Diagnostic Microbiology and Infectious Disease 76 (2013) 80–85

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

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☆,☆☆

Renata C. Picão [a,b,*], Juliana P. Cardoso [a], Eloiza H. Campana [a], Adriana G. Nicoletti [a], Fernanda V.B. Petrolini [a], Diego M. Assis [c,d], Luiz Juliano [c], Ana C. Gales [a]

[a] Laboratório ALERTA, Divisão de Infectologia do Departamento de Medicina da Universidade Federal de São Paulo, São Paulo, Brazil
[b] Instituto de Microbiologia Paulo de Góes da Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[c] Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, Brazil
[d] Laboratório de Microbiologia Aplicada da Fundação Parque Zoológico de São Paulo, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 9 December 2012
Received in revised form 31 January 2013
Accepted 1 February 2013
Available online 9 March 2013

Keywords:
Carbapenemases
bla_{KPC} dissemination
Wastewater

ABSTRACT

We investigated the antimicrobial resistance profile and the occurrence of *Klebsiella pneumoniae* carbapenemase (KPC)–producing Gram-negative rods in sewage samples obtained from a Brazilian teaching hospital and from the wastewater treatment plant (WWTP) that receives it for treatment. We identified multidrug-resistant bacteria as well as KPC-2–producing *Aeromonas* spp. and several Enterobacteriaceae species, including *Kluyvera* spp., in the hospital effluent and in different sites of the WWTP. Most isolates showed the bla_{KPC-2} gene harbored on a transposon that was carried by conjugative plasmids. The presence of KPC production among *Aeromonas* spp., *Kluyvera* spp., and other Enterobacteriaceae indicates the adaptability of such isolates to aquatic environments, not only in the hospital effluent but also throughout the WWTP. Although secondary treatment seems to decrease the amount of KPC producers in sewage, multidrug-resistant isolates are continually disposed in the urban river. Thus, sewage treatment regulations are urgently needed to decelerate the evolution of antimicrobial resistance beyond hospitals.

1. Introduction

Klebsiella pneumoniae carbapenemase (KPC)–producing bacteria has been isolated in microbiology laboratories from health institutions worldwide. This β-lactamase is more often identified in *Klebsiella* spp. clinical isolates, whereas it has been found among other Enterobacteriaceae, *Pseudomonas* spp., and *Acinetobacter* spp. as well (Walsh, 2010). The successful dissemination of KPC among Gram-negative rods is attributed to the mobility of the genetic elements associated with bla_{KPC}, transposons, and broad-host-range plasmids (Li et al., 2011; Naas et al., 2008).

Differently from what has been observed for New Dehli metallo-β-lactamase (NDM)–producing bacteria in India, where researchers evidenced their spread throughout the community (Walsh et al., 2011), KPC-producing bacteria in Brazil is so far restricted to the hospital environment (Gales et al., 2012). However, it is well established that colonized and infected patients may disseminate KPC-producing bacteria in excreta, which is usually accompanied by active antimicrobials. In this context, the hospital sewage may constitute the perfect scenario for exchange of resistance genes between clinical pathogens and environmental bacteria due to the presence of broad-spectrum antimicrobials along with such bacteria (Brown et al., 2006; Martinez, 2009; Taylor et al., 2011). Indeed, KPC-producing Enterobacteriaceae was identified from hospital effluents in Brazil and in China (Chagas, 2011; Zhang et al., 2012). However, sanitary regulations were not developed considering the threat of antimicrobial resistance. Thus, in Brazil, as in many other countries, several hospitals do not treat their wastewater before disposing it into the general sewage collecting system.

Wastewater treatment in large urban areas often applies the activated sludge process. Briefly, after a primary treatment consisting of grading to remove bulky materials, wastewater is aerated. In this step, microorganisms including many Gammaproteobacteria metabolize the

☆ Funding. This work was supported by Fundação Apoio à Pesquisa do Estado de São Paulo, Brazil (FAPESP, grant number 2009/11051-0 to R.C.P.). A.C.G. and L. J. are researchers from the National Council for Science and Technological Development (CNPq), Ministry of Science and Technology, Brazil (grant numbers: 307816/2009-5 and 302981/2011-0).

☆☆ Transparency declarations: ACG has received research funding and/or consultation fees from Sanofi-Aventis, Thermo Fisher, and bioMérieux. Other authors have nothing to declare.

* Corresponding author. Tel.: +55-21-2560-8344; fax: +55-21-256083-44.
E-mail address: renata.picao@micro.ufrj.br (R.C. Picão).

