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Multidrug resistance and diversity of resistance profiles in carbapenem-resistant Gram-negative bacilli throughout a wastewater treatment plant in Colombia

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ABSTRACT

Objectives: Carbapenem-resistant Gram-negative bacilli (CRGNB) have been reported in different wastewater treatment plants (WWTPs) throughout the world; however, few studies have described the antimicrobial resistance profile in different CRGNB throughout WWTPs, information that would identify points of selection of resistant bacteria. The objective of this work was to characterize the resistance profile of CRGNB harbouring *bla*KPC-2 from a Colombian WWTP.

Methods: Six samples were taken from four points of a WWTP. CRGNB were selected in chromID® CARBA and identified by 16S rRNA. Carbapenemases were determined by polymerase chain reaction (PCR), and susceptibility was assessed using VITEK2.

Results: One hundred and forty-two CRGNB harbouring *bla*KPC-2 were detected: 41% corresponded to *Aeromonas* spp. (n = 58) and 59% to *Enterobacteriaceae*. To establish the resistance profile, 50% of the isolates were selected proportionally by family and sampling point (26 *Aeromonadaceae* and 45 *Enterobacteriaceae*). All *Enterobacteriaceae* showed resistance to carbapenems and penicillins + inhibitors, high percentages of resistance to ceftriaxone (88.9%), and ciprofloxacin (44.4%), and low resistance to other antibiotics (>30%). In *Aeromonadaceae*, 76.9% were resistant to ceftriaxone, 58% to carbapenems, and 65.4% to ciprofloxacin. Twenty-one resistance profiles were observed, the most common of which were resistant to penicillins + inhibitor, cephalosporins (third to fourth generation), and carbapenems (19%). The percentage of multidrug resistance was 91% and was similar at all points of the WWTP.

Conclusions: The high frequency of multidrug resistance and great diversity of resistance profiles observed throughout the WWTP is of concern, and shows the role of WWTP as a reservoir and dissemination source of antimicrobial resistance to water sources.

1. Introduction

Bacterial resistance to antibiotics is a natural phenomenon that can be accelerated by the selection pressure exerted by substances such as antibiotics, biocides, and heavy metals [1]. In recent decades, this phenomenon has been increasing due to the excessive and inappropriate use of antibiotics in humans, animals, and agriculture, which constitutes a threat to public health [2].

Beta-lactams are one of the most used and useful families of antibiotics in the treatment of human infections. Among them, carbapenems constitute the last resort treatment for clinically important Gram-negative bacillus infections [3], such as those belonging to the *Enterobacteriaceae* family, for instance, *Klebsiella* spp., *Escherichia coli*, *Serratia* spp. and *Proteus* spp., and to non-fermenters such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. In recent years, a worrying increase in carbapenem resistance has been reported in these microorganisms, usually due to the presence of genes in mobile genetic elements that encode enzymes that degrade the antibiotic, called carbapenemases, mainly *bla*KPC, *bla*NDM, *bla*VIM, *bla*OXA-48 types. Additionally, these elements often carry resistance mechanisms to other antibiotic families such as quinolones, aminoglycosides, and sulfonamides, which gives these Gram-negative bacilli (GNB) a multidrug resistance (MDR) profile.

Due to the ability of these microorganisms to cause serious infections, and the difficulty of their therapeutic management because of MDR, the World Health Organization (WHO) classified

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the carbapenem-resistant Gram-negative bacilli (CRGNB) as of critical priority [2,4].

The description of the CRGNB and other families of antibiotics is most often done in the hospital setting, where they represent a serious problem either by causing infection or colonizing [5,6]. However, the alarming resistance situation points to the possibility of other sources or reservoirs of antibiotic-resistant bacteria (ARB) such as the environment and the community [5,6].

In this sense, it has been pointed out how wastewater treatment plants (WWTPs) can play an important role in the appearance and dissemination of ARB [7], because in WWTPs bacteria and antibiotics from different origins: human, veterinary, and industrial, converge giving rise to selection processes [7]. Most antibiotics used in human and veterinary clinical practice are not fully metabolized, so they are excreted in urine and faeces, which subsequently go to municipal wastewater and WWTP [8]. In wastewater and WWTPs antibiotic concentrations, even below the minimum inhibitory concentration (MIC), appear to be sufficient to favour the selection or transfer of resistance genes between bacteria [7,9]. Additionally, ARB that can colonize the human or animal gastrointestinal tract can reach the WWTPs by being eliminated through excreta [10].

In general, the description of CRGNB and their antimicrobial resistance profiles from environmental samples is more limited [11], and few studies have described the resistance profile to other antibiotics in these isolates and their behaviour throughout the treatment, information that would show the simultaneous presence of resistance mechanisms to different families of antibiotics and the hotspots of exchange or selection of resistant bacteria in the WWTP. Taking into account that Colombia has described a high frequency of CRGNB isolates, exceeding the percentages reported in other Latin American countries such as Argentina, Chile and Brazil [12], and that consumption of antibiotics is high, it is necessary to describe the resistance profile of GNB resistant to these antibiotics from a WWTP in Colombia, which will provide relevant information on the degree of spread of resistance outside clinical environments and its impact.

2. Materials and methods

2.1. Wastewater treatment plant and sampling

The study was conducted in a WWTP in Antioquia, Colombia, which collects domestic, industrial, and hospital wastewater from four municipalities in the region (614 000 inhabitants). The WWTP uses an activated sludge process with 4-h hydraulic retention time and receives an average water flow of 1.8 m^3/s, but has a maximum flow rate of 3.6 m^3/s. The resulting final effluent is discharged to the Medellin River.

In total, six samplings were made between January and July 2017. On each visit, 500 mL of water was collected in sterile Schott's glass, at four points in the plant: raw influent, aeration tanks, recycled activated sludge, and final effluent, for a total of four samples per samplings. To avoid effects associated with organic loading fluctuations, samples were collected every month on the same day between 14:00 and 16:00 hours. After collection, the samples were transported to the laboratory at 4 °C. The microbiological analysis was performed within 4 h after the samples were collected.

2.2. Phenotypic identification of carbapenem-resistant Gram-negative bacilli

For the phenotypic identification of CRGNB, the ChromID® CARBA chromogenic medium (BioMérieux, Marcy l'Etoile, France) was used [13,14]. In this medium, 100 μL of the sample was seeded by confluence, then incubated at 35 °C ± 2 °C for 24 h.

In this study, due to the high bacterial growth and the interest in selecting clinical and environmental carbapenem-resistant bacteria, five colonies from the three morphotypes that grew on ChromID CARBA chromogenic medium were randomly selected. Every colony was subcultured on MacConkey agar (Merk Millipore, Burlington, Massachusetts, United States) and nutrient agar (Merck Millipore) at 37 °C for 24 h. The oxidase test was also done [14,15].

