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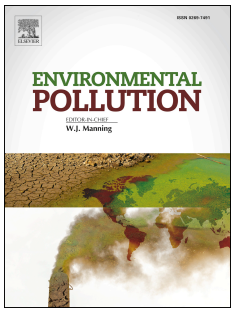

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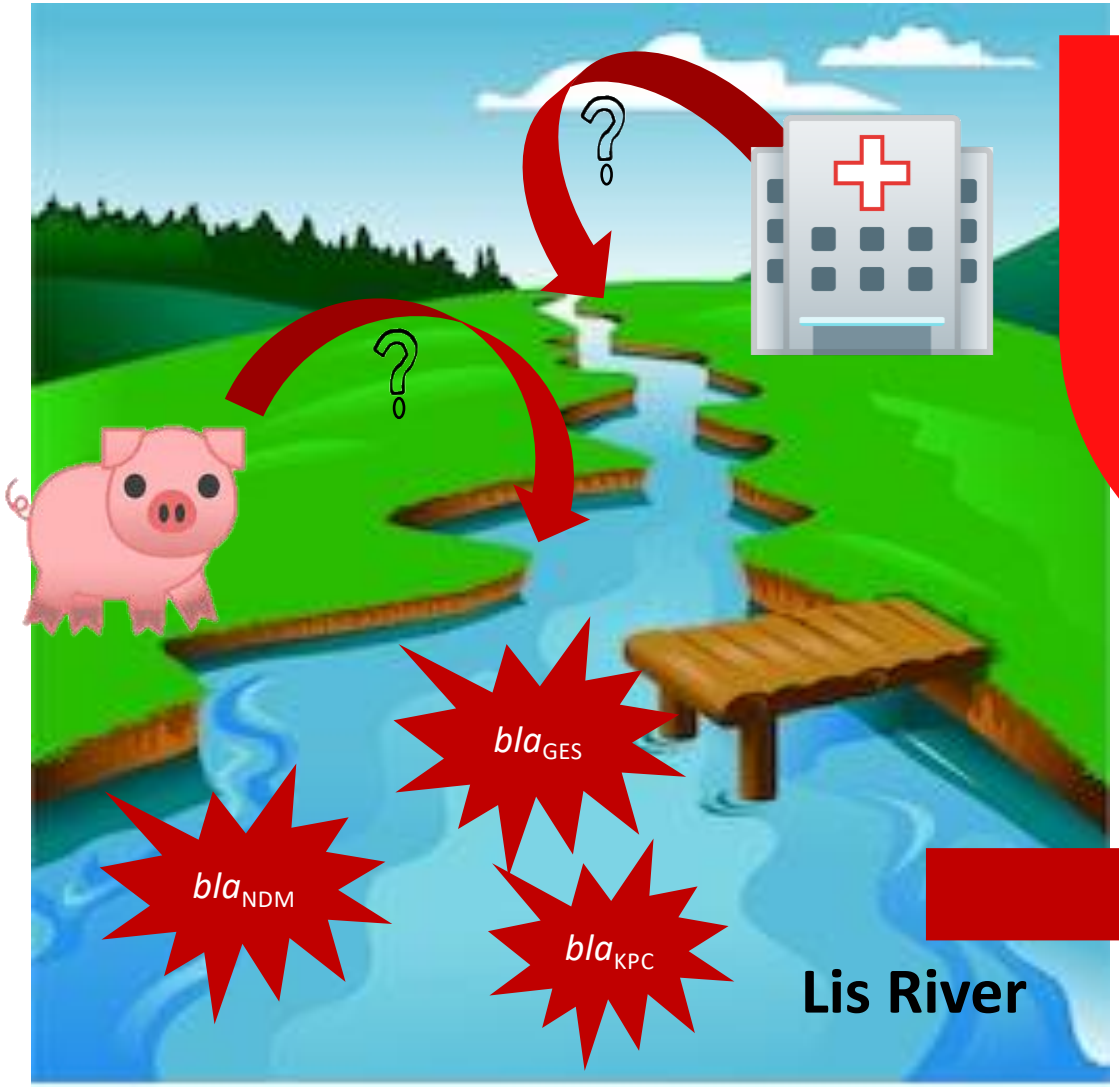

Enterobacteriaceae

K. pneumoniae
(n=9)
C. freundii (n=1)
E. roggenkampii
(n=3)

Occurrence of carbapenemase-producing *Enterobacteriaceae* in a Portuguese river: bla_{NDM}, bla_{KPC} and bla_{GES} among the detected genes

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Abstract

Carbapenems are used as last-resort drugs to treat infections caused by multidrug-resistant bacteria. Despite the increasing number of reports of carbapenem-resistant *Enterobacteriaceae* (CRE), there is still limited information on their distribution or prevalence in the environment. Our aim was to assess the occurrence of CRE in the Lis river (Portugal) and to characterize the genetic platforms linked to carbapenemase genes. We collected six water samples from sites near a wastewater treatment plant (n=4 samples) and livestock farms (n=2). Twenty-four CRE were characterized by BOX element-polymerase chain reaction (BOX-PCR), and thirteen representative isolates were analysed by Pulsed-Field Gel Electrophoresis (PFGE) and by sequencing the 16S rRNA gene. Antimicrobial susceptibility testing, PCR screening for carbapenemase-encoding genes, conjugation experiments and plasmid analysis were performed. Four isolates were chosen for whole-genome sequencing. All water samples contained CRE (4.0 CFU/mL on average). Representative isolates were multidrug-resistant (resistant to ciprofloxacin, trimethoprim-sulfamethoxazole and to all β-lactams tested) and were identified as *K. pneumoniae*, *Enterobacter* and *Citrobacter*. Isolates carried plasmids and harboured carbapenemase-encoding genes: $bla_{\text{KPC-3}}$ in *K. pneumoniae* (*n*=9), $bla_{\text{NDM-1}}$ in *Enterobacter* (n=3) and $bla_{\text{GES-5}}$ in *Citrobacter* (n=1). Conjugation experiments were successful in two *Klebsiella* isolates. *Enterobacter* PFGE profiles grouped in one cluster while *Klebsiella* were divided in three clusters and a singleton. Whole-genome sequencing analysis revealed $bla_{\text{GES-5}}$ within a novel class 3 integron (In3-16) located on an IncQ/pQ7-like plasmid in C*itrobacter freundii* CR16. $bla_{\text{KPC-3}}$ was present on IncFIA-FII pBK30683-like plasmids, which were subsequently confirmed in all *K. pneumoniae* (*n*=9). Furthermore, $bla_{\text{KPC-3}}$ was part of a genomic island in *K. pneumoniae* CR12. In *E. roggenkampii* CR11, $bla_{\text{NDM-1}}$ was on an IncA/C_2 plasmid. The carbapenemase-encoding plasmids harboured other resistance determinants and mobile genetic elements. Our results demonstrate that Lis river is contaminated with CRE, highlighting the need for monitoring antibiotic resistance in aquatic environments, especially to last-resort drugs.