0732-8893 © 2013 Elsevier Inc. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.001

suspended and soluble organic matter, a flocculent sludge is formed, and, after settling, the biomass is removed from the liquid stream (Chasick, 1969; Kim and Aga, 2007). The secondarily treated sewage is then discharged into rivers, estuaries, or oceans.

The bacterial load in the secondarily treated sewage is decreased compared to the incoming sample. However, a significant number of bacteria still remain, especially those that are resistant to antimicrobials (Galvin et al., 2010; Kim and Aga, 2007). However, in several previous papers regarding this issue, the resistance determinants involved as well as their genetic background were not disclosed. In the present work, we assessed the presence of antimicrobial-resistant Gram-negative rods, focusing on KPC producers, in the hospital effluent and through the wastewater treatment plant (WWTP) that receives it for treatment.

2. Materials and methods

2.1. Sewage sampling and culture

A single wastewater sample was collected from the effluent of a tertiary teaching hospital located in São Paulo, Brazil, and also from the affluent, aeration tank, activated sludge, and effluent of the WWTP that receives this hospital effluent. The plant is a secondary treatment facility that serves 4.4 million people, located 35 km apart from the hospital studied. During 2011, the plant showed an average hydraulic loading of 9.7 L/s. Four waterways usually release the treated sewage into an urban river. During the time the sewage samples were being collected, the WWTP was working over its capacity and was unable to treat all received sewage. Thus, one of the waterways was releasing sewage that had only received primary treatment. Thus, 100 mL of sewage samples collected from each waterway releasing secondarily treated sewage was combined and named as “secondary effluent”, while the sample recovered from the remaining waterway was named “primary effluent”. The samples were collected in 100-mL sterile flasks, conserved on ice during transportation, and then processed within 6 h. A 500-μL aliquot of 1:50 diluted samples was inoculated in chromogenic agar (BBL CHROMagar Orientation, Becton Dickinson, Cockeysville, MD, USA) plates (140 × 15 mm) with or without imipenem (1 μg/mL). To avoid the selection of only carbapenem-resistant bacteria, the hospital sewage was only cultured without any antimicrobial. From each plate, we subcultured a single colony among those sharing the same appearance by visual inspection. Grown colonies were then stored at – 20 °C in trypticase soy broth containing 15% glycerol (v/v).

2.2. Bacterial identification

Bacterial genus was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), using the MALDI Biotyper 2.0 software (BrukerDaltonik, Bremen, Germany), following the manufacturer's recommendations. KPC producers had their identification confirmed by 16S rDNA sequencing. Only Gram-negative rods were studied.

2.3. Susceptibility testing

The antimicrobial susceptibility profile was assessed by the Clinical Laboratory Standards Institute (CLSI) disk diffusion method and confirmed by agar dilution. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls, and the results were interpreted according to CLSI guidelines (CLSI, 2008, 2012a,b).

2.4. Investigation of bla_{KPC-2}, its genetic context, and transfer

bla_{KPC} detection was performed for all Gram-negative rods recovered, as previously described (Yigit et al., 2001). The genetic context of bla_{KPC} was determined by polymerase chain reaction (PCR) using primers anchoring in bla_{KPC-2} and in the IS*Kpn*6, IS*Kpn*7, and IS*Kpn*8 structures and in the *tnp*A or *tnp*R genes, as previously described (Li et al., 2011; Naas et al., 2008). Sequencing was carried out using the Big Dye Terminator Cycle Sequencing (Applied Biosystems, Carlsbad, CA, USA) and ABI PRISM 3130 (Applied Biosystems) apparatus. Transfer of the bla_{KPC-2} gene was assessed by mating out assays using the azide-resistant *E. coli* J-53 as the recipient strain. Transconjugants were selected with ticarcillin (50 μg/mL) and sodium azide (100 μg/mL), and the presence of bla_{KPC} in such isolates was confirmed by PCR.

3. Results

A total of 312 Gram-negative bacilli were recovered from the hospital sewage ($n = 130$, 41.6%) and from the WWTP (182, 58.4%). The most represented family in the hospital sewage was Enterobacteriaceae (61.2%), followed by Aeromonadaceae (18.6%), which was similar to that observed for the WWTP sewage samples cultured under imipenem selective pressure (70.6% and 10.6%, respectively). Samples from the WWTP cultured without imipenem showed the opposite effect, with predominance of Aeromonadaceae (58.8%), followed by Enterobacteriaceae (35.5%). Moraxellaceae and Pseudomonadaceae were isolated less frequently with culture conditions applied in this study. The distribution of genera in the samples evaluated varied according to the collection site and selective pressure applied, as shown in Fig. 1.

