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

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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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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