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**Presence of carbapenemase-producing Enterobacteriaceae in the River Lambro basin, Italy:
might sediment represent an important resistance reservoir?**

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Running head: KPC Enterobacteriaceae in the River Lambro, Italy

Keyw ords: River Lambro; Carbapenemase; Enterobacteriaceae, bla_{KPC}, *Klebsiella*.

ABSTRACT

In the last years, the rapid spread in anthropized ecosystems of pathogens which are resistant to carbapenem antibiotics has raised great concern. In this study, KPC-producing *Klebsiella pneumoniae* was found in the River Lambro in June 2019, whereas KPC-producing *Klebsiella oxytoca* and *Citrobacter braakii* were identified in untreated wastewaters. Susceptibility profiles indicated resistance to imipenem, ertapenem and meropenem. Different carbapenamase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{OXA-48}) were also found in the River Lambro, although not associated to living bacteria. The presence of a wide set of carbapenemase genes and resistant pathogens show that river sediments could act as a reservoir of antibiotic resistance potentially threatening human health.

INTRODUCTION

In the last decade, carbapenemase-producing Enterobacteriaceae (CPE) have become globally and sadly known because of their ability to cause healthcare-associated (nosocomial) infections that are resistant to antibiotics (Bonomo *et al.*, 2018). In addition, the fact that CPE are being identified with

increasing frequency not only within healthcare facilities but also in the environment, is of great concern. Carbapenemases (e.g., KPC, IMI, SME, GES, NDM, VIM, IMP, OXA-48) can hydrolyse not only carbapenems but almost all β -lactam antibiotics. KPC, NDM, VIM and OXA-48 represent some of the most common carbapenemases, whose genetic traits are commonly located on plasmids, a condition which favours the transfer of resistance genes among bacterial cells. Moreover, the resistances to β -lactams, biocides and metals often coexist on the same plasmid and can be co-selected and co-transmitted, increasing the spread of multi drug resistant (MDR) bacteria. Although the detections of carbapenemase coding genes in natural environments are increasing, their origins, transmission cycles and risks to humans are still limitedly understood (Khan *et al.*, 2018; Proia *et al.*, 2018). However, concern for the spread in natural environments of MDR pathogens carrying carbapenemases (*Klebsiella pneumoniae* (Schroeter, 1886) Trevisan, 1887, *Pseudomonas aeruginosa* (Schröter 1872), Migula 1900 and *Acinetobacter baumannii* Bouvet and Grimont 1986 in particular) is raising (Mills and Lee, 2019).

The main purpose of this study was to investigate the occurrence of a set of carbapenemase genes and related pathogen strains in sediments of the River Lambro and in untreated wastewaters pertaining the same river basin. Draining one of the most densely inhabited areas in Europe (~1500 inhabitants/km²), this river is a tributary of the main Italian watercourse, the River Po, and contributes significantly to its contamination up to the Adriatic Sea (Viganò *et al.*, 2015). In this regard, previous studies have demonstrated several impacts on the biotic communities exposed to the contaminants transported by the River Lambro (Viganò *et al.*, 2016).

METHODS

Using a Ponar grab sampler, a composite sediment sample (100 mL) was collected in the River Lambro in June, 2019, about 250 meters upstream of the confluence with the River Po (32° 54' 29.0587 E, 49° 58' 68.570 N) (Fig. 1). The sediment sample was kept refrigerated at 4°C in sterile PPT bottles until processing. We also collected a sample of suspended solids (sample Bi, 50 mL) from the raw wastewater of a treatment plant (WWTP), and a river sediment sample downstream of the same WWTP (sample Bd, 50 mL) discharging into the River Lambro. Both samples were kept in sterile PPT bottles at 4°C and immediately transferred to the Clinical Microbiology, Virology and Bioemergency Laboratory of ASST Fatebenefratelli Sacco (Milan, Italy) for subsequent analyses. Bacterial strains were obtained from microbiological cultures on agar plates selective for Gram positive and Gram-negative bacteria (Mannitol Salt Agar and MacConckey Agar plates, respectively) and on CARB agar to phenotypically highlight the resistance to carbapenems

(Biomérieux, France). Plates were incubated overnight at 37°C. The identification of species was obtained by Vitek MS (BioMérieux). The susceptibility profiles were determined by Vitek2 System (BioMérieux) according to the manufacturer protocol and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2019 breakpoints.