Keywords: *Enterobacteriaceae*; Carbapenemases; Environment; Whole-genome sequencing.

Capsule

Carbapenemase-producing *Enterobacteriaceae* were detected in a Portuguese river, carrying $bla_{\text{GES-5}}$, $bla_{\text{KPC-3}}$ and $bla_{\text{NDM-1}}$.

Introduction

During the last decades, we have been witnessing the escalation of antibiotic-resistant infections, a phenomenon that poses a serious threat to public health (O 'neill, 2016; World Health Organisation, 2018). Infections that were once easy to treat are now becoming fatal, leading to higher mortality rates and higher costs to healthcare systems (Center for Disease Dynamics Economics & Policy, 2015; Nadimpalli et al., 2017). A report published in 2016 by the UK government estimates that, by the year of 2050, 10 million lives will be at risk due to drug-resistant infections (O 'neill, 2016). Even last-resort antibiotics such as carbapenems, which are administered in cases of severe infections, are starting to lose their efficacy (European Centre for Disease Prevention and Control, 2017).

In Gram-negative bacteria, carbapenem resistance derives mainly from the production of carbapenemases. The first carbapenemase in *Enterobacteriaceae* was described in 1993 (*NmcA*) (Naas and Nordmann, 1994) and since then a large variety of carbapenemases have emerged and are now spreading worldwide at an alarming rate (Bonomo et al., 2018; Logan and Weinstein, 2017). Many carbapenemase-encoding genes are located on plasmids and linked with various mobile genetic structures, which further enhance their dissemination potential (Nordmann et al., 2012).

Reports of CRE have increased significantly in the past 20 years (Nordmann et al., 2011; Potter et al., 2016). In 2017, the World Health Organization (WHO) listed CRE as critical priority pathogens for R&D of new antibiotics (World Health Organisation, 2017).

The environment is currently seen as a vast reservoir of resistant organisms and their associated genes (Larsson et al., 2018). There is accumulating data revealing an interexchange of these genes between wildlife, livestock and humans (Wellington et al., 2013). CRE isolates have been found in samples of different origins in many countries in recent years, including wastewaters (de Oliveira et al., 2017; Gomi et al., 2018), vegetables (Liu et al., 2018), animals (García-Cobos et al., 2015; Webb et al., 2016), drinking water (Henriques et al., 2004; Saavedra et al., 2003), wells and river water (Migliavacca, 2017; Tacão et al., 2015; Zurfluh et al., 2013). Rivers are remarkable hotspots of antibiotic resistance, especially when exposed to human activities (Surette and Wright, 2017; Tacão et al., 2012; Zhang et al., 2009).

The Lis river (Central Portugal) has suffered several ecological disasters during the last 30 years, mainly due to piggery untreated wastewater discharges (J. Vieira et al., 2012). Several reports described poor water quality (Vieira et al., 2013; J. S. Vieira et al., 2012), including water contamination with veterinary antibiotics such as sulfamethazine (Paíga et al., 2016). Discharges from livestock farming are considered sources of antibiotic resistance genes in freshwater bodies (Nnadozie and Odume, 2019). Nonetheless, this river has never been inspected for the presence of antibiotic-resistant bacteria and resistance genes. Thereby, we sampled at Lis river in order to examine the occurrence of CRE and to characterize the genetic platforms linked to carbapenemase genes. With this study we also intend to contribute for the surveillance of CRE dissemination in Portuguese freshwater environments, hoping to raise awareness on potential human health risks.

Materials and Methods

Sampling and Bacterial Isolation

Water samples were collected in 2017 from Lis river urban area in four sites. Two sites were in the vicinity of a municipal wastewater treatment plant (UWWTP): UW (upstream the plant) (39°44'14.2"N;8°47'43.5"W) and DW (downstream the plant) (39°47'57.2"N;8°50'12.6"W). Two sites were near the entrance of the brook of Milagres (known as a hotspot for discharges from pig production facilities (J. Vieira et al., 2012)): UM (upstream the entrance) (39°46'28.7"N;8°50'01.0"W) and DM (downstream the entrance) (39°47'57.2"N;8°50'12.6"W). Sites UM and DM were sampled only once, while sites UW and DW were sampled twice, with a three-month interval, making a total of 6 water samples.

Water was collected in sterile bottles (7 L) and kept on ice for transportation. We filtered the water samples in triplicate through sterile 0.45-µm-pore membranes (400 mL each) (Pall Life Sciences) and placed them on Chromocult agar (Merck) supplemented with 4 µg/mL of imipenem. Chromocult agar without antibiotic was used to determine the proportion of imipenem-resistant bacteria by filtering also in triplicate 1mL of each water sample. Plates were incubated at 37 ºC and counts were performed after 24 h.

Individual imipenem-resistant colonies were purified and stored in 20% glycerol at -80 ºC.

Typing and Identification of CRE isolates

We used BOX-PCR, which is a PCR-based method of fingerprinting that targets repetitive sequences present throughout the genome of diverse bacterial species, to type all isolates as previously described (Tacão et al., 2012). Isolates representing each BOX profile were then selected and identified by 16S rRNA gene sequencing (Tacão et al., 2012). Representative CRE isolates were further analysed by PFGE of XbaI digested DNA, using the CHEF-DR II System (Bio-Rad Laboratories) as previously described (Ribot et al., 2006). PFGE is also a DNA fingerprinting method used for the purpose of subtyping bacteria and determine clonal relatedness (Tenover et al., 1995). We analysed the restriction patterns with GelCompar II (Applied Maths) in accordance to the criteria of Tenover and colleagues (Tenover et al., 1995).

