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Occurrence and persistence of carbapenemases genes in hospital and wastewater treatment plants and propagation in the receiving river

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ABSTRACT

This study aims to investigate the prevalence of clinically relevant carbapenemases genes (bla_{KPC}, bla_{NDM} and bla_{OXA-48}) in water samples collected over one-year period from hospital (H), raw and treated wastewater of two wastewater treatment plants (WWTPs) as well as along the Zenne River (Belgium). The genes were quantified in both particle-attached (PAB) and free-living (FLB) bacteria. Our results showed that absolute abundances were the highest in H waters. Although absolute abundances were significantly reduced in WWTP effluents, the relative abundance (normalized per 16S rRNA) was never lowered through wastewater treatment. Particularly, for the PAB the relative abundances were significantly higher in the effluents respect to the influents of both WWTPs for all the genes. The absolute abundances along the Zenne River increased from upstream to downstream, peaking after the release of WWTPs effluents, in both fractions. Our results demonstrated that bla_{KPC}, bla_{NDM} and bla_{OXA-48} are widely distributed in the Zenne as a consequence of chronic discharge from WWTPs. To conclude, the levels of carbapenemases genes are significantly lower than other genes conferring resistance to more widely used antibiotics (analyzed in previous studies carried out at the same sites), but could raise up to the levels of high prevalent resistance genes.

1. Introduction

Antibiotics have saved millions of human lives since their discovery and application for treating bacterial infections. However, its extensive use has led to the emergence, and spread of antibiotic resistance among bacterial pathogens thus posing a risk to human health [1].

Carbapenems are β-lactam antibiotics with a broad spectrum of activity that are usually considered drugs of last resort because they can be effective against severe hospital- and community-acquired infections caused by multidrug-resistant Gram-negative pathogens [2,3]. However, an increasing number of reports indicate that some β-lactamases can efficiently hydrolyze carbapenems [3]. These enzymes, called carbapenemases, hydrolyze carbapenems and belong to molecular class A (KPC and some GES variants), B (IMP, NDM and IMP metallo-β-lactamases) or D (OXA-48-like) of beta-lactamases according to the classification of Ambler [4]. Several studies have demonstrated that carbapenemases-producing pathogens cause serious infections in immunocompromised patients associated with high mortality rates due to limited treatment options [5,6]. This alarming situation is even worsened by the lack of new antibiotics at/or near clinical approval that are active against multidrug-resistant pathogens [3].

Carbapenemases of most clinical relevance are those whose respective genes are carried on plasmids thus favoring their maintenance and spread among Gram-negative bacterial species [7]. Consequently, the genes encoding for those carbapenemases could easily spread conferring resistance to environmental bacteria, thus increasing the possibility of dissemination of resistance genes to pathogens [8]. In particular KPC, NDM, and OXA-48-type enzymes have been widely described as the clinically most important carbapenemases and the persistence of the respective genes in environmental bacteria is of general concern due to its implications to public health [1,2]. In fact there is growing evidence that antibiotic resistance genes (ARGs) in clinical isolates are closely related to those found in their environmental counterparts [9].

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Wastewaters treatment plants (WWTPs) are considered hotspots for the acquisition and spread of antibiotic resistance in aquatic systems and three major reasons are often put forward to sustain this idea: i) the chronic discharge of antibiotic residues, antibiotic-resistant bacteria (ARB), and ARGs collected in the municipal and clinical sewer systems; ii) the favorable conditions for both selection and/or spread of ARGs among bacterial cells in WWTPs; and iii) the widespread observation that WWTP effluents contain high concentrations of different ARGs conferring resistance to widely used and last-resort antibiotics [10–12] that are consequently detected in aquatic ecosystems [13]. As a consequence, WWTP effluents are among the most important conduits for the spread of ARGs in aquatic environments. Receiving systems, and particularly urban rivers, may play an important role in driving the persistence and spread of ARGs within bacterial communities. In fact, rivers (particularly urban ones) provide a setting in which the horizontal exchange of mobile genetic elements encoding antibiotic resistance between bacteria can take place [14,15].

Carbapenem resistance has become a worldwide concern, and studies on the detection of carbapenemase-producing isolates in clinical settings are increasingly being reported [16]. Recently, several studies also started to investigate carbapenemase-producing bacteria and ARGs conferring resistance to carbapenems in non-clinical environments such as WWTPs [1,16,17], coastal recreational water [18], rivers [8,19,20] and lakes [21]. However none of these studies investigated at the same time the prevalence of the three most relevant genes conferring resistance to carbapenems (i.e. bla_{KPC}, bla_{NDM} and bla_{OXA-48}) in a complex, urban-impacted river. Our study aims to investigate the prevalence and spread of carbapenemases genes (CGs) from its source in hospital wastewater to the river water after passing through Brussels WWTPs [22] distinguishing particle-attached bacteria (PAB) from free-living bacteria (FLB). The different behaviors of the ARGs depending on the lifestyle of bacteria could have implications for the spread of resistance in aquatic ecosystems. In fact, PAB are subjected to sedimentation processes and consequently, depending on the river flow, they are not expected to travel downstream as rapidly as FLBs are expected to do. However, resistant bacteria colonizing particles could both fall on the benthic compartment, favoring the spread of resistance in biofilms, and be re-suspended, as a consequence of flood events, consequently delivering resistance downstream. The study of the different behaviors of CGs in PAB and FLB could also provide useful information for wastewater treatment management in order to reduce the input of resistance determinants in aquatic ecosystems. To our knowledge this is the first study reporting the occurrence and abundance of ARGs conferring resistance to carbapenems in Belgian aquatic systems.