The carbapenemase gene presence was confirmed directly from samples and from colonies growth on selected agar by Xpert Carba-R test (GeneExpert, Cepheid), in accordance with the manufacturer protocol. Before amplification, DNA was extracted by using the UltraClean microbial DNA isolation kit or the Power-Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) for the isolated strains, or for 0.25 g of the environmental samples respectively, according to the manufacturer protocols. The quality of DNA was assessed by agarose gel electrophoresis.

Xpert Carba-R test is able to detect the *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{OXA} (variants 48, 162, 163 and 204) and *bla*_{IMP} (variants 1, 3, 6, 10, 25 and 30) genes. The isolates were subjected to whole genome sequencing (WGS) typing. Genomic libraries were produced and sequenced using Ion Torrent Personal Genome Machine (PGM, Ion Torrent, Thermo Fisher, Italy) with a 2 by 250 paired-end run. The final assemblies were based on 96 to 237 contigs (400 bps/read), with N50 values comprised between 68,340 and 151,723, thus covering ~5.8 Mb of the bacterial genome.

The in silico MLST analysis was made by comparing the whole-genome sequences against the MLST 2.0.0. and the *K. pneumoniae* Pub MLST database (<http://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>) in order to assign allelic numbers to all ST loci. The resistome of antimicrobial resistance genes was analysed using ResFinder-2.1 software (default identity thresholds [ID] 98%) provided by the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>) and the resources of the Pasteur MLST *K. pneumoniae* database. PlasmidFinder-1.3 (ID 98%; <http://www.genomicepidemiology.org>) was used to define the content of the plasmid replicon types. Resistance genes were considered hosted on plasmids when located on contigs identified as plasmids in the PlasmidFinder results.

RESULTS AND DISCUSSION

An MDR isolate of *K. pneumoniae*, showing a high resistance level to all carbapenems, cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole by Vitek2 System and confirmed as KPC-positive by Xpert Carba-R test (sample A, Tab. 1) was isolated from the River Lambro sediment. The carbapenem MIC values was >8 mg/L for imipenem, >16 mg/L for ertapenem and >8 mg/L for meropenem. The WGS analysis indicated that this pathogen belongs to the sequence type ST-35, and that some resistance genes (including *dfrA25*, *tet(A)* and *sul1*) were

located on plasmid IncN. However, carbapenamase resistance genes were not associated to any plasmid, and could be harboured in other genetic mobile elements. To our knowledge, this is the first report of a KPC producing *K. pneumoniae* in the sediment of a watercourse in Italy. MDR *K. pneumoniae* is considered among the most worrisome resistant bacteria on a global scale, an opportunistic species and an effective source of resistance genes to other pathogens (Wyres and Holt, 2018). Only limited information is available on the diffusion of carbapenamase resistant pathogens in the Italian environment, and particularly in surface waters. In an area of the River Po basin, for example, *K. pneumoniae* carrying bla_{KPC-2} and bla_{KPC-3} were found in a wastewater treatment plant and in a well, but not in the associated river network (Caltagirone *et al.*, 2017). In Central Italy, *K. pneumoniae* carrying bla_{KPC-3} was also found in WWTPs, and resulted to be related to the clinical isolate ST-512 (Perilli *et al.*, 2013). In this context, our identification of *K. pneumoniae* bla_{KPC-2} ST-35 in Lambro sediments is even more noteworthy. In fact, although it is considered a worldwide spread variant, in Europe it has been identified only a few times and in nosocomial samples (Shen *et al.*, 2020).