2.3. Molecular identification using the RNAr 16s gene

DNA was extracted from suspected CRGNB, which were selected by ChromID CARBA and MacConkey agar using the DNA Wizard Genomic Purification Kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

Bacterial identification was carried out using the detection of the 16S ribosomal RNA gene by simple polymerase chain reaction (PCR), using universal primers 27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT, according to the protocol described by Dunbar et al. [16]. The amplification products were sequenced, and the sequences were analysed in the Geneious software version R8 (https://www.geneious.com) [17]. They were subsequently compared with sequences available in GenBank, using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information of the United States (NCBI) (www.ncbi.nlm.nih.gov/BLAST).

2.4. Carbapenemases detection and sequencing

The most clinically important carbapenemases: *bla*KPC, *bla*VIM, *bla*IMP, *bla*NDM and *bla*OXA-48, were evaluated by multiplex PCR, according to the protocol described by Poirel et al. and Ellington et al. [18,19].

Subsequently, the detected carbapenemase genes were sequenced to determine their respective variants. The sequences obtained were analysed in the forward and reverse directions and compared with sequences available in GenBank, using the BLAST program of NCBI (www.ncbi.nlm.nih.gov/BLAST) and, to establish the variant of each gene detected, were compared with the Lahey database (https://www.lahey.org/studies/).

2.5. Antimicrobial susceptibility

GNB positive for any of the carbapenemases evaluated were tested for antimicrobial susceptibility using the automated system Vitek2 (BioMérieux, France) [20,21]. The antibiotics evaluated were ampicillin/sulbactam (SAM), piperacillin/tazobactam (PTZ), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), doripenem (DOR), ertapenem (ETP), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (GN), ciprofloxacin (CIP), tigecycline (TGC), and colistin (COL).

The susceptibility test results were interpreted according to the M100 Performance Standards for Antimicrobial Susceptibility Testing, 27th Edition [22] and the M45-A2 Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria, 2nd Edition [23] both of Clinical and Laboratory Standards Institute (CLSI).

The isolates of intermediate result were considered resistant. TGC susceptibility was only interpreted for bacteria of the *Enterobacteriaceae* family according to EUCAST 2018 [24].

The intrinsic resistance and antibiotics without card data or interpretation by CLSI were excluded from the analysis of antibiotic resistance. The antibiotic ETP was excluded from the study because the ability of Vitek's AST N272 card to detect

resistance in the majority of *Enterobacteriaceae* in the study is unknown (BioMérieux, France).

MDR was defined as the resistance of an isolate to three or more categories of antibiotics. The categories were established as proposed by Magiorakos et al. [25] which for *Enterobacteriaceae* indicate as categories: aminoglycosides, carbapenems, third and fourth generation cephalosporins, cephamycins, fluoroquinolones, glycylcyclines, penicillins + inhibitor and polymyxins. These same categories were applied to the family *Aeromonadaceae*.

The analysis by sampling point was performed by antibiotics for *Enterobacteriaceae* and *Aeromonadaceae*, taking into account the antibiotics evaluated for each family.

The antimicrobial resistance profile of the CRGNB was performed on isolates that had complete data for the antibiotics evaluated.

2.6. Data analysis

The information was analysed using SPSS 24 software (IBM SPSS Statistics, IBM Corporation, Somer, NY) and Microsoft Office Excel (Microsoft Corporation (Redmond, Washington, United States)).

3. Results

3.1. Detection of carbapenemase genes and identification of carbapenem-resistant Gram-negative bacilli

From the ChromID CARBA chromogenic medium, 360 isolates suspected of presenting resistance to carbapenems were obtained, of which in 142 (39.4%) the presence of the *bla*KPC gene was confirmed by PCR. The other genes coding for *bla*VIM, *bla*NDM, and *bla*OXA-48 carbapenemases were not detected. Likewise, the sequencing of the *bla*KPC gene showed that every one of them was harbouring variant 2. On average in the plant, 21–26 CRGNB harbouring *bla*KPC were detected per month.

According to the identification of the 16S gene, the genus harbouring *bla*KPC-2 found were *Aeromonas* spp. (n = 58; 41%), *Enterobacter* spp. (n = 38; 27%), *Klebsiella* spp. (n = 11; 8%), *Citrobacter* spp. (n = 8; 6%), *Pantoea* spp. (n = 7; 5%), *Kluyvera* spp. (n = 4; 3%), *Raoultella* spp. (n = 12; 9%), *Escherichia* spp. (n = 3; 2%) and *Pseudomonas* spp. (n = 1; 1%) (Fig. 1).

Thirty-one percent of the isolates came from the raw influent (n = 44), 20% from the aeration tanks (n = 28), 21% from the recycled sludge (n = 30) and 28% from the final effluent (n = 40), with a variable distribution according to the genus of the microorganism.

3.2. Antimicrobial resistance

For the susceptibility analyses, 50% of the isolates harbouring *bla*KPC-2 were selected (n = 71), taking into account a proportional allocation by genus and sampling point.

The 71 bacteria harbouring *bla*KPC-2 selected corresponded to 26 *Aeromonas* spp. (36.6%), 17 *Enterobacter* spp. (24%), 7 *Klebsiella* spp. (9.8%), 5 *Citrobacter* spp. (7%), 5 *Pantoea* spp. (7%), 4 *Kluyvera* spp. (5.6%), 4 *Raoultella* spp. (5.6%) and 3 *Escherichia* spp. (4.2%). Twenty of these isolates were distributed in the raw influent (28%), 12 in the aeration tanks (17%), 14 in the recycled activated sludge (19.7%), and 25 in final effluent (35%).

3.2.1. Susceptibility in Enterobacteriaceae

A total of 45 isolates harbouring *bla*KPC-2 belonging to the *Enterobacteriaceae* family were selected (45/71). Per sampling point in the plant, it was observed that 29% (n = 13) were found in the raw influent, 18% (n = 8) in the aeration tanks, 11% (n = 5) in the recycled sludge, and 42% (n = 19) in the final effluent (Fig. 1).

In general, the isolates of the *Enterobacteriaceae* family analysed were resistant to SAM and PTZ, mostly with a MIC $\geq$32 mg/L and $\geq$128 mg/L, respectively. Cephalosporin resistance was highest at CRO 89% (n = 40) followed by CAZ in 42% (n = 19), and to a lesser extent for FEP 17.7% (n = 8). Likewise, it was observed that 40% of the *Enterobacteriaceae* evaluated were resistant to CAZ and CRO simultaneously (Fig. 2).

Regarding carbapenems, it was observed that the resistance frequency obtained was 100% for IMP and 98% for MEM and DOR. Likewise, 89% (n = 40) of the isolates evaluated were resistant to all three carbapenems simultaneously.