2. Material and methods

2.1. Study site and sampling strategy

In this study, the two WWTPs located in the Brussels Capital Region (Belgium) and the Zenne River, in which they discharge treated sewage waters, were investigated as well as the sewage waters of the UZ Brussels Hospital. The Brussels South (BS) WWTP (360,000 equivalent-inhabitants) is in operation since the year 2000 whereas the Brussels North (BN) WWTP (1.1 million equivalent-inhabitants) operates since 2007 and receive, among others, the untreated waters from the UZ Brussels Hospital. The two WWTPs function on different technologies. The BS WWTP treatment line includes a primary settling stage (to remove suspended solids) and a secondary biological treatment (an activated sludge process to remove biodegradable organic matter). At the BN WWTP there is a biological line including a primary settling stage followed by a modern tertiary treatment technology (simultaneous removal of biodegradable organic carbon, nitrogen, and phosphorus by an activated sludge process; Azenit P® technology). The second treatment line only uses a primary settling process a and runs in parallel to the biological line when the volume of the influent is too high during storms. On an annual basis, the volume treated in the biological line accounts for roughly 90% of the total volume reaching the WWTP.

In the BS WWTP, average daily samples were collected with refrigerated automatic samplers, whereas at the BN WWTP grab samples were collected in the morning. Since sampling campaigns were conducted under dry weather conditions, all data from the BN WWTP presented in this paper concern the biological line. In addition, a grab sample was collected at the outlet of the UZ Brussels Hospital (H), which, according to the 2012 annual report, has 721 beds, 29,239 hospitalizations per year, and 23,692 day hospitalizations per year (excluding minimum flat rates). The effluents of both WWTPs are released in the Zenne River, a small urban river running through the city of Brussels [22,23].

Seven stations were sampled along the Zenne River. A kilometric scale along the river was defined; the zero is arbitrarily set at station Z1 and increased from upstream to downstream sites. Stations Z1 (0 km) and Z3 (13 km) are located upstream from Brussels. Stations Z4 (19 km) and Z5 (20 km) are located upstream and downstream from the BS WWTP effluent discharge point, respectively. Stations Z8 (33 km) and Z9 (34 km) are located upstream and downstream from the BN WWTP, respectively, and Station Z11 (41 km) is downstream from Brussels area (Fig. 1).

Four sampling campaigns were conducted in 2016, one per season (January, April, June, and November). During the sampling campaigns, triplicate samples were collected at all the sites described above, stored in sterile 1000 mL bottles and kept at 4 °C until analysis.

2.2. Chemical analysis of carbapenems

The treatment of water samples was carried out as described elsewhere [23]. The final extracts were analyzed by means of liquid chromatography coupled to mass spectrometer triple quadrupole in tandem. The separation of 20 µL of extract was achieved with a Luna® (2 × 150 mm, 5 µm particle size, 100 Å pore size; Phenomenex) column with a mobile phase of water and acetonitrile flowing at 0.25 mL min^{-1}. Each gradient lasted 15 min. Detection was carried out using a spectrometer with a triple quadrupole analyser TSQ Quantiva (Thermo Fischer Scientific) equipped with a heated electrospray ionisation (H-ESI) source, operating in positive polarity with the following parameters: principal, auxiliary and sweep gas flows: 35 a.u., 9 a.u. and 1 a.u., respectively; voltage: 3300 V; capillary and vaporizer temperatures: 325 °C and 275 °C. The selected transitions for quantification (in bold) and confirmation were **300** > **142** and 300 > 98, for imipenem, and **384** > **114** and 384 > 100, for meropenem. The entire system was controlled by Xcalibur software v.2.2. The recoveries of the entire process ranged from 77.9% to 86.4% for meropenem and from 70.3% to 73.0% for imipenem, with good limits of detection in each case (see Table 1).

2.3. Extraction of DNA

The bacterial biomass was collected from water samples and concentrated by filtration. An aliquot (from 0.25 L to 1.5 L) of each replicate was filtered to collect two different bacterial fractions. Particle-attached bacteria (PAB) were collected by filtering water on 5-µm pore-size, 47-mm-diameter polycarbonate filters (Millipore, Billerica, MA, USA). Filtrates were then filtered through 0.22-µm pore-size 47-mm-diameter polycarbonate filters to retain free-living bacteria (FLB). Filters were kept at −80 °C until extraction. Extractions were performed following the same protocol described in [24]. DNA concentration and purity were determined using a NanoDrop ND-2000 UV Vis spectrophotometer (ThermoFisher, Scientific Inc., Wilmington, Delaware, USA).