The suspended solids sample from untreated wastewater hosted MDR *Citrobacter braakii* Brenner *et al.* 1993 and *Klebsiella oxytoca* (Flügge 1886) Lautrop 1956 (sample Bi, Tab. 1), which grew on selective CARB agar plates. The carbapenem MIC values was >8 mg/L for imipenem, >16 mg/L for ertapenem and >8 mg/L for meropenem for both the isolates from sample Bi, and the carbapenamase gene production was confirmed as KPC-positivity by Xpert Carba-R test. *P. aeruginosa* was also identified in the Lambro sediment collected downstream of the WWTP discharge (sample Bd), but in this case no *bla*-genes were detected, and no WGS was done. The isolated *K. oxytoca* strain carried the bla_{KPC-2} and bla_{OXY-1-7} resistance genes, and belonged to the ST-146 strain (sample Bi, Tab. 1). This ST was originally described in Polish patients (Izdebski *et al.*, 2018), and then in Australia (Kwong *et al.*, 2018), but never before in Italy. Resistant genes were not harboured in any of the detected plasmids (Tab. 1), but could be hosted in other mobile elements. Resistant *K. oxytoca* was already found in two small rivers in Northern Italy, but sequence type was not determined (Caltagirone *et al.*, 2017). The KPC-producing *C. braaki* (sample Bi, Tab. 1) belonged to an unknown sequence type, and the resistant bla_{KPC-2} gene was harboured in the plasmid IncN. However, other resistance genes, not associated with plasmids, were also found (bla_{CMY-76} and aph(3')-Ia).

The genus *Citrobacter* is highly diverse, and many STs have been described internationally, and grouped into five main lineages (Liu *et al.*, 2020). Probably, many more *C. braaki* STs exist, but they are still undescribed. Thus, it is not surprising that the ST identification of the *C. braaki*

isolated in this study was not possible. ESBL genes are rarely carried by this species complex, and bla_{KPC-2} positivity was signalled only for *Citrobacter koseri* Frederiksen, 1970. Here, interestingly, we first documented a KPC resistance associated to *C. braaki* sampled in untreated wastewater. This species is known to cause bacteremia in immune compromised patients with comorbidities, although with low virulence and incidence. Antibiotic resistance to quinolone is commonly present in *Citrobacter*, associated ESBL, and is often mediated by plasmids (Liu *et al.*, 2020). Interestingly, our analysis did not find any resistance to quinolone in the isolated ST of *C. braaki*.

Finally, different bacteria such as *Clostridium tertium* (Henry 1917) Bergey *et al.* 1923, *Bacillus megaterium* de Bary 1884, *Stenotrophomonas acidaminiphila* Assih *et al.* 2002, *Ochrobactrum tritici* Lebuhn *et al.* 2000 were also found in River Lambro sediment (sample A), which are probably environmental bacteria rarely of concern for human health. No carbapenem resistance genes were detected in these bacteria. On the contrary, Xpert Carba-R test on the same sample A revealed also the presence of different genes such as bla_{NDM}, bla_{VIM}, bla_{OXA-48} and bla_{KPC} which were not associated with the bacteria isolated on selective agar plates. Previous studies showed that cultivable bacteria could represent only a small portion of the environmental microbioma, which includes a far higher number of species. Indeed, it has been estimated that only 5-10% of the total number of species assumed to be present in wastewater and wastewater treatment plants could be successfully cultivated on selective media (Kümmerer, 2004). It is therefore possible that the resistance genes that we have not found to be associated with the isolated resistant bacteria belong to microorganisms that could not grow in vitro, at least under our experimental conditions. The other hypothesis is that they are traits of dispersed gene fragments, therefore no longer associated with specific microorganisms.

CONCLUSIONS

This preliminary survey identified carbapenemase-producing *K. pneumoniae* in the River Lambro sediments and other MDR Enterobacteriaceae which are potentially of concern for human health. Our findings suggest that resistant pathogens may enter surface waters directly from untreated or inefficiently treated wastewaters, as well as from combined sewer overflows (Rimoldi *et al.*, 2020). Alternatively, the transfer of resistance genes to human pathogens mediated by mobile elements could have occurred either in wastewaters or in the receiving freshwater environments. Both the hypothesis deserve further investigations, also addressing the most likely origins of the resistance spread, and the health risk associated with its spatial range along the river network.

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Tab. 1. Antibiotic resistance genes and plasmids found in the bacteria isolated from River Lambro sediment and in suspended solids from untreated wastewaters, as indicated by whole genome sequencing. Genbank codes for genome sequencing are also reported.