Antimicrobial Susceptibility Testing and Detection of Resistance Genes

Antimicrobial susceptibility was tested by the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing, 2017). The antibiotics tested were Amikacin (AK), Aztreonam (ATM), Cefepime (FEP), Cefotaxime (CTX), Ceftazidime (CAZ), Ciprofloxacin (CIP), Ertapenem (ETP), Gentamicin (CN), Imipenem (IPM), Meropenem (MEM), Piperacillin (PRL), Piperacillin/tazobactam (TZP), Ticarcillin (TIC), Ticarcillin/clavulanic acid (TTC), Tigecycline (TGC) and Trimethoprim/sulfamethoxazole (SXT) (Oxoid). We used *Escherichia coli* ATCC 25922 as quality control.

The CRE isolates were screened for carbapenemase-encoding genes bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{GES}, bla_{OXA-48} using primers and conditions previously described (Table S1) (Tacão et al., 2015). We also inspected the presence of bla_{CTX-M} and *mcr-1* (Tacão et al., 2017). Amplicons were confirmed by sequencing.

Conjugation Experiments and Plasmid Analysis

We examined the transfer of carbapenem resistance genes by the broth culture conjugation method using *E. coli* J53 as recipient (Tacão et al., 2017). Briefly, donors and recipient strains were mixed at a ratio of 1:1 in broth culture and grown 24 h at 37 ºC without agitation. Transconjugants were selected on Plate Count Agar (PCA) containing imipenem (4 μg/mL) and sodium azide (200 μg/mL) and confirmed by BOX-PCR. We confirmed the presence of bla_{NDM}, bla_{KPC} and bla_{GES} genes in transconjugants by PCR and determined Minimal inhibitory concentrations (MICs) in Mueller-Hinton agar according to EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2016). The transfer frequency was also calculated as the number of transconjugants per recipient cell.

Plasmids were extracted using the NZYMiniprep kit (NZYTech) and examined by electrophoresis. We assigned incompatibility groups by the PCR-based replicon typing scheme (Carattoli et al., 2005). The IncX, IncI2 and IncR replicons were detected according to previous methods (Chen et al., 2013; Compain et al., 2014). We evaluated the presence of pBK30661-

like and pBK30683-like plasmids by PCR in *bla*$_{KPC}$-harbouring *K. pneumoniae* isolates (Chen et al., 2014).

Whole Genome Sequencing (WGS) and Analyses

We selected four CRE isolates for WGS, considering the PFGE and plasmid profiling results. Genomic DNA was purified using the Wizard Genomic DNA Purification kit (Promega) and paired-end libraries were generated using Illumina HiSeq 2500 platform (Illumina) according to the manufacturer's protocol. We checked raw reads quality using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and subjected them to a trimming process to remove those with a phred quality score below 20 by applying Trimmomatic (version 0.36, parameters: illuminaclip on, slidingwindow 4:15, leading , trailing 3, crop off, minlen 36) (Bolger et al., 2014). The assembly was performed with SPAdes version 3.11.0 (Bankevich et al., 2012).

We annotated the assembled draft genomes using Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008). Species identification was confirmed by calculating the average nucleotide identity (ANIb) values using the online ANI calculator tool (Rodriguez-R and Konstantinidis, 2016), and digital DNA-DNA hybridization (dDDH) values using the Genome-to-Genome Distance Calculator 2.1 (Meier-Kolthoff et al., 2013). Finally, we considered the G+C% divergence in the overall genome relatedness analysis (Meier-Kolthoff et al., 2014).

WGS data was analysed using tools to determine multi-locus sequence types (MLST), presence of resistance genes (Resfinder 3.0) and plasmids (PlasmidFinder 1.3), all available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). We also used the comprehensive antibiotic resistance database (CARD) to predict the resistomes and the genomic island prediction software (GIPSy) to predict genomic islands (GIs) associated with antibiotic resistance (McArthur et al., 2013; Soares et al., 2016). Integrons were compared against the integron database INTEGRALL (http://integrall.bio.ua.pt) (Moura et al., 2009).

We verified contig arrangements of *bla*$_{NDM-1}$-, *bla*$_{KPC-3}$- and *bla*$_{GES-5}$-carrying plasmids by PCR and Sanger sequencing to close gaps between contigs. For this, specific primers were designed (Table S1).

Nucleotide Sequence Accession Number

The whole genome sequences were submitted at DDBJ/ENA/Genbank under the accession numbers RBMO00000000 (*E. roggenkampii* CR11), RBWG00000000 (*K. pneumoniae* CR12), RBWI00000000 (*C. freundii* CR16) and RBWH01000000 (*K. pneumoniae* CR20).

Results

Occurrence and Diversity of Imipenem-resistant *Enterobacteriaceae*

Carbapenem (imipenem)-resistant *Enterobacteriaceae* were detected in all samples. Colony counts on Chromocult were on average $2.33\times10^{3}\pm2.78\times10^{2}$ CFU/mL of riverine water (Table 1). In medium supplemented with imipenem, average colony counts of 22.0±7 CFU/mL were obtained, corresponding to approximately 0.85% of total coliforms.

Twenty-four isolates were analysed by BOX-PCR and thirteen isolates, with distinct profiles from each sampling site, were identified by 16S rRNA gene sequence analysis as *Klebsiella pneumoniae* (n=9), *Citrobacter* (n=1) and *Enterobacter* (n=3) (Table 2).

PFGE analysis divided the nine *K. pneumoniae* isolates into three clusters and one singleton (Figure S1A). All *Enterobacter* shared an identical pattern and were obtained from sites UW (CR7; CR8) and UM (CR11) (Figure S1B).

Antibiotic Susceptibility Patterns and Carbapenemase genes

CRE isolates were multidrug-resistant (resistant to three or more classes of antibiotics; Table 2) (Magiorakos et al., 2012). Isolates were resistant to ciprofloxacin, trimethoprim-sulfamethoxazole and to all β-lactams tested. The only exceptions were the *Enterobacter* isolates, which were susceptible to aztreonam as well as the *Citrobacter* isolate, which was

susceptible to aztreonam, cefotaxime, cefepime and amikacin. All *Enterobacter* isolates were resistant to amikacin and gentamicin. Five *K. pneumoniae* isolates and the *Citrobacter* isolate were resistant to gentamicin. No isolate was resistant to tigecycline.

All 13 CRE isolates carried one carbapenemase-encoding gene: bla_{KPC-3} was found in *K. pneumoniae* isolates, bla_{NDM-1} was found in *Enterobacter* isolates and the *Citrobacter* isolate harboured a bla_{GES-5} (Table 2). bla_{CTX-M} and *mcr-1* were not detected.

