Basel, including sites sampled upstream and downstream of the wastewater discharge of the University Hospital of Basel, a tertiary care centre admitting more than 32 000 adult patients per year. Wastewater accrues from the city of Basel, which has a current population of 190 000, as well as 11 surrounding communities, excluding wastewater from the chemical and pharmaceutical industry, which is treated in a separate chemical wastewater treatment plant (WWTP). The WWTP processes 100 million litres of raw sewage per day using a primary sedimentation basin, secondary biological treatment tanks and a final secondary sedimentation basin. Treated water is released directly into the River Rhine.

Sampling took place on seven occasions between June 2015 and March 2016. Sewage water samples were taken from a total of 10 sites, representing four different types of wastewater: four sampling sites (denoted as sites I–IV), representing MWW, were located ca. 250, 330, 500 and 730 m upstream of four sewage discharge sites of the University Hospital Basel. Four sampling sites (denoted as V–VIII), representing HWW, were located immediately downstream of the four hospital discharge sites. Sampling site IX, representing wastewater TPI, was located at the inlet of the WWTP, at a distance of ca. 4000 m from sites V–VIII. Sampling site X, representing TPE, was situated at the final outlet channel of the WWTP.

For analysis of MWW and HWW, samples from sites I–IV and sites V–VIII, respectively, were pooled on each sampling occasion.

2.2. Bacterial isolation

For each water sample, 50 mL was passed through a 0.45 μm filter (Millipore, Billerica, MA). Filters were incubated for 24 h at 37 °C in 10 mL of Enterobacteriaceae enrichment broth (Becton Dickinson, Heidelberg, Germany) for enrichment. Screening for CPE was performed by streaking one loopful of enriched culture onto chromID® CARBA SMART and one loopful onto chromID® OXA-48 (bioMérieux, Marcy-l'Étoile, France). Screening for aminoglycoside-resistant Gram-negative bacteria was conducted using Luria Bertani agar (Difco Laboratories, Franklin Lakes NJ) containing amikacin (200 mg/L), vancomycin (10 mg/L) and amphotericin B (5 mg/L). All colonies with different morphologies were picked, subcultured and purified on chromID® CARBA SMART/chromID® OXA-48 or on selective medium with amikacin, respectively. Oxidase-negative, presumptive carbapenemase-producers were further subcultured on Müller–Hinton agar (Becton Dickinson, Allschwil, Switzerland) plates and were subsequently screened for carbapenemase activity using the RAPIDEC® CARBA NP test (bioMérieux). Isolates were subjected to identification by protein profiling using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (AXIMA Confidence; Shimadzu-Biotech Corp., Kyoto, Japan) using SARAMIS™ Database (Spectral Archive and Microbial Identification System; AnagnosTec, Potsdam-Golm, Germany) and PAPMID Database (Mabritec SA, Riehen, Switzerland). Strains yielding doubtful results were subjected to genetic identification based on sequencing of the *rpoB* gene fragment [11].

2.3. Phylogenetic classification of Escherichia coli isolates

Each *E. coli* isolate was assigned to one of the four phylogenetic groups designated A, B1, B2 or D using PCR as described previously [12], whereby group A and B1 typically contain commensal *E. coli* strains, whilst groups B2 and D consist of virulent extraintestinal strains.

2.4. Multilocus sequence typing of Escherichia coli and Klebsiella pneumoniae

For MLST of *E. coli* isolates, internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified by PCR and were sequenced as described by Wirth et al [13]. Sequences were imported into the *E. coli* MLST database website (http://mlst.ucc.ie/mlst/dbs/Ecoli) to determine sequence types (STs).

MLST of the *K. pneumoniae* isolates was performed by PCR amplification and sequencing of seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) according to previously described methods [14]. STs were determined according to the MLST database (http://bigsdb.pasteur.fr/klebsiella/).

Alleles and STs that had not been previously described were submitted to the curators of the databases and were assigned new designations.

2.5. Detection of antimicrobial resistance genes

DNA was extracted by a standard heat lysis protocol and was analysed by PCR for the presence of antimicrobial resistance genes. Synthesis of primers and custom DNA sequencing were carried out by Microsynth (Balgach, Switzerland). Purification of amplicons was performed using a PCR purification kit (Sigma-Aldrich, Buchs, Switzerland). All presumptive carbapenemase-producers were screened for the presence of bla_{VIM}, bla_{KPC}, bla_{NDM-1} and bla_{OXA-48} using previously described primers [15,16].