Bacteria	ST	Beta-lactam	Sulph	Tetra	Quin	Trimeth	Fosfo	Aminogl	Plasmids	Genbank
<i>Klebsiella pneumoniae</i> (River Lambro, sample A)	35	bla _{KPC-2} bla _{SHV-33}	sul1 ^a	tet(A) ^a	oqxA oqxB	dfrA25 ^a	fosA	-	IncFIB(K) IncFII(K) IncN	BioProject: PRJNA736865 BioSample: SAMN19667786
<i>Klebsiella oxytoca</i> (Untreated WW, sample Bi)	146	bla _{KPC-2} bla _{OXY-1-7}	-	-	-	-	-	aph(3')-Ia	IncFIB(K) IncFII(K) IncN	BioProject: PRJNA736865 BioSample: SAMN19667787
<i>Citrobacter braakii</i> (Untreated WW, sample Bi)	-	bla _{KPC-2} ^a bla _{CMY-76}	-	-	-	-	-	aph(3')-Ia	IncHI1A(CIT) IncHI1B(CIT) IncN	BioProject: PRJNA736865 BioSample: SAMN19667788

Sulph, Sulphonamide; Tetra, Tetracycline; Quin, Quinolone; Trimeth, Trimethoprim; Fosfo, Fosfomycin; Aminogl, Aminoglycoside; ^aResistance genes hosted on plasmid incN.

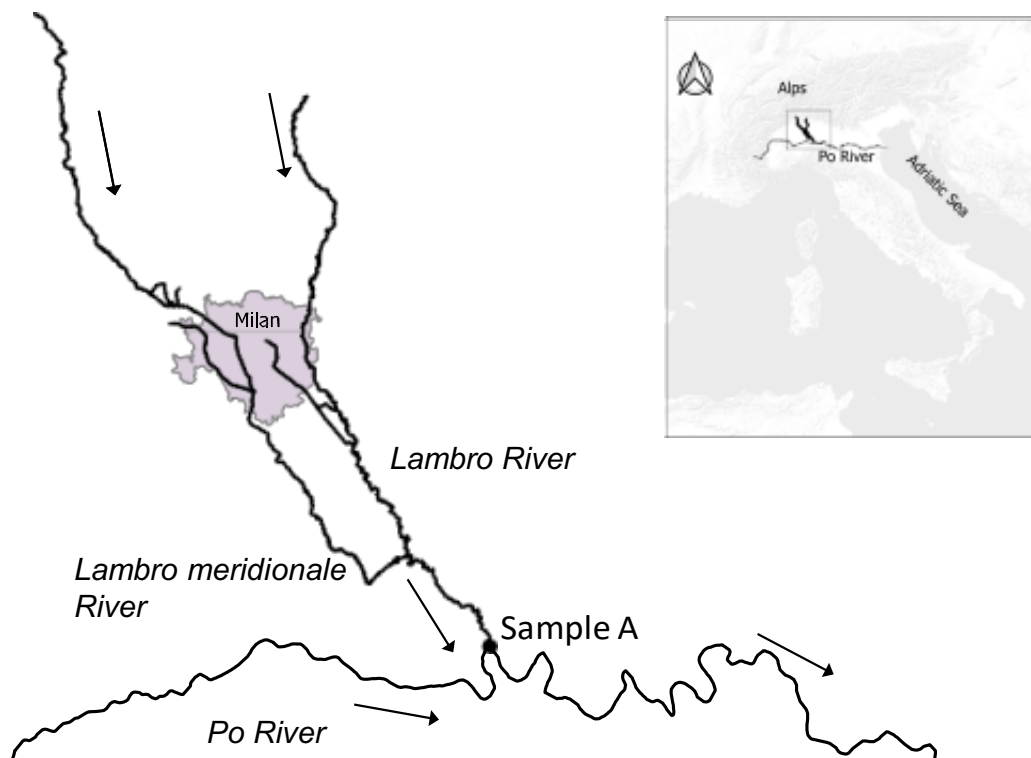


Fig. 1. Map of River Lambro and sample A location at the basin closure. The extension of the city of Milan, and the River Lambro Meridionale, which collects most of the treated wastewaters of the city, are also indicated. On the inset, the location of River Lambro in the River Po basin is shown on a wide scale.