Plasmids and Conjugation

The presence of plasmids was confirmed in all isolates (Figure S2). Most *Klebsiella* isolates shared the same plasmid profile while isolates CR17 and CR20 displayed distinct profiles (Figure S2). Among the *Enterobacter* isolates, CR7 and CR8 had the same plasmid profile while CR11 exhibited a different pattern. Replicon IncY was detected by PCR in 8 *K. pneumoniae* isolates, whereas IncR was found in the *Citrobacter* isolate (Table 2).

Transconjugants were successfully obtained from isolates *K. pneumoniae* CR18 and CR21. Transconjugants carried bla_{KPC} and exhibited significant MICs increase for carbapenems (from 24 to 428 times) and cephalosporins (from 128 to 250 times) (Table 3). The transfer frequencies (transconjugants per recipient cell) of pBK30683-like plasmids to *E. coli* J53 were 5.16×10^{-7} for CR18 and 6.88×10^{-7} for CR21.

WGS Analysis

Based on PFGE and plasmid profiles, 4 CRE isolates were selected for WGS (Table S2). The single *Citrobacter* isolate (CR16) was chosen since it is the only representative of this genus. Although all three *Enterobacter* isolates shared the same PFGE profile, isolate CR11 exhibited a singular plasmid profile and was chosen for WGS. Regarding the *Klebsiella* isolates, CR12, representative of the dominant PFGE profile and CR20, displaying unique PFGE and plasmid profiles, were the ones selected for WGS.

Genome sequence-based methods (ANIb and dDDH values) were congruent in the identification of both *Klebsiella* isolates as *Klebsiella pneumoniae*. The genome sequence of *Enterobacter* CR11 was 98.6% identical by ANIb to the genome of *Enterobacter roggenkampii* DSM 16690 while the sequence of *Citrobacter* CR16 was 99.0% identical by ANIb to the genome of *Citrobacter freundii AR_0116* (Table S3). dDDH values of *Enterobacter* CR11 and *Citrobacter* CR16 with the mentioned type strains were well above 70%, indicating that they belong to the same species (Chun et al., 2018).

K. pneumoniae **CR12 and CR20**

K. pneumoniae CR12 and CR20 belonged to sequence types ST231 and ST147 (Table 4). In *Klebsiella* CR12, the PCR-based protocol proposed by Chen et al.(Chen et al., 2014) was positive for all eight PCRs, confirming the genetic context of bla_{KPC-3} within Tn*4401d* in a FIA-FII pBK30683-like plasmid (GenBank accession number KF954760) (Chen et al., 2014). After further assembly based on PCR and Sanger sequencing, analysis unveiled that the bla_{KPC-3} containing contig (size 27,843 bp; Figure 1) was 100% identical in terms of nucleotide sequence to the corresponding region of plasmid pBK30683 from *K. pneumoniae* BK30683, isolated in two hospitals in the US (Table 4) (Chen et al., 2014). Other resistance determinants were detected on the same contig that are also present in pBK30683, including bla_{TEM-1}, bla_{OXA-9} (encoding β-lactam resistance), *aacA4*, *aadA1*, *strB* (aminoglycoside resistance), *sul2* (sulphonamide resistance), *dfrA14* (trimethoprim resistance) and an operon encoding resistance to nickel (Table S4). Further analysis of the genome of *K. pneumoniae* CR12 led to the identification of five GIs ranging from 7 to 28kb, harbouring genetic determinants related with resistance (Table S5). Sequence analysis of each region revealed that two of these structures harbour the genes encoding resistance-nodulation-division (RND)-type multidrug efflux pumps (GI-2) and the porin encoding gene *ompF* (GI-3). CR12 GIs analysis also revealed that the nested transposon Tn*4401*/Tn*1331*, where bla_{KPC} is located, is part of GI-5 that also harbours *sul*, *strA,B* and the nickel-resistance protein *nirB.*

In *K. pneumoniae* CR20, a cointegrated IncFIA/FII plasmid was also confirmed by the same method (Chen et al., 2014) and the bla_{KPC-3}-containing contig (size=10,058 bp) was 100% identical in terms of nucleotide sequence to the corresponding region of plasmid pBK30683 (Table 4). The PCR scheme was positive for all reactions except for the one targeting Tn*4401* conserved sequences located downstream bla_{KPC}. Further contig assembly revealed that in CR20 bla_{KPC-3} gene is directly flanked by the insertion sequence IS26, confirming the absence of the region between these two genetic elements described in pBK30683 plasmid (Figure 1). Accordingly, all the resistance genes located in this region, including *aacA4*, *aadA1*, bla_{TEM-1}, bla_{OXA-9} and *sul2*, were absent in *K. pneumoniae* CR20. GIPSy analysis resulted in the identification of one GI of 27kb in *K. pneumoniae* CR20 containing genes that encode for RND multidrug efflux proteins (Table S5).

Other resistance determinants were detected in both *Klebsiella* isolates (Table S4), namely the fosfomycin resistance gene *fosA* and the naturally occurring β-lactamase gene bla_{SHV-1} in CR12 and bla_{SHV-11} in CR20. Mutations reported in fluoroquinolone-resistant *K. pneumoniae* (Correia et al., 2017) were detected, including S80I in *parC* (in both isolates) and S83I in *gyrA* (in CR20). Regarding plasmids, an IncFIB replicon was predicted in both *Klebsiella* isolates and a Col440II plasmid was predicted in CR20. The remaining *K. pneumoniae* isolates (n=7) were inspected by PCR and the presence of pBK30683-like plasmids was confirmed.

***E. roggenkampii* CR11**

PCR mapping of the bla_{NDM-1}-surrounding sequences revealed its association with an IncA/C_2 plasmid. A BLAST search using the assembled contig (size 33,823 bp) indicated that pCR11_A/C_2 shares a high degree of similarity with other IncA/C_2 plasmids (Figure 2). Indeed, this contig was 99.85% identical with the corresponding region of pNDM15-1078, a plasmid from a clinical *E. coli* isolate in Canada (accession no. CP012902.1) (Table 4). The same contig shared 99.99% identity with plasmid pNDM-CIT from *C. freundii* STE (accession no.

JX182975.1), a clinical isolate obtained from a hospitalized patient with an urinary infection in south-eastern India (Dolejska et al., 2013).