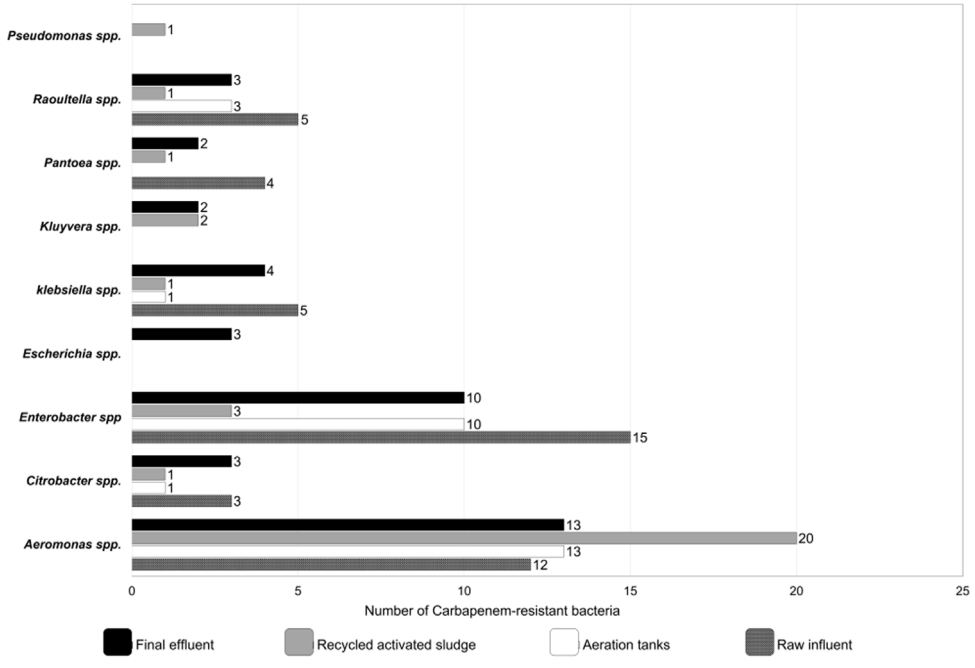

Fig. 1. Number of carbapenem-resistant bacteria harbouring *bla*KPC in different sampling sites of WWTP.

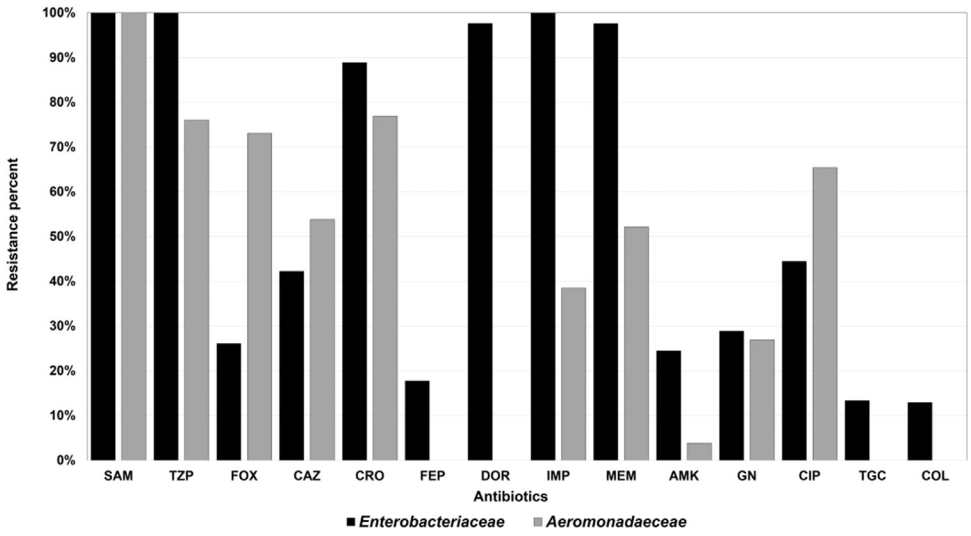

Fig. 2. Percentage of resistance in carbapenem-resistant *Enterobacteriaceae* and *Aeromonadaceae* isolated from wastewater treatment plant. FEP, DOR, TGC and COL were not evaluated for *Aeromonas* spp. AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CRO, ceftriaxone; DOR, doripenem; FEP, cefepime; FOX, cefoxitin; GN, gentamicin; IMP, imipenem; MEM, meropenem; SAM, sulbactam/ampicillin; TGC, tigecycline; TZP, tazobactam/piperacillin.

Similar resistance percentages, 24% and 28%, respectively, were observed for AMK and GN. In the case of CIP, 44% of the isolates were resistant to this antibiotic. For TGC and COL, a resistance percentage of 13% was observed (Fig. 2).

3.2.1.1. Enterobacteriaceae susceptibility analysis by genus. Of note, in the susceptibility results found in the *Enterobacteriaceae* by genus, 40% of *Klebsiella* spp. and 100% *Escherichia* spp. tested positive for extended spectrum beta-lactamase (ESBL). On the other hand, *Enterobacter* spp. and *Pantoea* spp. were resistant to all antibiotics evaluated (Fig. 3). However, *Raoultella* spp. was only resistant to beta-lactam antibiotics.

3.2.1.2. Antimicrobial resistance in Enterobacteriaceae per sampling plant point in the WWTP. When the antimicrobial resistance of the *Enterobacteriaceae* per sampling plant point was analysed, it was found that all isolates evaluated were resistant to SAM, PTZ, and IMP. Similarly, it was found that all strains were resistant to MEM and DOR, except in the final effluent isolates, where one reduction in the number of isolates resistant to MEM and DOR was evidenced.

Interestingly, in sludge or tanks, a high number of isolates resistant to FOX, CAZ, CRO, and FEP were observed in comparison with the number of resistant strains to these antibiotics from other points of the plant. Likewise, it was found that in most cases, the

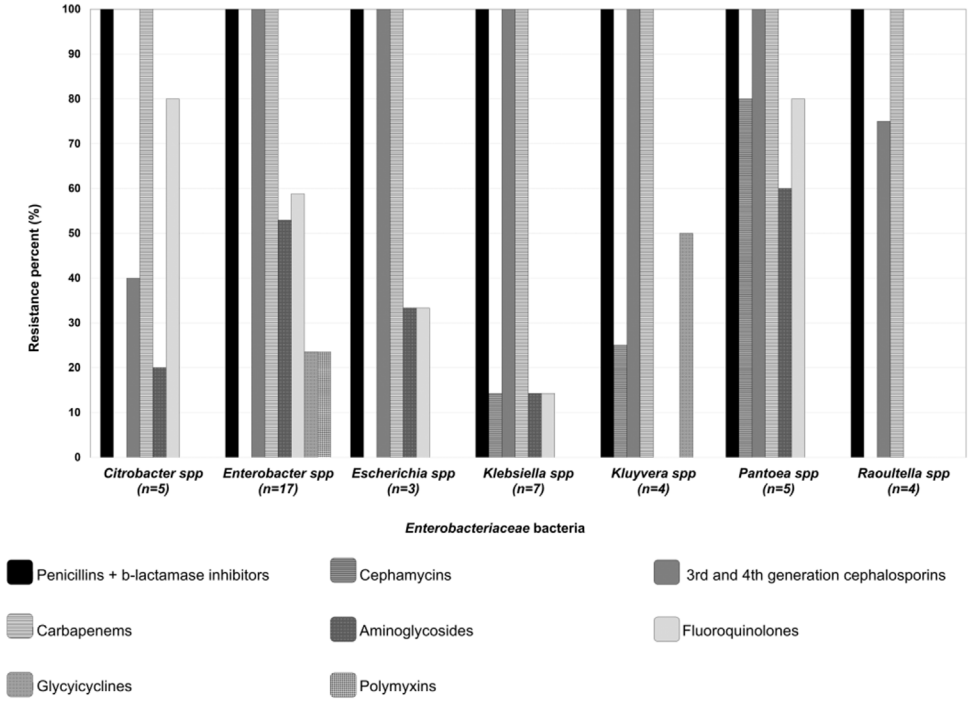

Fig. 3. Percentage of resistance in carbapenem-resistant *Enterobacteriaceae* family by category of antibiotics. Colistin was only evaluated for *Enterobacter*, *Escherichia*, *Klebsiella* and *Raoultella*. Intrinsic resistance were excluded for analysis.

number of isolates resistant to these antibiotics decreased in the final effluent.