Resistance rates observed among isolates obtained from sewage samples are presented in Table 1. Overall, *Aeromonas* spp. recovered in the absence of selective pressure showed a resistance rate above 50% for third-generation cephalosporins, except for ceftazidime (33.3%). Aminoglycosides were the most active agents against *Aeromonas* spp. (Table 1). When samples were cultured without selective pressure, carbapenem-resistant *Aeromonas* spp. were only obtained from the hospital effluent. Eight *Aeromonas* spp. were recovered from WWTP under imipenem selective pressure and showed resistance rates of ≥50.0% for most tested antimicrobials, with the exception of sulfamethoxazole–trimethoprim, ciprofloxacin, and tetracycline (Table 1). It is important to emphasize that 42.9%, 22.2%, and 7.9% of *Aeromonas* spp. isolated from the WWTP without selective pressure showed resistance to ceftriaxone, ceftazidime, and ciprofloxacin, respectively.

Similarly to what was observed for *Aeromonas* spp., most Enterobacteriaceae isolates recovered showed high resistance rates to cefoxitin (60.7%) and to third-generation cephalosporins (resistance rates ≥55.0%). Cefepime, imipenem, and meropenem showed a similar activity, with 18.8%, 20.5%, and 16.2% resistance rates, respectively. Resistance rates for ciprofloxacin, gentamicin, amikacin, sulfamethoxazole–trimethoprim, and tetracycline were 12.8%, 17.1%, 29.1%, 22.6%, and 20.8%, respectively. The 53 enterobacterial isolates recovered from WWTP samples cultured in the presence of imipenem showed higher resistance rates to carbapenems (above 95%) than to ceftazidime and cefepime (both 49.1%). These isolates also showed high resistance rates to aminoglycosides (56.6%), whereas only 17% were resistant to ciprofloxacin (Table 1). Among Enterobacteriaceae recovered from the WWTP in the absence of imipenem, 28.9%, 15.8%, 10.5%, and 7.9% were resistant to ceftriaxone, ceftazidime, ciprofloxacin, and cefepime, respectively. Surprisingly, 2 *Enterobacter* spp. isolates, 1 from the aeration tank and another from the primary effluent of the WWTP that were recovered without selective pressure, showed either imipenem or meropenem resistance and were later found to be KPC producers.

The antimicrobial resistance rates observed for *Acinetobacter* spp. and *Pseudomonas* spp. are also presented in Table 1.

A total of 62 bacterial isolates showed the bla_{KPC-2} gene. These isolates had their carbapenemase activity confirmed by spectrophotometric