Further analysis revealed that *bla*$_{NDM-1}$ was located on a Tn125-like transposon together with a *ble*$_{MBL}$ (resistance to bleomycin) and a truncated phosphoribosylanthranilate isomerase gene (Bontron et al., 2016). This region is flanked by two identical class 1 integrons comprising an *intl1*, a *dfrA12-aadA2* array (encoding trimethoprim and aminoglycoside resistance), *qacEΔ1* (quaternary ammonium compounds), and *sul1* (sulphonamide resistance), which are flanked upstream by ISCR1 insertions. Directly upstream the *bla*$_{NDM-1}$ gene, an IS26-insertion sequence was identified. A region that is described in pNDM-CIT (Dolejska et al., 2013) containing other relevant resistance genes including *armA* (aminoglycosides), *mph2* and *mel (*macrolides), and the *mer* operon (mercury), was also detected in this isolate, located in a 8,202 bp contig which is probably part of pCR11_A/C$_2$ (Table S4).

An IncQ2 and a *CoIRNAI* plasmids were predicted in *E. roggenkampii* CR11. The IncQ2 plasmid consisted on an 8,382 bp contig exhibiting high similarity with pQ7 plasmid (99.79% nucleotide identity) (Guillard et al., 2015). The main difference in pCR11_IncQ2 plasmid was the presence of a novel class 3 integron (In3-17) where the *bla*$_{GES-1}$ cassette is absent and a *bla*$_{OXA-677}$ replaces the fused *bla*$_{OXA}$/*aac* cassette (Figure 2). GIPSy analysis unveiled that the *bla*$_{OXA-677}$ gene is also located on GI-5 in the pCR11_IncQ2 plasmid. Moreover, genes that encode for tripartite multidrug resistance systems proteins were also present in GI-3 and GI-4 in *E. roggenkampii* CR11 (Table S5).

An amino acid substitution (S83I) previously reported in the *gyrA* quinolone resistance-determining region of *Enterobacter cloacae* isolates (Guillard et al., 2015), was detected in CR11. Finally, the *in silico* analysis revealed the presence of an *ampC* gene (*bla*$_{MIR-22}$), intrinsic of the *E. cloacae* complex (Jacoby and Tran, 1999; Reisbig and Hanson, 2004).

***C. freundii* CR16**

Citrobacter freundii CR16 was assigned to a new sequence type (ST429). The bla_{GES-5} gene was detected on a fully assembled IncQ2 plasmid of 8,950 bp, with a GC content of 61.7%. This plasmid shares high similarity with pQ7 (accession no.FJ696404) described in *E. coli* TB7 (Poirel et al., 2010), with a query coverage of 99% and nucleotide identity of 99.89% (Table 4). A similar plasmid was present in *Enterobacter* CR11, as described above. Comparing to pQ7, the major difference was the presence of a novel class 3 Integron (In3-16) with a nucleotide substitution in the bla_{GES} gene that results in a different variant, bla_{GES-5} (Figure 3).

The integron *intI1-qnrB4-* bla_{DHA} was detected in *Citrobacter* CR16, in a contig which exhibited 99.9% nucleotide identity and a query coverage of 94.0% with plasmid pMPDHA, from a *Klebsiella oxytoca* clinical isolate found in France (Verdet et al., 2006). Additional resistance genes present in pMPDHA such as *sul1*, *ereA2* (macrolide resistance), *aacA4* and *aac(3')-IIb* (aminoglycosides), were also detected in *Enterobacter* CR11 with the same arrangement, indicating their probable presence in a pMPDHA-like plasmid. The *qnrB4* gene is also located in GI-4. Other GIs related to resistance traits are present in CR16, namely, GI-1 and GI-2, comprising genes that encode for RND multidrug efflux proteins (Table S2).

Other resistance determinants were found, including the chromosomal bla_{CMY-2} and genes encoding macrolide (*mph(A)*, *mrx*) and chloramphenicol (*catII*) resistance (Table S4). IncX5, IncHI2 and IncR plasmids were predicted in this isolate. In *gyrA*, an amino acid substitution (S83I) known to reduce fluoroquinolone susceptibility was detected.

Discussion

The global rise of resistance to carbapenems in *Enterobacteriaceae* poses one of the most pressing public health threats related to antibiotic resistance. However, the number of studies investigating aquatic environments as potential reservoirs of CRE is still very limited.

In this study, we report the occurrence of CRE in water from Lis river, Portugal. A low percentage of imipenem-resistant *Enterobacteriaceae* (approximately 0.85%) was determined, which might be explained by the restrictive rules in the administration of carbapenems in Portugal (Tacão et al., 2015). As reported in other studies (Bonomo et al., 2018), production of carbapenemases seems to be the major contributing mechanism for carbapenem-resistance. The occurrence of *Enterobacteriaceae* that harbour carbapenemase-encoding genes in aquatic systems is of great concern since these antibiotics are reserved to treat the most severely ill patients in hospitals (Nicolau, 2008). Reports of CRE recovered from aquatic systems are increasing worldwide and have already been reported in countries from all continents, except for Australia (Mills and Lee, 2019; Woodford et al., 2014). The Indian subcontinent is one of the most worrying cases, where the gene encoding NDM-1 is widely disseminated (Walsh et al., 2011). In a recent work where the distribution of carbapenem-resistant *E. coli* was analyzed in the Indian aquatic environment, Akiba and co-workers reported the occurrence of twenty isolates resistant to imipenem in a total collection of 446 isolates (4.49%) (Akiba et al., 2016). In Portugal, Tacão and co-workers accessed the prevalence and diversity of carbapenem-resistant bacteria in the Vouga river basin and determined a low percentage of imipenem-resistant isolates (0.19%) of which only 2.7% were members of the *Enterobacteriaceae* family (Tacão et al., 2015). Also, Poirel, Kieffer and colleagues found KPC-, VIM- and IMP-producing *E. coli* isolates in a river crossing the city of Santo Tirso, north Portugal (Kieffer et al., 2016; Poirel et al., 2012). Most of these reports describing the occurrence of carbapenemases in bacteria isolated from aquatic environments are still largely observational and lack sufficient depth or quantification in order to make comparative analysis. Nonetheless, the variety of the carbapenemase genes harboured by *Enterobacteriaceae* found in the Lis river and their antimicrobial susceptibility patterns are alarming.