Isolates exhibiting an ESBL phenotype were screened for bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes using previously published primers [17,18]. Presumptive 16S rRNA methylase-producers were analysed for the presence of *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* as described previously [19]. All isolates were screened for the presence of the plasmid-mediated colistin resistance gene *mcr-1* using primers published recently [20]. Nucleotide sequences were analysed with CLC Main Workbench 7.7 (CLC bio, Aarhus, Denmark). Database searches were performed using the BLASTN program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/).

2.6. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) of imipenem, ertapenem and meropenem for CPE and of amikacin, kanamycin and gentamicin for MPB were determined by Etest (bioMérieux) according to the manufacturer's instructions and Clinical and Laboratory Standards Institute (CLSI) evaluation criteria [21]. All isolates were tested for susceptibility to 13 antimicrobial agents by the disk diffusion method. Zone diameters were determined and evaluated according to CLSI protocols and criteria [21]. The antibiotics tested were ampicillin, amoxicillin/clavulanic acid, cefalotin, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, kanamycin, streptomycin, sulfamethoxazole, trimethoprim, chloramphenicol and tetracycline (Becton Dickinson, Heidelberg, Germany). Multidrug

resistance was defined as resistance to three or more classes of antimicrobials.

3. Results

3.1. Detection and characterisation of bacteria

Presumptive carbapenemase-producers were recovered from all wastewater types within the sewage disposal network (Table 1; Supplementary Table S1). A total of 2 CPE isolates were retrieved from MWW, 23 from HWW, 10 from TPI and 14 from TPE. In total 49 CPE isolates were retrieved from the chromID® CARBA SMART/chromID® OXA-48 plates, including 21 *E. coli*, 12 *K. pneumoniae*, 8 *Citrobacter freundii*, 1 *Citrobacter youngae*, 5 other *Citrobacter* spp., 1 *Enterobacter aerogenes* and 1 *Enterobacter cloacae*. Typing of the *E. coli* isolates placed 10 (47.6%) within phylogenetic group A and clonal complex (CC) 10, and 9 (42.9%) within phylogenetic group D and CC38. The remaining isolates belonged to various STs (Table 1). Typing of the *K. pneumoniae* isolates identified six (50%) belonging to CC258. Other STs, including two new types ST2256 and ST2257, are listed in Table 1. No *E. coli* B2:ST131 clones were identified in any of the wastewater samples.

Isolates expressing high resistance to aminoglycosides were recovered from HWW, TPI and TPE using the selective medium with amikacin. These isolates were identified as *E. coli* ($n = 8$), belonging to ST10, ST635, ST648 and ST1266, and *Citrobacter* spp. ($n = 2$) (Table 1).

3.2. Characterisation of antimicrobial resistance genes

All detected resistance genes are listed in Supplementary Table S1. The majority of CPE isolates (35/49; 71.4%) from the chromID® CARBA SMART/chromID® OXA-48 plates harboured bla_{OXA-48}. Six (12.2%) of the isolates were bla_{NDM}-producers (two bla_{NDM-1}, three bla_{NDM-5} and one bla_{NDM-9}). Five (10.2%, all *K. pneumoniae*) isolates tested positive for bla_{KPC}, three (6.1%) contained $bla_{OXA-181}$ and four (8.2%) harboured bla_{VIM} (Table 1). Four (8.2%) of the isolates produced more than one carbapenemase: one *E. coli* D:ST393 and one *C. freundii* strain possessed both bla_{OXA-48} and bla_{VIM-1}. Similarly, two *Citrobacter* spp. carried both bla_{OXA-48} and bla_{NDM-1}. Twenty-five (51.0%) of the CPE isolates also contained bla_{CTX-M} genes. Of these, 14 (56.0%) were $bla_{CTX-M-15}$, 6 (24.0%) were $bla_{CTX-M-3}$, 3 (12.0%) were $bla_{CTX-M-24}$ and 2 (8.0%) were $bla_{CTX-M-14}$. Two (4.1%) of the 49 CPE contained bla_{SHV-12}. The genes $bla_{CTX-M-3}$ and bla_{SHV-12} were observed only in *Citrobacter* spp. (Supplementary Table S1). The two bla_{NDM-1}-harbouring *Citrobacter* spp. isolates were additionally tested for the presence of 16S rRNA methylase genes, and *armA* was detected (Table 1).

Of the eight methylase-producing *E. coli* strains isolated from the selective medium with amikacin, five (62.5%) harboured *rmtB* and three (37.5%) contained *armA* (Table 1). Furthermore, one isolate contained bla_{NDM-5}, one possessed $bla_{CTX-M-65}$ and two isolates contained $bla_{CTX-M-55}$ and $bla_{CTX-M-65}$, respectively (Table 1 and Supplementary Table S1). None of the CPE or MPB possessed the colistin resistance gene *mcr-1*.