Concerning the distribution of CIP resistant isolates per sampling plant point, a high number of isolates were found in the raw influent in comparison with other points of the plant. In addition, in the isolates from the final effluent, a slight increase in resistance to CIP was observed.

On the other hand, the number of isolates resistant to TGC, AMK, GN, FOX, and FEP was variable throughout the WWTP but were not observed in the recycled sludge (n = 5). Likewise, the presence of COL-resistant isolates was observed in the raw influent strains, aeration tanks, and recycled sludge (Fig. 4).

3.2.2. Susceptibility in Aeromonadaceae

Twenty-six isolates of the *Aeromonadaceae* family harbouring *bla*KPC were evaluated (Fig. 2), in which 100% (n = 26) showed resistance to SAM, while 76% (n = 19) of the isolates were resistant to PTZ. With respect to third-generation cephalosporins and FOX, it was observed that both FOX and CRO showed high resistance percentages: 73.1% (n = 19) and 76.9% (n = 20), respectively, compared to CAZ, where resistance was observed in only 53.8% (n = 14) of the isolates. It was noted that all in the isolates with resistance to CRO the MIC was $\geq$64 mg/L. In relation to carbapenems, resistance to MEM of 52.2% (n = 12) and to IMP 47.8% (n = 10) was observed, while in 34.6% (n = 9) of the isolates there was no resistance to any carbapenems, and 27% (n = 7) of the isolates were resistant to both carbapenems simultaneously.

As for the other antibiotics evaluated, it was observed that 26.9% of *Aeromonadaceae* were resistant to GN, 3.8% (n = 1) to AMK, and 65.4% to CIP.

3.2.2.1. Antimicrobial resistance in Aeromonadaceae per sampling plant point in the WWTP. When observing the frequency of resistant *Aeromonadaceae* for each sampling point, it was observed that the distributions of isolates were seven (27%) in the raw influent, four (15%) in the aeration tanks, nine (35%) in the recycled sludge, and six (23%) in the final effluent (Fig. 1).

The percentage of SAM resistance in *Aeromonadaceae* was the same in all sampling points. With respect to other antibiotics, in the aeration tanks or the recycled sludge, a high number of strains resistant to PTZ, FOX, CAZ, CRO, IMP, MEM, and GN were found. However, the number of isolates resistant to these antibiotics decreased in the final effluent.

Likewise, a higher frequency of CIP-resistant strains was found in the raw influent; which decreased along the WWTP, except in the final effluent, where a slight increase in resistance to this antibiotic was found. In the same way, the only isolate resistant to AMK was detected in the activated sludge (Fig. 5).

3.3. Analysis of antimicrobial resistance profiles by antibiotic category

Of the 71 strains used for susceptibility analyses, 64 isolates had all data of susceptibility for each antibiotic evaluated. Therefore, the analysis of antimicrobial resistance profiles and MDR was carried out on 64 isolates.

In the analysis of antimicrobial resistance profiles by antibiotic category, 21 different profiles were detected, 24% (n = 5) in *Aeromonadaceae*, 52% (n = 11) in *Enterobacteriaceae*, and the remaining 24% (n = 5) were shared between the two bacterial families. Ninety-one percent of the isolates in both groups of bacteria presented MDR (38 of *Enterobacteriaceae* and 20 of *Aeromonadaceae*), which were distributed between 18 different profiles. The most commonly observed profile in the two bacterial families was resistance to penicillins + inhibitor, third and fourth generation cephalosporins, and carbapenems (n = 12; 19%), which was isolated from all sampling months. The second most observed profile was resistance to penicillin/inhibitor + cephamycins + third and fourth generation cephalosporins + carbapenems + aminoglycosides + fluoroquinolones with 11% (n = 7), which was detected in the isolates of months 1, 4, 5, and 6. The two profiles were present in the four points of the plant. The other profiles did not present a significant frequency for the study, and some of them were only present in one sampling. In general, this work did not find a tendency in MDR behaviour related to sampling month (Supplementary Fig. S1).

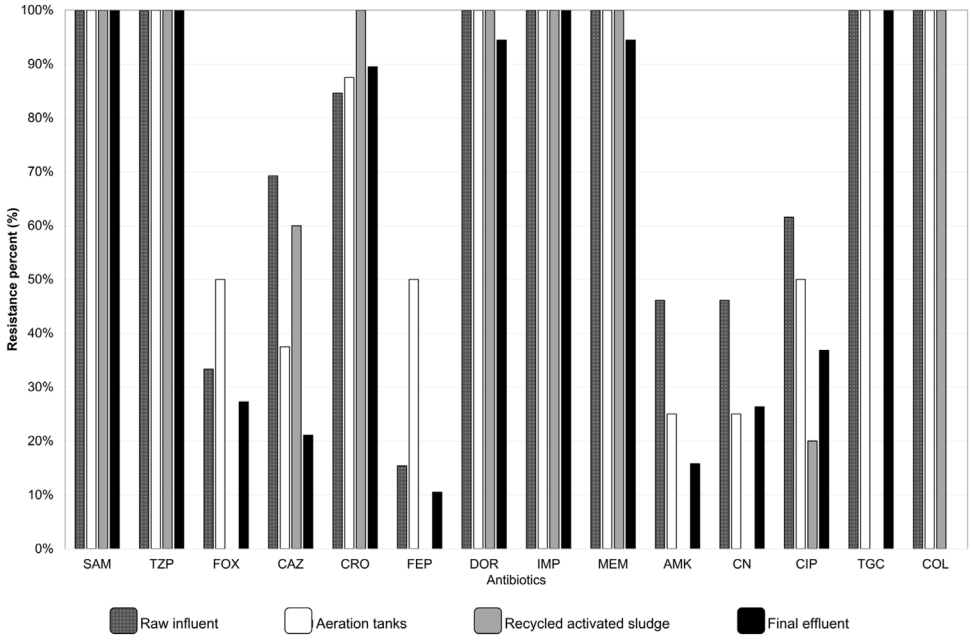

Fig. 4. Percentage of resistance in carbapenem-resistant *Enterobacteriaceae* family isolated at each sampling point (raw influent, aeration tanks, recycled activated sludge, and final effluent). AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; CRO, ceftriaxone; DOR, doripenem; FEP, cefepime; FOX, cefoxitin; GN, gentamicin; IMP, imipenem; MEM, meropenem; SAM, sulbactam/ampicillin; TGC, tigecycline; TZP, tazobactam/piperacillin.