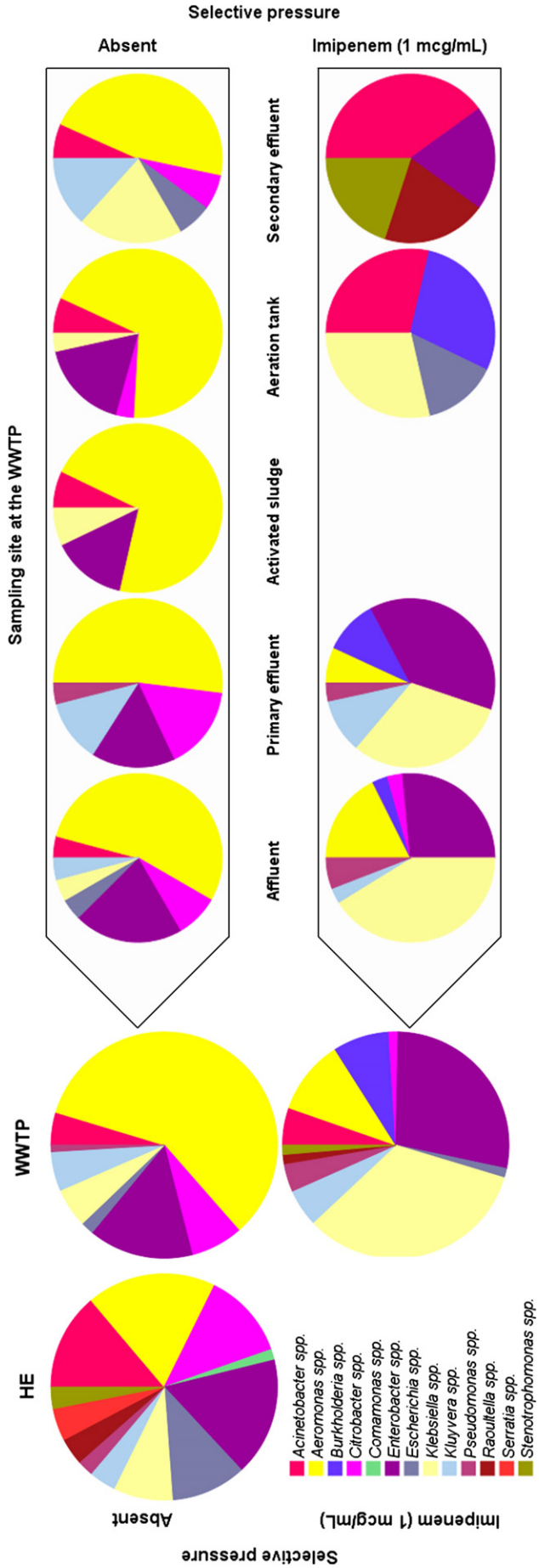

Fig. 1. Distribution of genera in sewage samples evaluated according to the collection site and selective pressure applied. HE = Hospital effluent; WWTP = wastewater treatment plant.

Table 1
Antimicrobial resistance rates for isolates recovered from sewage samples, according to the sampling site.

Group of isolates (n)	Sampling site (n_{total}/n_{IPM}[a])		Resistance rates for selected antimicrobials[c] (%)															
			TIC	TIM	FOX	CRO	CTX	CAZ	FEP	ATM	IPM	MEM	NA	CIP	CN	AK	TOB	TE	SXT
Aeromonas spp.	All samples (n = 95/8)		ND[d]	ND[d]	58.9	52.6	54.7	34.7	15.8	23.2	10.5	10.5	ND[d]	16.8	17.9	13.7	ND[d]	13.7	32.6
	Hospital effluent (n = 24/0)		ND[d]	ND[d]	58.3	70.8	79.2	62.5	41.7	45.8	16.7	16.7	ND[d]	33.3	45.8	37.5	ND[d]	20.8	33.3
	WWTP[b]	Affluent (n = 19/6)	ND[d]	ND[d]	68.4	68.4	63.2	52.6	10.5	21.1	21.1	21.1	ND[d]	21.1	21.1	10.5	ND[d]	15.8	42.1
		Activated sludge (n = 10/0)	ND[c]	ND[c]	90.0	20.0	20.0	10.0	0.0	0.0	0.0	0.0	ND[c]	0.0	0.0	0.0	ND[c]	0.0	10.0
		Aeration tank (n = 20/0)	ND[c]	ND[c]	50.0	45.0	50.0	20.0	5.0	15.0	0.0	0.0	ND[c]	15.0	0.0	0.0	ND[c]	20.0	35.0
		Primary effluent (n = 15/2)	ND[d]	ND[d]	46.7	60.0	60.0	20.0	13.3	26.7	13.3	13.3	ND[d]	6.7	13.3	13.3	ND[d]	0.0	20.0
		Secondary effluent (n = 7/0)	ND[d]	ND[d]	42.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND[d]	0.0	0.0	0.0	ND[d]	14.3	57.1
		Total WWTP (n = 71/8)	ND[d]	ND[d]	59.2	46.5	46.5	25.4	7.0	15.5	8.5	8.5	ND[d]	11.3	8.5	5.6	ND[d]	11.3	32.4
Enterobacteriaceae	All samples (n = 170/53)		84.1	57.1	72.4	65.9	72.9	35.3	28.2	54.7	44.7	41.2	67.1	14.1	29.4	37.6	57.6	25.9	28.8
	Hospital effluent (79/0)		88.6	51.9	62.0	68.4	72.2	35.4	24.1	57.0	29.1	22.8	74.7	13.9	22.8	39.2	59.5	39.2	44.3
	WWTP	Affluent (35/25)	85.7	77.1	94.3	71.4	80	45.7	42.9	62.9	71.4	71.4	60.0	20.0	51.4	45.7	65.7	14.3	17.1
		Activated sludge (3/0)	66.7	33.3	33.3	33.3	66.7	33.3	33.3	66.7	0.0	0.0	66.7	0.0	33.3	66.7	66.7	0.0	0.0
		Aeration tank (10/3)	40.0	30.0	70.0	40.0	80.0	20.0	20.0	40.0	20.0	30.0	60.0	0.0	20.0	30.0	30.0	20.0	0.0
		Primary effluent (34/23)	78.9	57.9	78.9	63.2	65.8	26.3	21.1	47.4	63.2	57.9	57.9	7.9	26.3	28.9	52.6	10.5	15.8
		Secondary effluent (9/2)	77.8	33.3	33.3	44.4	44.4	33.3	33.3	22.2	22.2	22.2	44.4	33.3	11.1	11.1	33.3	22.2	22.2
		Total WWTP (91/53)	80.2	61.5	81.3	63.7	73.6	35.2	31.9	52.7	58.2	57.1	60.4	14.3	35.2	36.3	56.0	14.3	15.4
Pseudomonas spp.	All samples (n = 7/3)		71.4	71.4	ND[d]	ND[d]	ND[d]	71.4	85.7	42.9	42.9	42.9	ND[d]	42.9	42.9	0.0	85.7	ND[d]	ND[d]
	Hospital effluent (n = 3)		66.7	66.7	ND[d]	ND[d]	ND[d]	66.7	66.7	33.3	0.0	0.0	ND[d]	0.0	0.0	0.0	100.0	ND[d]	ND[d]
	WWTP	Affluent (n = 2/2)	100.0	100.0	ND[d]	ND[d]	ND[d]	100.0	100.0	50.0	100.0	100.0	ND[d]	100.0	100.0	0.0	100.0	ND[d]	ND[d]
		Primary effluent (n = 2/1)	50.0	50.0	ND[d]	ND[d]	ND[d]	50.0	100.0	50.0	50.0	50.0	ND[d]	50.0	50.0	0.0	50.0	ND[d]	ND[d]
		Total WWTP (n = 4/3)	75.0	75.0	ND[d]	ND[d]	ND[d]	75.0	100.0	50.0	75.0	75.0	ND[d]	75.0	75.0	0.0	75.0	ND[d]	ND[d]
Acinetobacter spp.	All samples (n = 27/4)		51.9	48.1	ND[d]	44.4	48.1	44.4	33.3	ND[d]	44.4	51.9	ND[d]	59.3	22.2	40.7	14.8	11.1	44.4
	Hospital effluent (n = 18)		66.7	61.1	ND[d]	44.4	50.0	44.4	38.9	ND[d]	55.6	55.6	ND[d]	ND[d]	16.7	38.9	11.1	5.6	50.0
	WWTP	Affluent (n = 1/0)	0.0	0.0	ND[d]	0.0	0.0	0.0	0.0	ND[d]	0.0	0.0	ND[d]	100.0	0.0	0.0	0.0	0.0	0.0
		Activated sludge (n = 1/0)	0.0	0.0	ND[c]	0.0	0.0	0.0	0.0	ND[c]	0.0	0.0	ND[c]	0.0	0.0	0.0	0.0	100.0	0.0
		Aeration tank (n = 4/2)	25.0	50.0	ND[c]	50.0	50.0	50.0	25.0	ND[c]	50.0	50.0	ND[c]	50.0	50.0	50.0	50.0	0.0	50.0
		Secondary effluent (n = 3/2)	33.3	0.0	ND[d]	66.7	66.7	66.7	33.3	ND[d]	0.0	66.7	ND[d]	33.3	33.3	66.7	0.0	33.3	33.3
		Total WWTP (n = 9/4)	22.2	22.2	ND[d]	44.4	44.4	44.4	22.2	ND[d]	22.2	44.4	ND[d]	44.4	33.3	44.4	22.2	22.2	33.3