3.3. Antimicrobial susceptibility profiles

MICs and the results of the disk diffusion tests for each isolate are listed in Supplementary Table S1. Regarding multidrug resistance, 39 (79.6%) of the 49 CPE and all 10 (100%) MPB were MDR. Six (28.6%) carbapenemase-producing *E. coli* (four NDM, one VIM-1 and one OXA-181 producer) and seven (58.3%) *K. pneumoniae* (five KPC, one OXA-48 and one NDM-9 producer) exhibited resistance to one or more carbapenems. Of the 35 OXA-48-producers, 5 (14.3%)

Table 1
Carbapenemase- and 16S rRNA methylase-producing Enterobacteriaceae detected in different sites and on different sampling occasions within the sewage network.

Species/phylogroup:ST; CC (n)	Carbapenemase or 16S rRNA methylase	Isolates (n)				Sampling occasions (n)
		MWW	HWW	TPI	TPE	
Escherichia coli						
A:ST10; CC10 (1)	OXA-48	–[a]	–	1	–	1
A:ST10; CC10 (3)	RmtB	–	–	2	1	2
A:ST215; CC10 (3)	OXA-48	–	2	–	1	1
A:ST617; CC10 (3)	NDM-5	1	–	1	1	1
A:ST410; CC23 (1)	OXA-181	–	–	–	1	1
A:ST635; CC399 (1)	RmtB	–	1	–	–	1
B1:ST940 (2)	OXA-181	–	–	1	1	1
B2:ST1266 (3)	ArmA	–	–	2	1	2
D:ST38; CC38 (9)	OXA-48	1	2	2	4	5
D:ST393; CC31 (1)	OXA-48, VIM-1	–	1	–	–	1
D:ST354 (354) (1)	OXA-48	–	–	1	–	1
D:ST648 (1)	RmtB, NDM-5	–	–	–	1	1
Klebsiella pneumoniae						
ST29 (1)	OXA-48	–	–	–	1	1
ST147 (1)	NDM-9	–	1	–	–	1
ST395 (1)	OXA-48	–	–	–	1	1
ST258; CC258 (2)	KPC-2	–	1	–	1	2
ST437; CC258 (2)	OXA-48	–	2	–	–	2
ST512; CC258 (2)	KPC-2	–	2	–	–	2
ST485 (1)	OXA-48	–	–	–	1	1
ST2256 (1)	KPC-2	–	1	–	–	1
ST2257 (1)	OXA-48	–	1	–	–	1
Citrobacter freundii (7)	OXA-48	–	4	2	1	4
Citrobacter freundii (1)	OXA-48, VIM-1	–	1	–	–	1
Citrobacter youngae (1)	OXA-48	–	1	–	–	1
Citrobacter spp. (2)	OXA-48, NDM-1, ArmA	–	1	–	1	1
Citrobacter spp. (2)	OXA-48	–	1	1	–	2
Citrobacter spp. (1)	VIM-1	–	1	–	–	1
Enterobacter cloacae (1)	OXA-48	–	1	–	–	1
Enterobacter aerogenes (1)	VIM-like	–	–	1	–	1

ST, sequence type; CC, clonal complex; MWW, municipal wastewater; HWW, hospital wastewater; TPI, treatment plant influent; TPE, treatment plant effluent.
[a] None detected.

were classified as resistant to one or more carbapenems according to current CLSI guidelines.

3.4. Isolation frequency for sampling occasions

A large proportion of the study isolates 25 of the CPE and 2 of the MPB) were retrieved sporadically (on one sampling occasion each). CPE that were detected on two occasions included *K. pneumoniae* belonging to CC258, RmtB-producing *E. coli* A:ST10 and ArmA-producing *E. coli* B2:ST1266 (Table 1). OXA-48-producing *C. freundii* were recovered on four sampling occasions and from HWW, TPI and TPE. The most frequently encountered clone, OXA-48-producing *E. coli* D:ST38, was isolated on five occasions and was the only clone detected simultaneously in MWW, HWW, TPI and TPE.