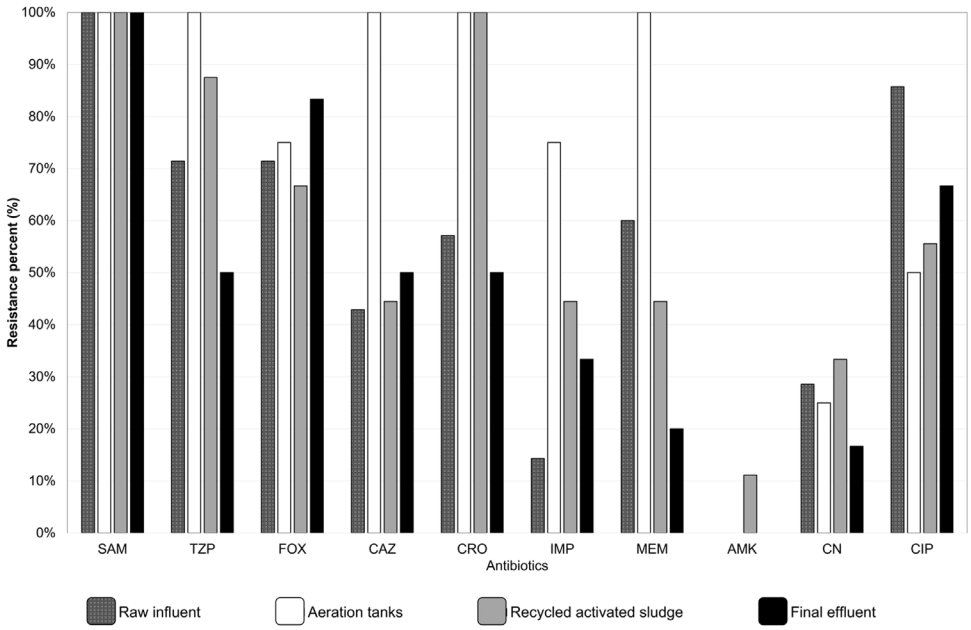

Fig. 5. Percentage of resistance in carbapenem-resistant *Aeromonadaceae* isolated at each sampling point (raw influent, aeration tanks, recycled activated sludge, and final effluent). AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; DOR, doripenem; FOX, cefoxitin; GN, gentamicin; IMP, imipenem; MEM, meropenem; SAM, sulbactam/ampicillin; TZP, tazobactam/piperacillin.

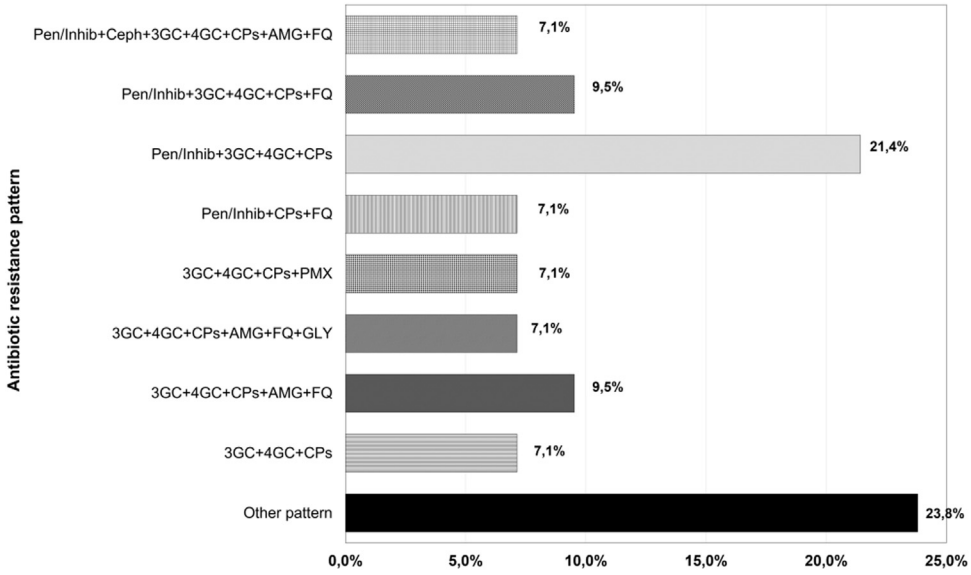

Fig. 6. Antimicrobial resistance profile in carbapenem-resistant *Enterobacteriaceae* family to different categories of antibiotics. 3GC, third generation cephalosporins; 4GC, fourth generation cephalosporins; AMG, aminoglycosides; Ceph, cephamycins; CPs, carbapenems; FQ, fluoroquinolones; GLY, glycylcyclines; Pen/inhib, penicillins/B-lactamase inhibitors; PMX, polymyxins. Other profiles: these represent the antimicrobial resistance profile found in less to 5% of isolates.

The antimicrobial resistance profiles in *Enterobacteriaceae* and *Aeromonadaceae* to different categories of antibiotics are shown in Figs. 6 and 7.

4. Discussion

The worldwide increase in antibiotic resistance has generated a significant impact on public health in the social and economic sphere, and its environmental impact has been reported in recent years [7]. The results of this study demonstrate an important frequency of multidrug-resistant CRGNB harbouring *bla*KPC-2 in a WWTP in Colombia. This resistance mechanism has been documented by our group in several hospitals in the city [3,12], and recently in local WWTP (unpublished data in preparation). These findings reflect the impact of resistance on our region and the risk of its dissemination to the environment and the community.

In recent years, CRGNB harbouring *bla*KPC have been increasingly described in municipal and hospital WWTPs, rivers, and lakes worldwide [26]. Most of these studies describe or isolate bacteria of clinical importance from the Enterobacteriaceae family such as *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *E. coli* [26,27], although they also describe the presence of other environmental bacterial genus such as *Raoultella* spp., *Kluyvera* spp. and