All *K. pneumoniae* isolates carried a $bla_{\text{KPC-3}}$ on a truncated nested transposon Tn*4401*/Tn*1331* located on pBK30683-like plasmids, highlighting the spread of the mobile genetic platform

375 *bla*$_{KPC-3}$-Tn*4401d* among *Enterobacteriaceae* in Portugal (Rodrigues et al., 2016). This platform
376 can be moved between strains and can insert into the chromosome or plasmids of already
377 multidrug-resistant strains (Cerqueira et al., 2017). For instance, the co-occurrence of *bla*$_{KPC-3}$-
378 Tn*4401d* with *mcr-1*-harboring plasmids has been recently reported in clinical *E. coli* in
379 Portugal (Tacão et al., 2017). In our study *bla*$_{KPC-3}$ transfer by conjugation was confirmed for
380 two *K. pneumoniae* isolates. The occurrence of *K. pneumoniae* environmental isolates
381 harbouring *bla*$_{KPC-3}$ is also alarming due to the increasing prevalence of KPC-3 producers among
382 *K. pneumoniae* in Portuguese hospitals (Manageiro et al., 2015a). Particularly, ST147 isolates
383 (of the same ST as isolate CR20) carrying this common platform were recently involved in
384 outbreaks in Portuguese hospitals (Rodrigues et al., 2016). ST147 has been associated with
385 carbapenem resistance worldwide due to production of VIM, KPC and NDM enzymes
386 (Manageiro et al., 2015a; Zowawi et al., 2015). There are also reports on the occurrence of
387 ST147 isolates on wastewater (Alouache et al., 2014; Zurfluh et al., 2018) and companion
388 animals (Ovejero et al., 2017).

389 *K. pneumoniae* CR12 (ST231), which shared the same PFGE profile with 3 isolates obtained
390 from different sampling sites in two different dates, also had the same *bla*$_{KPC-3}$-Tn*4401d*-IncFIA
391 structure in its genome. Furthermore, this structure is part of a genomic island, which enhances
392 the probability of horizontal gene transfer events (Juhas et al., 2009). *K. pneumoniae* ST231
393 carrying this platform have been linked to community invasive infections and its high content in
394 virulence factors and antimicrobial resistance genes has also been highlighted, including bla$_{GES-5}$
395 and *bla*$_{SHV-12}$ in Portugal (Manageiro et al., 2015a; Rodrigues et al., 2016). Furthermore, clinical
396 isolates of *K. pneumoniae* ST231 producing NDM-1 have been identified in the UK (Giske et
397 al., 2012).

398 Of extreme relevance is the identification of four *Enterobacter* isolates carrying the *bla*$_{NDM-1}$
399 carbapenemase gene. This is one of the most clinically significant carbapenemases which has
400 become widely distributed in recent years, particularly in India (Nordmann and Poirel, 2014;
401 Walsh et al., 2011).

The presence of *bla*$_{NDM-1}$ in a broad-host range plasmid, which carries other relevant resistance genes, is of great concern and the inclusion of several mobile genetic platforms on this plasmid renders an even higher risk of transimission to strains of clinical relevance (Sugawara et al., 2017). Similar plasmids have been detected in multidrug-resistant isolates in the UK and India, including from water samples in New Delhi (Carattoli et al., 2011). Reports of NDM-producing bacteria in Europe are mostly from clinics, while environmental niches comprise only sporadic cases, as a NDM-1-producing *Salmonella enterica* isolated from a wild bird in Germany and a NDM-9-producing *K. pneumoniae* from wastewater in Switzerland (Fischer et al., 2013; Zurfluh et al., 2018). The gene *bla*$_{NDM}$ has been detected in WWTPs receiving hospital effluents in Belgium and Spain (Proia et al., 2018; Subirats et al., 2017). To the best of our knowledge, this is the first report on the presence of *bla*$_{NDM}$-harbouring *Enterobacteriaceae* in an environmental setting in Portugal. Till date, the only published report of a NDM-1 producer in Portugal is a *Providencia stuartii* strain isolated during a hospital outbreak (Manageiro et al., 2015b).

E. roggenkampii is a recently-described species that has a similar gene content and ANI characteristics as previously defined species in the *E. cloacae* complex (Sutton et al., 2018). The high similarity between these species often results in the misidentification of *E. roggenkampii* strains as *E. cloacae* (Sutton et al., 2018).

Similarly to *Klebsiella* and *Enterobacter* isolates, *Citrobacter* CR16 also displayed a relevant antibiotic resistance arsenal. The location of *bla*$_{GES-5}$ as part of a novel class 3 integron on an InQ2 mobilisable plasmid is of particular emphasis (Poirel et al., 2010). These IncQ-type plasmids are known to play an important role in the dissemination of antibiotic resistance genes (Poirel et al., 2010).

The GES-5 variant is the main carbapenem-hydrolysing GES-type enzyme identified in *Enterobacteriaceae* and possesses significant higher carbapenemase activity than GES-1 (Poirel et al., 2009). This variant is also disseminated quite widely, being found not only in nosocomial

settings but also in the environment (Nordmann and Poirel, 2014; Poirel et al., 2009). In Portugal, an *IntI3-bla*$_{GES-5}$ structure has already been described in *K. pneumoniae* ST231 clinical isolates, belonging to the same sequence type of *Klebsiella* CR20 (Manageiro et al., 2015a). Also in Portugal, *bla*$_{GES-5}$ was detected in four *K. pneumoniae* isolates obtained from water streams (Manageiro et al., 2014).

Overall, our results highlight the propagation of acquired carbapenemase genes outside the clinic and in river environments, that may be related to the location of these genes in conjugative plasmids also harbouring transposable elements.

Intensive livestock activities have a serious impact on Lis river water quality (Vieira et al., 2013) and may contribute to the high coliform counts determined in the river water (2.3×10^3 CFU/mL on average). There are also several studies providing evidence that piggery manure is a hotspot of transferable plasmids carrying antibiotic resistance genes, correlating the use of antibiotics in food animals and antibiotic resistance among bacteria isolated from humans (Cerqueira et al., 2017; Manageiro et al., 2015a; Rodrigues et al., 2016; Webb et al., 2016; Zowawi et al., 2015). Besides livestock activities, municipal and hospital wastewater treatment plants are considered a major source of antibiotic-resistant bacteria, which is ultimately released in the environment (Gomi et al., 2018; Pärnänen et al., 2019). Although wastewater treatment processes normally result in significant reductions in the bacterial load, the percentage of antibiotic-resistant bacteria and antibiotic resistance genes are not reduced to the same extent and can even increase (Alouache et al., 2014; Giske et al., 2012; Ovejero et al., 2017).

In order to obtain a more detailed understanding of the Lis river resistome and to track the source of contamination, larger and more detailed studies are needed encompassing more sampling sites. Although animal agriculture discharges seem to have a significant impact on the microbial load in water from Lis river, our results are not conclusive in linking imipenem-resistant strains to piggeries-associated pollution.