4. Discussion

This study was carried out to determine the occurrence of CPE and MPB at different sites of the sewage disposal network of the city of Basel, Switzerland, including before and after influx of wastewater from the hospital into the sewage network and the WWTP. Whilst the presence of CPE and other antimicrobial-resistant bacteria in HWW and within treatment plants is reported in the literature [9,22,23], little is known so far about the presence of CPE in MWW. In this study, MWW contained NDM-5-producing *E. coli* A:ST617. NDM-5 was first described in a clinical *E. coli* clone ST648 isolate in the UK in 2011 [24]. To our knowledge, NDM-5 has not been described in ST617 before and its identification not only in MWW but also in TPI and TPE discharged into the River Rhine indicates that NDM-5-producers may be widespread in the community and in the aquatic environment. Furthermore, OXA-48-producing *E. coli* D:ST38 was isolated from MWW. Its detection in MWW as well as HWW, TPI and TPE provides further evidence for the spread of this clone in the community and healthy carriers, as described recently [25]. HWW contained a higher number of CPE isolates compared with MWW, including KPC- and OXA-48-producing *K. pneumoniae* belonging to CC258, a lineage that is the most prevalent cause of MDR *K. pneumoniae* infections in many countries [26,27]. HWW also contained a large number of OXA-48-producing *Citrobacter* spp. The occurrence of OXA-48-producing *Citrobacter* spp. in HWW over a period of several months may be due to their regular discharge from the hospital or to the persistence of these isolates in the wastewater environment. Of note are two *Citrobacter* spp. isolates (one from HWW and one from TPE) with an interesting genotypic and phenotypic similarity to a *C. freundii* urinary clinical isolate of a patient hospitalised previously in the University Hospital of Basel [28]. These isolates co-harboured bla_{OXA-48}, bla_{NDM-1}, bla_{SHV-12}, $bla_{CTX-M-15}$ and *armA* and were resistant to β-lactams, the carbapenem compound ertapenem, aminoglycosides, fluoroquinolones and folic acid pathway inhibitors. For further comparison and clarification of the species identification, whole-genome sequencing is currently in progress and will be reported on later. This study has several limitations. First, not all morphologically identical colonies that were recovered from the water samples on selective plates were analysed. Thus, some resistant strains may have been missed. Therefore, the actual occurrence of CPE and MPB in wastewater may be even higher than observed. Second, it was not possible to directly compare the wastewater isolates with clinical isolates from the hospital, as no monitoring data or isolates from clinical cases from this hospital were available. The current findings highlight the need for further studies dedicated to correlating the presence of wastewater-associated CPE with human clinical isolates. Testing municipal and hospital wastewater for CPE or other antimicrobial-resistant bacteria may also represent an approach to recognise emerging trends in resistance and the burden of faecal carriage in the population.

In TPI and TPE, carbapenemase-producing and methylase-producing isolates of clonal types that were identical to those found in MWW or HWW (mainly OXA-48-producing *E. coli* ST38 and OXA-48-producing *Citrobacter* spp.) were detected. This finding indicates that these isolates possess the ability to persist within wastewater and to survive the wastewater treatment process. Furthermore, some of the clones detected in TPE in this study have been described previously in clinical case reports or hospital outbreaks, such as OXA-181-producing *E. coli* ST410 [29] and OXA-48-producing *K. pneumoniae* ST29 and ST395 [30,31]. Other clones identified in this study such as *E. coli* ST393 and ST354 have been reported previously as ESBL-producers [32] but not, to our knowledge, as carbapenemase-producers. As mentioned in Section 3.1, a considerable fraction of CPE and MPB from the wastewater analysed in this study were commensal *E. coli* belonging to CC10. Strains belonging to this clonal complex have been observed to contribute significantly to the spread of ESBLs [33,34]. Hence, the detection of carbapenemases and 16S rRNA methylases in CC10 isolates in wastewater is of concern and supports the hypothesis of horizontal gene transmission by plasmid-mediated mechanisms among Enterobacteriaceae [32]. In contrast, no *E. coli* B2:ST131 isolates were detected. There is growing concern that this international extraintestinal pathogenic clone may be currently involved in the global dissemination of KPC-2 and other carbapenemases [35]. Data from the current study indicate that to date, the wastewater and TPE analysed in this study do not represent a reservoir for KPC-2-producing *E. coli* ST131. Although several studies have described KPC- and VIM-producers in HWW [36], the current data indicate that OXA-48-producing Enterobacteriaceae, in particular *E. coli* and *Citrobacter* spp., may represent the greater threat to public health and environmental integrity.

In conclusion, wastewater is a reservoir of viable CPE and methylase-producing clones harbouring clinically relevant carbapenemases. Moreover, the purification process employed in the WWTP does not sufficiently reduce the burden of such MDR isolates, which are subsequently released into the aquatic environment. Additional wastewater treatment processes are urgently required in order to reduce MDR bacteria of anthropogenic origin in wastewater, in particular primary treatment of hospital effluents prior to discharge into the sewage network.

Acknowledgements

The authors wish to thank the staff of the municipal works services of the city of Basel (Switzerland) for their help with sample collection.

Funding: This work was partly supported by the Swiss Federal Office of Public Health, Division Communicable Diseases.

Competing interests: None declared.

Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2017.04.017.

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