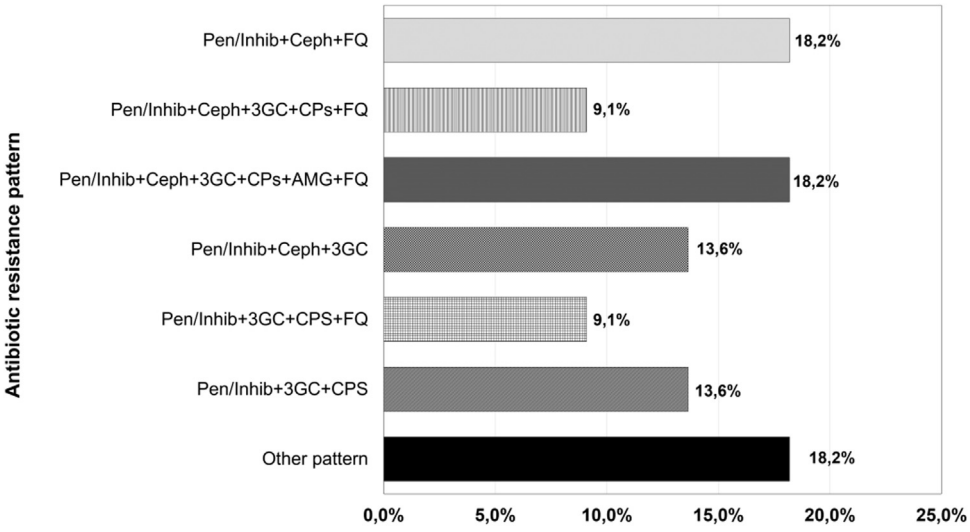

Fig. 7. Antimicrobial resistance profile in carbapenem-resistant *Aeromonadaceae* family to different categories of antibiotics. The fourth generation cephalosporins were not tested in this family. Other profiles: these represent the antimicrobial resistance profile found in less to 5% of isolates. 3GC, third generation cephalosporins; 4GC, fourth generation cephalosporins*; AMG, aminoglycosides; Ceph, cephamycins; CPs, carbapenems; FQ, fluoroquinolones; GLY, glycylcyclines; Pen/inhib, penicillins/B-lactamase inhibitors; PMX, polymyxins.

Aeromonas spp. [26,28]. These microorganisms coincide with those detected in this work, where the most frequent CRGNB harbouring *bla*KPC-2 were *Aeromonas* spp. and *Enterobacter* spp.

Likewise, the results of the study related to resistance profiles highlight the high percentage of multidrug-resistant isolates (91%), which were found distributed in all the sampling points of the plant. Multidrug-resistant microorganisms have been described more frequently in hospital settings [3,12,29]; however, this finding gives rise to greater concern since a dissemination of multidrug-resistant bacteria is being carried out in the environment, with all the implications that this entails.

In addition to the high percentage of MDR among the bacterial genus found, great diversity in resistance profiles was observed, which may be a consequence of the pressure of antibiotics on these environments and of the success in the transmission of resistance mechanisms between these microorganisms [29]. These findings confirm, as has been documented in other studies, the importance of WWTPs as reservoirs of resistant bacteria and the need to search for alternatives to try to contain the spread such as the use of ultraviolet light, hydrogen peroxide, persulfate or methods of oxidation [30]. The high MDR and the great diversity of profiles imply that there is a constant pressure of selection and dissemination of resistance mechanisms that, as a consequence, result in different bacterial populations with the capacity to harbour and maintain these mechanisms within the WWTP [7,29].

Additionally, the dissemination of ARB and antibiotic resistance gene throughout the environment can affect the health of the community: because these emerging pollutants have the ability to persist for a long time in aquatic environments, they can be released into the effluents and reach rivers, whose waters are used for agriculture and/or livestock [29,31].

The most frequently found bacterial genus harbouring *bla*KPC in the study was *Aeromonas*. Although these bacteria are mainly described in the environment, they are also clinically important because they can cause infections that are difficult to treat [32]. Interestingly, it was observed that *Aeromonas* spp. isolates, despite harbouring the gene for *bla*KPC, showed low resistance to the evaluated carbapenems (58%), and some isolates were even sensitive by both Vitek and Kirby-Bauer. This behaviour can be because the *bla*KPC carbapenemase of *Aeromonas* spp. may not be expressed due to the biological cost of having this active resistance mechanism and/or probably not requiring it to be expressed.

Another explanation may be related to technical difficulties, as described for the detection of carbapenem resistance by *CphA* carbapenemase, a substrate-specific metallobetalactamase in that its detection may be affected by the use of the standard inoculum of bacteria, requiring higher inoculums [33]. This result highlights the importance of the use of molecular methods for screening carbapenem-resistant bacteria in the environment.

The detected isolates of *Aeromonas* spp. generally showed greater than 50% resistance to beta-lactams except for IMP (38.5%); *Aeromonas* spp. carries an inducible chromosomal ampC, allowing it to hydrolyse penicillins and first, second and third generation cephalosporins, including cephamycins [34]. This is consistent with total SAM resistance and MICs >128 mg/L a PTZ, in addition to high percentages of the other non-carbapenemic beta-lactams observed in the study, and which have also been documented in similar studies [26].

For the isolates of the genus *Raoultella*, resistance only to beta-lactam antibiotics was observed, unlike a study in China where resistance to non-beta-lactam antibiotics was found [15]. This may indicate that bacteria of this genus should be considered as a potential reservoir for carbapenem resistance and could be linked to the clinic [15,26].

All isolates belonging to the *Enterobacteriaceae* family obtained total resistance to the carbapenemic and penicillin/inhibitor categories, but a high susceptibility to FOX (74%), CAZ (58%) and FEP (82%) antibiotics was observed, possibly due to the fact that the *bla*KPC-2 variant may have a weak hydrolysis on FOX and CAZ [35,36]. This is contrary to what was reported in a study in Brazil, where percentages of susceptibility from 35.4% to FEP, 19.3% to CAZ and a low susceptibility to MEM and IMP carbapenems (12.9% and 4.8%, respectively) were found [26].

Generally, in CRGNB harbouring *bla*KPC, resistance to other antibiotics such as quinolones and aminoglycosides can be observed [36,37]. In the isolates detected in this study, it is observed that after beta-lactam resistance, the resistance to fluoroquinolones was the next highest, both in *Enterobacteriaceae* (44.4%) and *Aeromonadaceae* (65.4%), in contrast to the other non-beta-lactam antibiotics such as aminoglycosides (33.3% in

Enterobacteriaceae and 26.9% in *Aeromonadaceae*) that did not have such a high resistance. The resistance in environmental isolates to fluoroquinolones and aminoglycosides has been consistent with what has been reported in similar studies where resistance to fluoroquinolones >50% and aminoglycosides >25% has been found [26,38]. This could indicate that the aminoglycosides having a low percentage of resistance could have an effective action against these positive *bla*KPC isolates [26].

According to several authors, this relationship of resistance to beta-lactam and quinolones evidenced in our study could indicate an association between the gene encoding resistance to beta-lactams, such as *bla*KPC, *bla*CTX-M and *bla*OXA, and the quinolone resistance genes, such as *gyr*, *qnr*, *aac (6′)-Ib-cr* in the same mobile genetic element of environmental isolates [7,28,39].

The percentage of antibiotic resistance found at each point of the treatment plant was very variable at all points of the plant, as described in other studies of *Enterobacteriaceae* and *Aeromonadaceae* in WWTPs [26,40]. With *Enterobacteriaceae*, a similar percentage of resistance to penicillin and carbapenems in all points of the plant could indicate the ability of these microorganisms to maintain and express carbapenem resistance. This is in contrast to *Aeromonadaceae*, where the resistance to these antibiotics was variable and can be explained by the low rate of expression of carbapenem resistance found in this study and described above.

It should be noted that a high expression of resistance to cephalosporins and carbapenems was found in *Aeromonadaceae* and *Enterobacteriaceae* from aeration tanks and/or recycled activated sludge. These results suggest that at this point in the plant, these bacteria could have a high selection pressure that favours a high expression of resistance to carbapenems. Another explanation could be the possibility of these isolates harbouring other beta-lactam resistance mechanisms that enhance expression resistance to these antibiotics at this point of the plant.

In the case of CIP, the results were similar to *Aeromonadaceae* and *Enterobacteriaceae*; there were a higher number of CIP-resistant isolates in the raw influent and a slight increase in resistance to this antibiotic at final effluent with respect to the secondary treatment. These results indicate how an important quantity of the CIP-resistant isolates arrive at the WWTP, probably due to the extensive use of these antibiotics in human and veterinary infections [41]. Concerning to the slight increase in resistance to this antibiotic in the raw influent, other authors have pointed out the decrease in resistance to this antibiotic in treatment, or its disappearance and its reappearance in samples of treated wastewater effluents. These results could be explained by the horizontal transfer of these genes between microbial communities [41].

Additionally, in *Enterobacteriaceae*, the resistance to TGC, AMK, and GN was not observed in the recycled activated sludge. Contrary to what happened to *Aeromonadaceae*, in which the resistance to GN was observed in greater proportion in the recycled sludge and to AMK in the aeration tanks. These results could indicate that resistance mechanisms to TGC, AMK, and GN are present in a variable way between bacterial groups throughout the plant. This could also be explained by the reorganization of microbial communities through the WWTP and/or horizontal gene transfer between bacterial groups [41].

In this study, the presence of *Enterobacter* spp., suspected of being COL-resistant, in the raw influent strains, aeration tanks, and recycled sludge was detected. The absence of COL-resistant isolates in the final effluent could suggest that the WWTP can reduce *Enterobacter* spp. with this type of mechanism of resistance, or that in secondary treatment the horizontal gene transference between other microbial communities not evaluated in the study could occur. Despite these findings, these results should be analysed carefully in future studies due to the low number of isolates detected (n = 4) and the need to confirm COL resistance by other methods.

5. Conclusion

The results obtained show how high percentages of multidrug-resistant CRGNB are present in all points of the plant, suggesting that non-beta-lactam antibiotic resistance mechanisms could share the same mobile genetic element in which the *bla*KPC gene is found. Similarly, the presence of phenotypically sensitive Gram-negative environmental bacilli such as *Pantoea* spp., *Raoultella* spp., *Kluyvera* spp. and *Aeromonas* spp. harbouring *bla*KPC shows how these microorganisms can harbour resistance genes without expressing them and act as reservoirs of resistance mechanisms to multiple antibiotics. These results demonstrate the importance of the health monitoring of potentially pathogenic and environmental microorganisms in water sources and highlight the importance of the use of phenotypic and molecular methods for screening carbapenem-resistant bacteria in the environment. Finally, the results obtained again indicate that WWTPs are a source of dissemination and a reservoir of multidrug-resistant bacteria, making it necessary to look for strategies aimed at mitigating the dissemination of antimicrobial resistance in these scenarios.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.02.033.

References

[1] Marti E, Balcázar JL. Antibiotic resistance in the aquatic environment. Compr Anal Chem 2013;62:671–84, doi:http://dx.doi.org/10.1016/B978-0-444-62657-8.00019-7.
[2] Lee HS, Loh YX, Lee JJ, Liu CS, Chu C. Antimicrobial consumption and resistance in five Gram-negative bacterial species in a hospital from 2003 to 2011. J Microbiol Immunol Infect 2015;48:647–54, doi:http://dx.doi.org/10.1016/j.jmii.2014.04.009.
[3] Vanegas JM, Parra OL, Jiménez JN. Molecular epidemiology of carbapenem resistant gram-negative bacilli from infected pediatric population in tertiary-care hospitals in Medellín, Colombia: an increasing problem. BMC Infect Dis 2016;16:463, doi:http://dx.doi.org/10.1186/s12879-016-1805-7.
[4] WHO. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. WHO Media Centre; 2017.
[5] Abbott IJ, Jenney AWJ, Spelman DW, Pilcher DV, Sidjabat HE, Richardson LJ, et al. Active surveillance for multidrug-resistant Gram-negative bacteria in the intensive care unit. Pathology 2015;47:575–9, doi:http://dx.doi.org/10.1097/PAT.0000000000000302.
[6] Wang H-P-P, Zhang H-J-J, Liu J, Dong Q, Duan S, Ge J-Q-Q, et al. Antimicrobial resistance of 3 types of Gram-negative bacteria isolated from hospital surfaces and the hands of health care workers. Am J Infect Control 2017;45:E143–7, doi:http://dx.doi.org/10.1016/j.ajic.2017.06.002.
[7] Marti E, Huerta B, Rodríguez-Mozaz S, Barceló D, Jofre J, Balcázar JL. Characterization of ciprofloxacin-resistant isolates from a wastewater treatment plant and its receiving river. Water Res 2014;61:67–76, doi:http://dx.doi.org/10.1016/j.watres.2014.05.006.

[8] Zheng S, Qiu X, Chen B, Yu X, Liu Z, Zhong G, et al. Antibiotics pollution in Jiulong River estuary: source, distribution and bacterial resistance. Chemosphere 2011;84:1677–85, doi:http://dx.doi.org/10.1016/j.chemosphere.2011.04.076.
[9] Novo A, André S, Viana P, Nunes OC, Manaia CM. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res 2013;47:1875–87, doi:http://dx.doi.org/10.1016/j.watres.2013.01.010.
[10] Diallo AA, Brugère H, Kérourédan M, Dupouy V, Toutain P-L-L, Bousquet-Mélou A, et al. Persistence and prevalence of pathogenic and extended-spectrum beta-lactamase-producing *Escherichia coli* in municipal wastewater treatment plant receiving slaughterhouse wastewater. Water Res 2013;47:4719–29, doi:http://dx.doi.org/10.1016/j.watres.2013.04.047.
[11] Hrenovic J, Ivankovic T, Ivekovic D, Repec S, Stipanicev D, Ganjto M. The fate of carbapenem-resistant bacteria in a wastewater treatment plant. Water Res 2017;126:232–9, doi:http://dx.doi.org/10.1016/j.watres.2017.09.007.
[12] Ocampo AM, Vargas CA, Sierra PM, Cienfuegos AV, Jiménez JN. Caracterización molecular de un brote de *Klebsiella pneumoniae* resistente a carbapenémicos en un hospital de alto nivel de complejidad de Medellín, Colombia [Molecular characterization of a carbapenem-resistant *Klebsiella pneumoniae* outbreak in a tertiary care hospital in Medellín, Colombia]. Biomédica 2015;35:24, doi:http://dx.doi.org/10.7705/biomedica.v35i4.2610.
[13] Antonelli A, D'Andrea MM, Vaggelli G, Docquier JD. Rossolini GM. OXA-372, a novel carbapenem-hydrolysing class D β-lactamase from a *Citrobacter freundii* isolated from a hospital wastewater plant. J Antimicrob Chemother 2015;70:2749–56, doi:http://dx.doi.org/10.1093/jac/dkv181.
[14] Ojer-Usoz E, Gonzâlez D, García-Jalón I, Vitas AI. High dissemination of extended-spectrum β-lactamase-producing Enterobacteriaceae in effluents from wastewater treatment plants. Water Res 2014;56:37–47, doi:http://dx.doi.org/10.1016/j.watres.2014.02.041.
[15] Yang F, Huang L, Li L, Yang Y, Mao D, Luo Y. Discharge of KPC-2 genes from the WWTPs contributed to their enriched abundance in the receiving river. Sci Total Environ 2017;581–582:136–43, doi:http://dx.doi.org/10.1016/j.scitotenv.2016.12.063.
[16] Dunbar J, Ticknor LO, Kuske CR. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Env Microbiol 2001;67:190–7, doi:http://dx.doi.org/10.1128/AEM.67.1.190-197.2001.
[17] Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012;28:1647–9, doi:http://dx.doi.org/10.1093/bioinformatics/bts199.
[18] Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 2011;70:119–23, doi:http://dx.doi.org/10.1016/j.diagmicrobio.2010.12.002.
[19] Ellington MJ, Kistler J, Livermore DM, Woodford N. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. J Antimicrob Chemother 2007;59:321–2, doi:http://dx.doi.org/10.1093/jac/dkl481.
[20] Alaidarous M, Alanazi M, Abdel-Hadi A. Isolation, identification, and antimicrobial susceptibility of bacteria associated with waterpipe contaminants in selected area of Saudi Arabia. Biomed Res Int 2017;2017:8042603, doi:http://dx.doi.org/10.1155/2017/8042603.
[21] Romeu Álvarez B, Salazar Jiménez P, Lugo Moya D, Rojas Hernández NM, Eslava Campos CA. Susceptibilidad antimicrobiana de aislamientos de *Escherichia coli* procedentes de ecosistemas dulceacuícolas. (Spanish) [Antimicrobial susceptibility of *Escherichia coli* isolated from river water ecosystems]. Rev Cubana Med Trop 2012;64:132–41.
[22] Clinical and Laboratory Standards Institute. M100 performance standards for antimicrobial susceptibility testing. 27th ed. Wayne, PA: CLSI; 2017.
[23] Clinical and Laboratory Standards Institute. M45-A2 methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline – second edition, Vol. 30. Wayne, PA: CLSI; 2010.
[24] The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters, version 80. . p. 0–77. https://www.eucast.org/.
[25] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268–81, doi:http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x.
[26] Picão RC, Cardoso JP, Campana EH, Nicoletti AG, Petrolini FVB, Assis DM, et al. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and Enterobacteriaceae in sewage. Diagn Microbiol Infect Dis 2013;76:80–5, doi:http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.001.
[27] Yang F, Mao D, Zhou H, Luo Y. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in northern China. PLoS One 2016;11:1–14, doi:http://dx.doi.org/10.1371/journal.pone.0156383.
[28] Montezzi LF, Campana EH, Corrêa LL, Justo LH, Paschoal RP, Da Silva ILVD, et al. Occurrence of carbapenemase-producing bacteria in coastal recreational waters. Int J Antimicrob Agents 2015;45:174–7, doi:http://dx.doi.org/10.1016/j.ijantimicag.2014.10.016.
[29] Santoro DO, Cardoso AM, Coutinho FH, Pinto LH, Vieira RP, Albano RM, et al. Diversity and antibiotic resistance profiles of *Pseudomonads* from a hospital wastewater treatment plant. J Appl Microbiol 2015;119:1527–40, doi:http://dx.doi.org/10.1111/jam.12936.
[30] Serna-Galvis EA, Salazar-Ospina L, Jiménez JN, Pino NJ, Torres-Palma RA. Elimination of carbapenem resistant *Klebsiella pneumoniae* in water by UV-C, UV-C/persulfate and UV-C/H_2O_2. Evaluation of response to antibiotic, residual effect of the processes and removal of resistance gene. J Environ Chem Eng 2020;8:102196, doi:http://dx.doi.org/10.1016/J.JECE.2018.02.004.
[31] Park JH, Kim YJ, Binn-Kim Seo KH. Spread of multidrug-resistant *Escherichia coli* harboring integron via swine farm waste water treatment plant. Ecotoxicol Environ Saf 2018;149:36–42, doi:http://dx.doi.org/10.1016/j.ecoenv.2017.10.071.
[32] Chen PL, Ko WC, Wu CJ. Complexity of β-lactamases among clinical *Aeromonas* isolates and its clinical implications. J Microbiol Immunol Infect 2012;45:398–403, doi:http://dx.doi.org/10.1016/j.jmii.2012.08.008.
[33] Wu CJ, Chen PL, Wu CJ, Yan JJ, Lee CC, Lee HC, et al. Distribution and phenotypic and genotypic detection of a metallo-β-lactamase, CphA, among bacteraemic *Aeromonas* isolates. J Med Microbiol 2012;61:712–9, doi:http://dx.doi.org/10.1099/jmm.0.038323-0.
[34] Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC beta-lactamases among *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates at a veterans medical center. J Clin Microbiol 2000;38:1791–6.
[35] Mehta SC, Rice K, Palzkill T. Natural variants of the KPC-2 carbapenemase have evolved increased catalytic efficiency for ceftazidime hydrolysis at the cost of enzyme stability. PLoS Pathog 2015;11:1–20, doi:http://dx.doi.org/10.1371/journal.ppat.1004949.
[36] Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect Dis 2009;9:228–36, doi:http://dx.doi.org/10.1016/S1473-3099(09)70054-4.
[37] Paterson DL. Resistance in gram-negative bacteria: Enterobacteriaceae. Am J Infect Control 2006;34:20–8, doi:http://dx.doi.org/10.1016/j.ajic.2006.05.238.
[38] Paiva MC, Reis MP, Costa PS, Dias MF, Bleicher L, Scholte LLS, et al. Identification of new bacteria harboring qnrS and aac(6′)-Ib/cr and mutations possibly involved in fluoroquinolone resistance in raw sewage and activated sludge samples from a full-scale WWTP. Water Res 2017;110:27–37, doi:http://dx.doi.org/10.1016/j.watres.2016.11.056.
[39] Varela AR, Nunes OC, Manaia CM. Quinolone resistant *Aeromonas* spp. as carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. Sci Total Environ 2016;542:665–71, doi:http://dx.doi.org/10.1016/j.scitotenv.2015.10.124.
[40] Ferreira Da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. FEMS Microbiol Ecol 2007;60:166–76, doi:http://dx.doi.org/10.1111/j.1574-6941.2006.00268.x.
[41] Conte D, Palmeiro JK, da Silva Nogueira K, de Lima TMR, Cardoso MA, Pontarolo R, et al. Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and river water. Ecotoxicol Environ Saf 2017;136:62–9, doi:http://dx.doi.org/10.1016/j.ecoenv.2016.10.031.