[a] n_{IMP} describes the number of isolates that were recovered under selective pressure exerted by imipenem (1 mg/L).
[b] WWTP = Wastewater treatment plant.
[c] TIC = tidarcillin; TIM = ticarcillin/clavulanic acid; FOX = cefoxitin; CRO = ceftriaxone; CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; ATM = aztreonam; ETP = ertapenem; IPM = imipenem; MEM = meropenem; AK = amikacin; DN = gentamicin; TOB = tobramycin; NA = nalidixic acid; CIP = ciprofloxacin; TE = tetracycline; SXT = sulfamethoxazole–trimethoprim.
[d] Not determined since there are no CLSI breakpoints available for interpreting susceptibility results.

assays performed as described previously (Picao et al., 2009), which were not susceptible to EDTA inhibition, indicating that bla_{KPC-2} was properly expressed by all isolates (data not shown). Among KPC-2 producers, representatives of the genera *Klebsiella* spp. (41.9%), *Enterobacter* spp. (24.2%), *Citrobacter* spp. (8.1%), *Serratia* spp. (4.8%), and, remarkably, also *Raoultella* spp. (6.4%), *Aeromonas* spp. (6.4%), and *Kluyvera* spp. (8.1%) were observed. KPC producers were present in the hospital effluent (37.1%, n = 23) as well as in the WWTP (62.9%, n = 39).