In conclusion, our findings depict aquatic environments as underappreciated reservoirs of clinical relevant *Enterobacteriaceae* resistant to carbapenems and other antibiotics, which may be spreading their resistance genes to different strains. These results emphasize the need to enhance and extend the systematic monitoring of carpapenemases dispersion in bacteria from non-human sources, specially in environments subjected to anthropogenic pressures.

Figures:

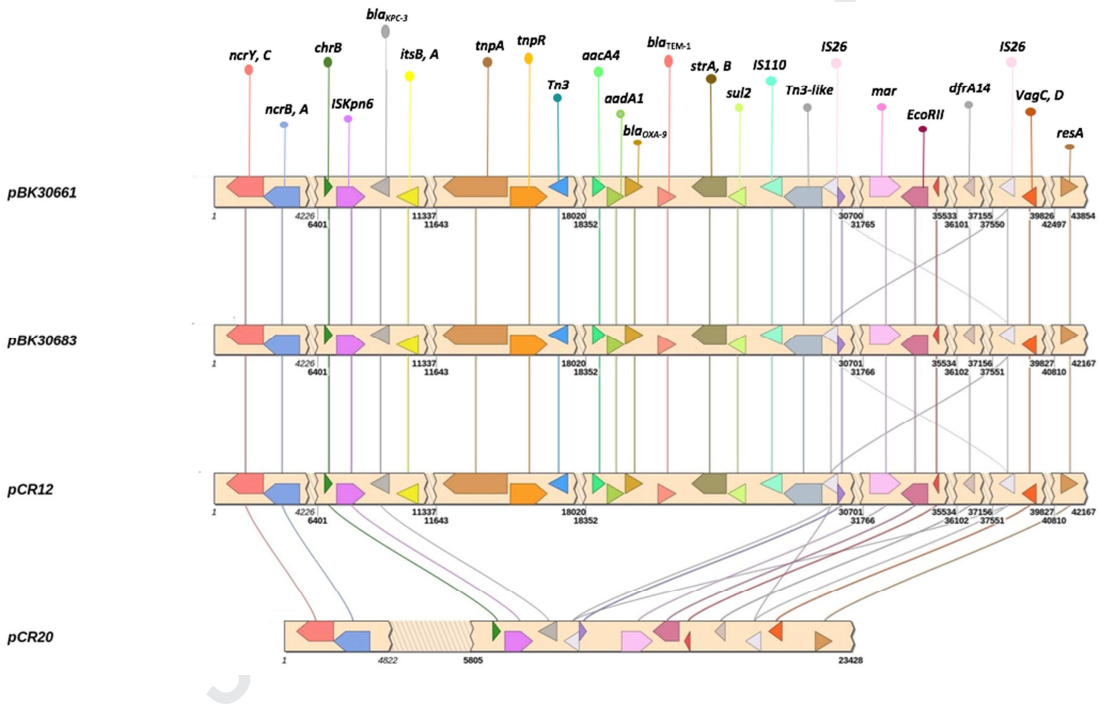

Figure 1. Synteny analysis of *bla*$_{KPC-3}$ gene context in IncFIA/FII plasmids from *K. pneumoniae* isolates BK30661 (GenBank accession no.KF954759), BK30683 (GenBank accession no.KF954760), CR12 (GenBank accession no.RBWG00000000) and CR20 (GenBank accession no.RBWH01000000). Lines indicate conserved genes.

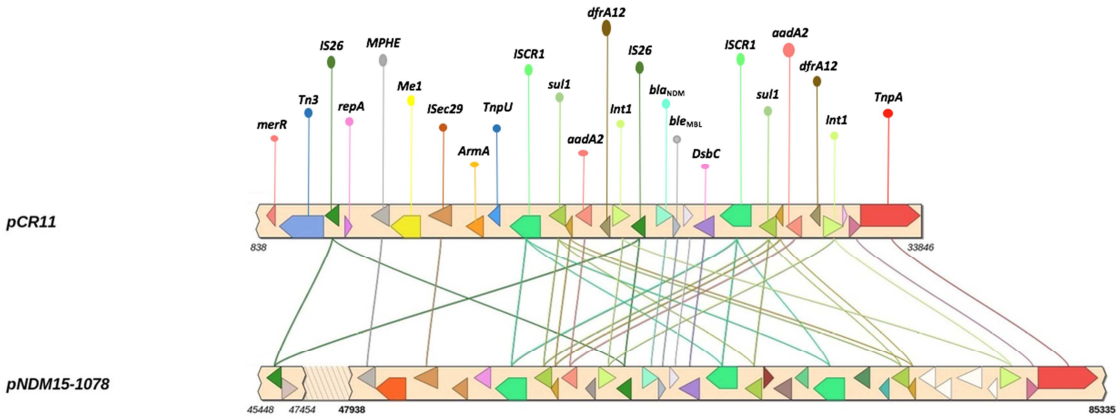

Figure 2. Major structural features of the region harbouring the *bla*$_{NDM-1}$ gene between the IncA/C_2 plasmid isolated in this study, pCR11, with similar backbone plasmid pNDM15-1078 (GenBank accession no.CP012902). "Flip" arrows indicate changes in gene direction.

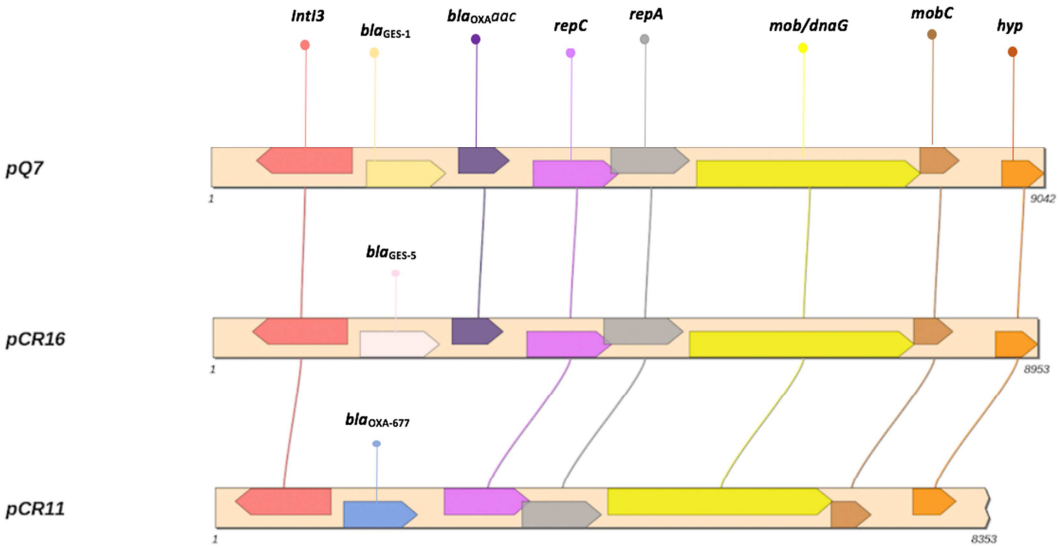

Figure 3. Alignment of IncQ2-typing plasmids to the reference plasmid pQ7 (GenBank accession no.FJ696404). Top row shows the reference plasmid and the rows below the reconstructed plasmids in *Citrobacter* CR16 (GenBank accession no. RBWI00000000) and *Enterobacter* CR11 (GenBank accession no. RBMO00000000).