The great majority of KPC-producing isolates from the WWTP were recovered when samples were cultured under imipenem selective pressure (56.5%, n = 35), mainly from the affluent (48.7%, n = 19) and from the primary effluent (38.5%, n = 15), whereas 2 KPC-producing *Klebsiella* spp. were also isolated from the aeration tank. Of note, 4 KPC-producing isolates were recovered from the WWTP from samples cultured in the absence of imipenem: 1 *Klebsiella* sp. from the affluent, 1 *Aeromonas* sp. and 1 *Enterobacter* sp. from the aeration tank, and 1 *Enterobacter* sp. from the activated sludge. None of the KPC producers was isolated from the secondary effluent. The distribution of KPC producers according to the sampling site and selective pressure applied is depicted in Fig. 2.

The most active antimicrobial against KPC producers was cefepime (35.4% susceptible; $MIC_{50/90}$, 32 and 256 μg/mL), followed by ciprofloxacin (33.8% susceptible; $MIC_{50/90}$, 4 and 16 μg/mL), gentamicin (24.2% susceptible; $MIC_{50/90}$, 32 and >128 μg/mL), and ceftazidime (19.3% susceptible; $MIC_{50/90}$, 16 and 64 μg/mL). The carbapenems were the least active drugs against KPC producers. Although the potency of these drugs against this set of isolates was similar ($MIC_{50/90}$, 64 and >64 μg/mL for both), meropenem showed a higher susceptibility rate than imipenem (12.9% and 4.8% susceptible, respectively).

The genetic environment of bla_{KPC-2} was investigated for all KPC producers. Forty-eight isolates harbored this gene as part of a Tn*4401* structure (all *Klebsiella* spp. and *Raoultella* spp., most *Enterobacter* spp. and *Citrobacter*, as well as 2 *Aeromonas* spp.), whereas 11 isolates had the Tn3-*tnp*R/IS*Kpn*8/bla_{KPC-2}/IS*Kpn*6 array (most *Kluyvera* spp. and 2 *Aeromonas* spp.). The genetic environment of bla_{KPC-2} could not be identified for 2 *Enterobacter* spp. and 1 *Serratia* sp. Of note, KPC

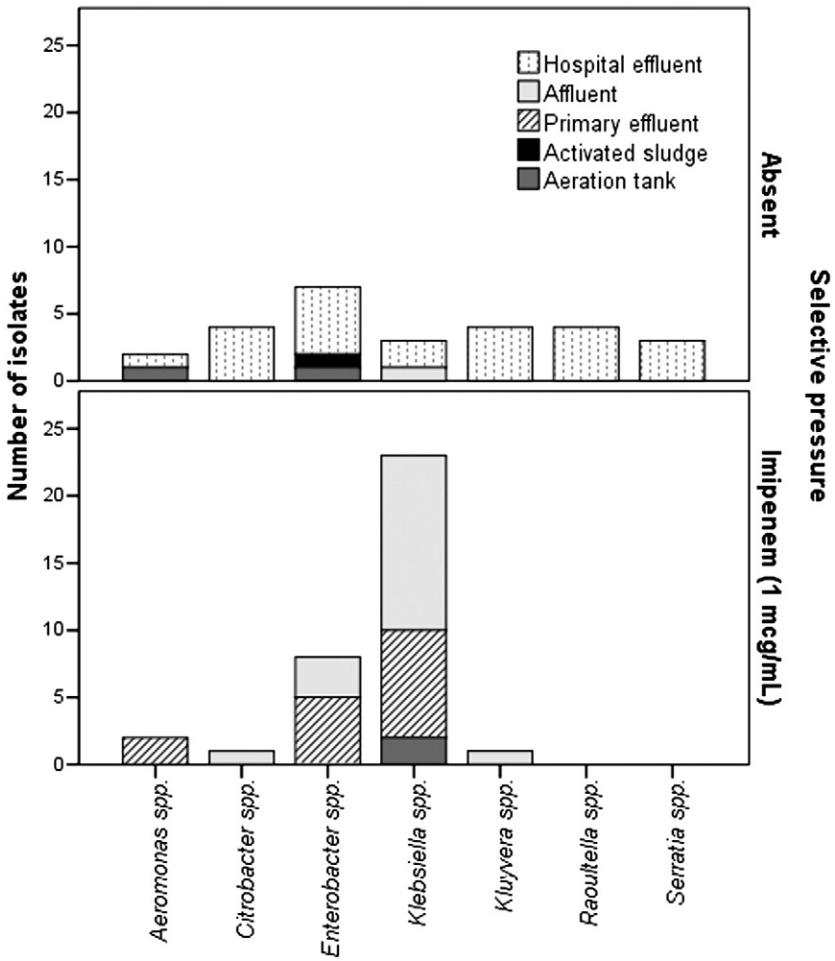

Fig. 2. Distribution of KPC producers according to the sampling site and selective pressure applied. Sampling sites in the wastewater treatment plant include the affluent, activated sludge, aeration tank, and primary effluent.

producers isolated from the hospital sewage carried either the Tn*4401* (50%) or the Tn3-*tnp*R/IS*Kpn*8/bla_{KPC-2}/IS*Kpn*6 array (36.3%), whereas 95% of KPC producers recovered from the WWTP had bla_{KPC} harbored on the Tn*4401* transposon.

The bla_{KPC-2} gene of every isolate recovered from the hospital sewage was successfully transferred by mating with *E. coli* J53 as the recipient strain, indicating that it was located either on conjugative or on mobilizable plasmids. bla_{KPC} transfer by mating-out assays was unsuccessful for only 7 KPC-producing isolates, which were recovered from the WWTP (*Enterobacter* spp., $n = 4$; *Klebsiella* spp., $n = 2$; and *Aeromonas* sp., $n = 1$). Further studies are needed to evaluate whether these isolates carried bla_{KPC-2} on a nonconjugative plasmid or on its chromosome.

4. Discussion

Hospital effluents represent a concern for the environmental dissemination of bacteria carrying critical antimicrobial resistance determinants (Chagas, 2011). In addition to resistant bacteria, hospital effluents are also characterized by the presence of significant levels of active antimicrobials (Brown et al., 2006; Kim and Aga, 2007; Verlicchi, 2012). Despite their specific nature, hospital effluents are frequently considered to be as pollutant as urban wastewaters, being treated together with other urban effluents and then discharged into natural environments (Verlicchi, 2012).

The discharge of multidrug-resistant bacteria including KPC producers into an urban river is worrisome, since these isolates could persist in the environment and act as opportunistic pathogens and/or resistance reservoirs that could accelerate the evolution of antimicrobial resistance in the community (Baquero et al., 2008; Kim and Aga, 2007; Martinez, 2009). For instance, Brazilian researchers have recently documented the presence of SPM-producing *P. aeruginosa* in the same urban river where the sewage studied here was discharged (Fontes et al., 2011).

In the present work, we have observed that Gram-negative rods recovered from the hospital sewage showed the highest resistance rates for most antimicrobials, which was expected for the reasons mentioned above. However, a significant portion of isolates recovered from the WWTP in the absence of selective pressure was also resistant to broad-spectrum antimicrobials. Since the hospital sewage was extensively diluted in the WWTP, these remarkable findings may suggest that domestic wastewater also contains a high amount of bacteria that are resistant to broad-spectrum antimicrobials, likely resulting from the selective pressure exerted by the use of antimicrobials and sanitizers in the community. Even though KPC producers were not recovered from secondarily treated sewage, it is noticeable that neither primary nor secondary treatment was efficient in eliminating multidrug-resistant bacteria from sewage, as previously observed (Galvin et al., 2010; Kim and Aga, 2007).

We have identified for the first time KPC production among *Aeromonas* spp. and *Kluyvera* spp., species considered to be predominantly environmental and notorious reservoirs of resistance genes (Picao et al., 2008; Rodriguez et al., 2004). During the period of study, neither *Aeromonas* spp., *Raoultella* spp., nor *Kluyvera* spp. clinical isolates that produce KPC were recovered from clinical specimens by our hospital clinical microbiology laboratory. Since bla_{KPC-2} was

identified on mobile genetic elements, our findings suggest that *Aeromonas* spp., *Raoultella* spp., and *Kluyvera* spp. might have acquired the bla_{KPC-2} in the hospital's sewage, which could have been favored by the selective pressure exerted by the antimicrobials usually present in such location (Brown et al., 2006). Of note, KPC-producing *Aeromonas* spp. was also recovered from the WWTP studied, indicating that this genus can represent a bla_{KPC} reservoir in the environment. The study design conducted in the present work was not quantitative. Although the real magnitude of this phenomenon has yet to unravel, the presence of bla_{KPC} in bacteria considered predominantly environmental is remarkable. In addition, the persistence of KPC-producing Enterobacteriaceae in sewage and in aquatic environments deserves attention.

Analysis of the genetic environment of bla_{KPC-2} showed that the great majority of isolates carried this gene on conjugative or mobilizable plasmids, harbored on a Tn*4401* structure. We speculate that this finding may reflect that plasmids carrying this transposon may encode functions conferring advantageous features for bacteria living in environments such as the one evaluated here, which may not be true for plasmids carrying the Tn3-*tnp*R/IS*Kpn*8/bla_{KPC-2}/IS*Kpn*6 array. These findings may encourage researchers to investigate this phenomenon and increase our understanding regarding the genetic elements driving the evolution of antimicrobial resistance in natural environments as well as in the community.

In an era when antimicrobial resistance evolution and dissemination are not accompanied by the development of new antimicrobials, controlling the dissemination of antimicrobial-resistant bacteria is absolutely necessary. In this context, hospital sewage treatment regulations regarding the spread of resistance bacteria and/or genes could contribute to decelerating the antimicrobial resistance evolution beyond hospitals.

Acknowledgments

The authors are very grateful to the SABESP personnel, especially to Américo Oliveira Sampaio, Marcelo Kenji Miki, Ana Lúcia Silva, for collecting wastewater samples, and to Lorena Correa Felhberg and Bruna Nonato for technical assistance.

References

Baquero F, Martinez JL, Canton R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 2008;19:260–5.

Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 2006;366:772–83.

Chagas TP, Seki LM, da Silva DM, Asensi MD. Occurrence of KPC-2-producing *Klebsiella pneumoniae* strains in hospital wastewater. J Hosp Infect 2011;77:281.

Chasick AH. Fundamentals of activated sludge. Water Environ Res 1969;41:1377–82.

Clinical and Laboratory Standards Institute (CLSI). Development of in vitro susceptibility testing criteria and quality control parameters; approved guideline-third edition, M23-A3. Wayne, Pa: CLSI; 2008.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 22th informational supplement (M100-S22). Wayne, PA: CLSI; 2012a.

Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, M7-A9. Wayne, PA: CLSI; 2012b.

Fontes LC, Neves PR, Oliveira S, Silva KC, Hachich EM, Sato MI, et al. Isolation of *Pseudomonas aeruginosa* coproducing metallo-beta-lactamase SPM-1 and 16S rRNA methylase RmtD1 in an urban river. Antimicrob Agents Chemother 2011;55:3063–4.

Gales AC, Castanheira M, Jones RN, Sader HS. Antimicrobial resistance among Gram-negative bacilli isolated from Latin America: results from SENTRY Antimicrobial Surveillance Program (Latin America, 2008–2010). Diagn Microbiol Infect Dis 2012.

Galvin S, Boyle F, Hickey P, Vellinga A, Morris D, Cormican M. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Appl Environ Microbiol 2010;76:4772–9.

Kim S, Aga DS. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. J Toxicol Environ Health B Crit Rev 2007;10:559–73.

Li G, Wei Q, Wang Y, Du X, Zhao Y, Jiang X. Novel genetic environment of the plasmid-mediated KPC-3 gene detected in *Escherichia coli* and *Citrobacter freundii* isolates from China. Eur J Clin Microbiol Infect Dis 2011;30:575–80.

Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut 2009;157:2893–902.

Naas T, Cuzon G, Villegas MV, Lartigue MF, Quinn JP, Nordmann P. Genetic structures at the origin of acquisition of the beta-lactamase bla KPC gene. Antimicrob Agents Chemother 2008;52:1257–63.

Picao RC, Poirel L, Demarta A, Silva CS, Corvaglia AR, Petrini O, et al. Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. J Antimicrob Chemother 2008;62:948–50.

Picao RC, Poirel L, Gales AC, Nordmann P. Diversity of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. Antimicrob Agents Chemother 2009;53:3908–13.

Rodriguez MM, Power P, Radice M, Vay C, Famiglietti A, Galleni M, et al. Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. Antimicrob Agents Chemother 2004;48:4895–7.

Taylor NG, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol 2011;26:278–84.

Verlicchi P, Al Aukidy M, Galletti A, Petrovic M, Barcelo D. Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. Sci Total Environ 2012;430C:109–18.

Walsh TR. Emerging carbapenemases: a global perspective. Int J Antimicrob Agents 2010;36(Suppl 3):S8-S14.

Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect Dis 2011;11:355–62.

Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2001;45:1151–61.

Zhang X, Lu X, Zong Z. Enterobacteriaceae producing the KPC-2 carbapenemase from hospital sewage. Diagn Microbiol Infect Dis 2012;73:204–6.