Tables:

476 **Table 1.** Prevalence of Imipenem-Resistant Coliforms in the water of four river sites (UM, DM,
477 UW, DW) sampled in 2017. Samples from sites UW and DW were collected twice. Three
478 replicas were plated for each site.

Sampling sites	*Total Coliforms (CFU/mL)*	*% of total culturable bacteria in Chromocult resistant to Imipenem*	*No. of selected Imipenem-resistant isolates*	*No of Imipenem-resistant strains with different BOX profiles*
UM	$3.7\times10^3\pm1.0\ x10^2$	0.8 ± 0.1	3	2
DM	$2.5\times10^3\pm7.8\ x10$	1.0 ± 0.0	3	2
UW1	$6.8\times10^2\pm8.0\ x10$	0.4 ± 0.2	8	3
DW1	$2.7\times10^3\pm6.4\ x10^2$	2.3 ± 0.9	1	1
UW2	$2.9\times10^3\pm5.9\ x10^2$	0.2 ± 0.1	4	3
DW2	$1.3\times10^3\pm1.6\ x10^2$	0.4 ± 0.1	5	2

479

480 **Table 2.** Identification, antibiotic susceptibility profiles and genotypes of CRE isolated from Lis
481 river (sites UM, DM, UW and DW) carrying carbapenemase genes.

					Resistance Profile															
Isolate	**Closest taxa identified by BLAST using 16S rDNA gene**	**Sample of Origin**	**Replicon typing**	**Carbapenemase gene**	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	CTX	FEP	CN	CIP	TGC	SXT
CR3	*K. pneumoniae*	UW1	IncY	bla_{KPC}													
CR7	*Enterobacter*	UW1	-	bla_{NDM}													
CR8	*Enterobacter*	UW1	-	bla_{NDM}													
CR9	*K. pneumoniae*	DW1	IncY	bla_{KPC}													
CR11	*Enterobacter*	UM	-	bla_{NDM}													
CR12	*K. pneumoniae*	UM	IncY	bla_{KPC}													
CR13	*K. pneumoniae*	DM	IncY	bla_{KPC}													
CR14	*K. pneumoniae*	DM	IncY	bla_{KPC}													
CR16	*Citrobacter*	UW2	IncR	bla_{GES}													
CR17	*K. pneumoniae*	UW2	IncY	bla_{KPC}													
CR18	*K. pneumoniae*	UW2	IncY	bla_{KPC}													
CR20	*K. pneumoniae*	DW2	IncR	bla_{KPC}													

CR21	*K. pneumoniae*	DW2	IncY	bla_{KPC}

PRL, Piperacillin; TZP, Piperacillin-tazobactam; TIC, Ticarcillin; TTC, Ticarcillin-clavulanic acid; ETP, Ertapenem; MEM, Meropenem; IPM, Imipenem; ATM, Aztreonam; TGC, Tigecycline; SXT, Trimethoprim-sulfamethoxazole; CAZ, Ceftazidime; CTX, Cefotaxime; FEP, Cefepime; AK, Amikacin; CN, Gentamicin; CIP, Ciprofloxacin (dark grey, resistant; light grey, intermediate; white, susceptible).

484 **Table 3**. MICs of carbapenems and cephalosporins for *K. pneumoniae* isolates CR18 and CR21,

485 transconjugants *E. coli* J53::KPC and recipient strain *E. coli* J53.

	MIC, mg/L (susceptibility)				
	K. pneumoniae CR18	*E. coli* J53 (18t)	*K. pneumoniae* CR21	*E. coli* J53 (21t)	*E. coli* J53
Ceftazidime	>256 (R)	64 (R)	>256 (R)	64 (R)	0.5 (S)
Cefotaxime	>256 (R)	24 (R)	>256 (R)	16 (R)	0.094 (S)
Ertapenem	>32 (R)	6 (R)	>32 (R)	12 (R)	0.016 (S)
Imipenem	>32 (R)	4 (IR)	>32 (R)	6 (IR)	0.19 (S)
Meropenem	>32 (R)	4 (IR)	>32 (R)	4 (IR)	0.015 (S)

(R), Resistant; (IR), Intermediate resistance; (S), Susceptible

487

		Carbapenemase-encoding genetic structure				
Isolate	*MLST*	*Gene*	*Closest Plasmid*	*Replicon type*	*Size (bp)*	*Genbank*
E. roggenkampii CR11	-	bla_{NDM-1}	pNDM15-1078	IncA/C2	176,048	CP012902.1
K. pneumoniae CR12	ST231	bla_{KPC-3}	pBK30683	FIA/FII	139,941	KF954760
C. freundii CR16	ST429	bla_{GES-5}	pQ7	IncQ2	9,042	FJ696404
K. pneumoniae CR20	ST147	bla_{KPC-3}	pBK30683	FIA/FII	121,769	KF954760

488 **Table 4**. Sequence type of CRE isolates and genetic context of carbapenemase encoding genes.

489

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Supplementary data

Tables S1-S5 and Figures S1-S2 are available as Supplementary data.

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Highlights:

- Carbapenem-resistant Enterobacteriaceae were detected in a Portuguese river
- Isolates carried *bla*$_{\text{GES-5}}$, *bla*$_{\text{KPC-3}}$ or *bla*$_{\text{NDM-1}}$ genes
- Genes were located on conjugative or mobilizable plasmids.
- *bla*$_{\text{GES-5}}$ was located in a novel class 3 integron
- First detection of *bla*$_{\text{NDM}}$ in a portuguese environmental setting.

Author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: