Antimicrobial resistance patterns and genotypic diversity between clinical and water systems isolates of *Pseudomonas aeruginosa* in Cali, Colombia

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Abstract. *Pseudomonas aeruginosa* is considered an opportunistic pathogen, causing a wide variety of infections in compromised hosts, also frequently develops multi-resistance to antibiotics and can colonize various habitats, including water systems. The main aim of this study was to investigate antibiotics susceptibility pattern, genotypic diversity and detection of resistence genes in *P. aeruginosa* isolates from clinical and aquatic environment sources. Of the 220 *P. aeruginosa* isolates examined, 48 were clinical isolates and 172 isolates from wastewater and freshwater. Susceptibility to eight antimicrobial agents was carried out by disk diffusion method. Clinical and environmental isolates were screened for the presence of the genes encoding bla_{KPC-2}, $bla_{CTX-M-9}$, bla_{PER-1}, bla_{OXA-10}, bla_{IMP-1}, bla_{VIM-2} and bla_{ampC} by polymerase chain reaction (PCR). Isolates were examined with PCR-SSCP analysis of partial DNAr 16S sequence. Isolates were mainly resistant to cefoxitin. Multidrug-resistant *P. aeruginosa* (MDRPA) strains were found in 70% and 90.3% of the clinical and environmental isolates, respectively. The prevalence rates of â-lactamase genes were recorded (bla_{KPC-2} 41.3%, bla_{VIM-2} 36.8%, bla_{IMP-1} 13.6%, $bla_{CTX-M-9}$ 10.9% and bla_{ampC} 10.5%,). The PCR-SSCP analysis showed three conformational patterns. All clinical isolates and most environmental isolates were grouped into a single cluster. In this study, we found that *P. aeruginosa* strains recovered from city water systems must be considered potential reservoir for ESBL genes, especially *bla*KPC-2 and *bla*VIM-2.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that has been emerging as a primary source of nosocomial infections, and can cause urinary and respiratory tract infections, soft tissue infections, endocarditis, and a variety of systemic infections, particularly in burned patients, with cancer, cystic fibrosis, or compromised immune systems (Giamarellou, 2002; Inglis *et al.*, 2010; Luna *et al.*, 2013; Allen *et al.*, 2014).

The treatment is often complicated, because this microorganism has intrinsic multidrug resistance and several strains have acquired resistance genes to a wide variety of antimicrobials, including to most commercially available β-lactams (Lambert, 2002, Rodríguez *et al.*, 2009; Jeannot *et al.*, 2013; Magiorakos *et al.*, 2012). Resistance to carbapenems is produced especially by the production of metallo-β-lactamases and serine enzymes (Lambert, 2002; Rodríguez *et al.*, 2009; Jeannot *et al.*, 2013).

In recent years the circulation of strains has increased resistant to carbapebemes and to other antimicrobials, has led to the emergence of multidrug-resistant *P. aeruginosa* (MDRPA).

P. aeruginosa can grow in many environments, it is found in water, soil, sewage, animal faeces, on vegetation and the human body and has a great capacity to adapt to the environment, survive extremes which includes temperatures ranging from 4°C to 43°C, and weak ion concentrations, among others (Wu *et al.*, 2012; Lalancette *et al.*, 2017).

Several Studies on the molecular epidemiology and population structure of Gram-negative bacteria have shown that incidence of the bacterium in the aquatic environment has increased as a consequence of the large-scale use of antibiotics (Baquero, *et al.*, 2008; Scotta *et al.*, 2011; Vaz-Moreira *et al.*, 2012; Akinpelu *et al.*, 2014). *P. aeruginosa* often is found in natural waters such as lakes and rivers in concentrations of 10/100 mL to >1 000/100 Ml (Vaz-Moreira *et al.*, 2012).

Some studies indicate that the environmental reservoirs of the *P. aeruginosa* can be a source of infection (Yang *et al.*, 2009; Scotta *et al.*, 2011; Breathnach *et al.*, 2012; Decker & Palmore 2013; Cortes *et al.*, 2013), thus, healthcare-associated infections can also be linked to contaminated water systems, especially among immunocompromised and severely ill patients (Yang *et al.*, 2009; Breathnach *et al.*, 2012; Decker & Palmore, 2013).

In Colombia, studies have focused on the characterization of MDRPA in clinical isolates from patients with *P. aeruginosa* infections (Crespo *et al.*, 2004; Cortes *et al.*, 2013; Vanegas *et al.*, 2014; Correa *et al.*, 2015), but little information is available on the distribution in the environment, especially in water systems. In this study, we evaluated the antibiotic resistance patterns of *P. aeruginosa* isolated from clinical samples of patients with nosocomial infection and water systems of the city of Cali, focusing on the identification of high-risk clones and of β-lactam resistance genes.

MATERIAL AND METHODS

Study area and sampling. The artificial waters samples were collected from the wastewater treatment plant "Cañaveralejo PTAR-C" (sampling site A), a rainwater channel (B), that receives discharges of domestic wastewater, and so-called "Canal Intersector (CVC) Sur" (sampling site C), that receives the waste load from the southeast of the Cali city (geographical coordinates: 3 ° 27'00 "N 76 ° 32'00" W, in southwestern Colombia). Similarly, the freshwater samples were collected from Cauca River at the height of the Puerto Mallarino plant (D) and Melendez River (E) at the points indicated on the map (Figure 1). The samples were obtained in two periods: during the dry season (January) and during the wet season (May) of 2018.

Wastewater effluent and receiving river water samples were collected from point collection in triplicate in 500 ml brown glass bottles protected with aluminum seal and / or Teflon cap, completely filled and kept at 4°C in an icebox containing ice during the transportation to the laboratory and processed immediately for the enumeration of bacteria.

In the case of clinical samples, 48 isolates were obtained from patients who were in the Intensive Care Unit (ICU) of the University Hospital San Juan de Dios in the city of Cali with invasive infection by *P. aeruginosa* between the months of January and May 2018. This invasive infection is defined as the isolation of bacteria from a normally sterile site in a patient with clinical signs and symptoms consistent with infection by the bacteria. Ten isolates were obtained from urine, sixteen from pus samples from deep wounds, thirteenfrom sputum and nine samples were obtained from blood.

Isolation and Processing of Samples
Freshwater and wastewater samples were serially diluted and 100 μL of the diluted samples were plated on Cetrimide agar (Merck, Germany). Clinical samples were plated directly on the Cetrimide agar. Samples were incubated at 42°C. *Pseudomonas*-like isolates were thereafter
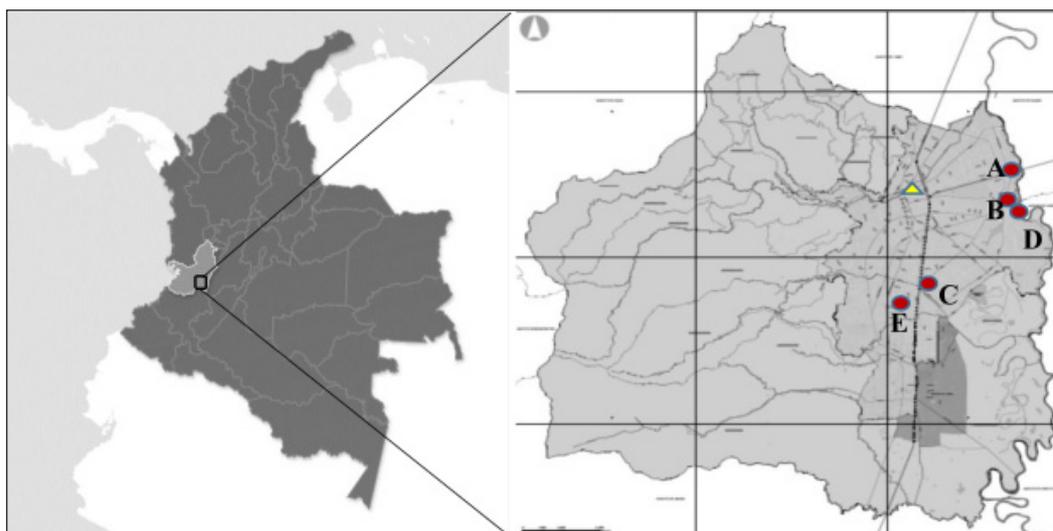
Figure 1. Geographical location of the sampling site.
(a) Department of Valle del Cauca, Colombia (b) City of Cali. (c). Red points show the sites where surface water samples were collected: Wastewater treatment plant "Cañaveralejo PTAR-C" (A), rainwater channel (B) near the wastewater treatment plant, Canal Intersector (CVC) Sur (C), water treatment plant "Puerto Mallarino" (D) and Melendez River (E), and the yellow triangle indicate the location of the hospital from which the clinical samples were obtained.

transferred into Nutrient agar (Oxoid Ltd., Hampshire, United Kingdom) plates and incubated overnight at 37°C and thereafter screened based on typical morphology, catalase and oxidase reactions. The purified isolates of *P. aeruginosa* were finally confirmed with a Vitek GNI+ system (bioMeriex Vitek Inc., Hazelwood, MO), which is a rapid and reliable identification method.

DNA Isolation and Extraction

DNA extraction was carried out from bacterial culture in LB (Luria-Bertani) broth, using the genomic DNA extraction kit (MOBIO Laboratories, QIAGEN).

The concentration of DNA in the samples was quantified by using a FOTO/Analyst® Investigator/FX Systems (FOTODYNE Incorporated).

Identification of Isolates by Polymerase Chain Reaction (PCR)

Confirmation of *P. aeruginosa* isolates was performed through DNAr 16S gene detection with Specific primers PA-GS-F (5'-TCAGTCACACTGGAACTGAG-3'), and

PA-GS-R (5'-GTAATTCCGAGGAACGCTTG-3') to amplify a fragment of approximately 240 bp, according to the protocol established by Rolain *et al.*, 2004. *P. aeruginosa* reference strain ATCC 27853 was used as positive control and a reaction mixture containing Nuclease free water as negative control. The amplified PCR products were analysed by gel electrophoresis in 1.2% agarose gels stained with ethidium bromide (EtBr) 0.5 mg/L, for 1 h at 100 V in $0.5 \times$ TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5).

Determination of antibiotic resistance profiles

Antibiotic resistance profiles were obtained from 220 isolates (84 environmental isolates obtained during the dry season, 88 isolated during the wet season and 48 clinical isolates). A standardized amount was inoculated (standard 0.5 of Mc Farland) and antimicrobial susceptibility testing was determined by disk diffusion method on Mueller-Hinton (MH) agar (Difco Laboratories, Detroit, MI, USA), using interpretative criteria of the Clinical and Laboratory

Standard Institute (CLSI) (Wanyne, 2015). The following antimicrobials were used: Trimetroprim/Sulfametoxazol (SXT, 23.75 µg + 1.25 µg), Gentamicin (GEN, 10 µg), Ciprofloxacin (CIP, 5 µg), Ceftazidime (CAZ, 30 µg), Cefoxitin (FOX, 30 µg), Cefotaxime (CTX, 30 µg), Meropenem (MEM, 10 µg), Piperacillin/Tazobactam (TZP, 40 µg), Cefixime (CFM, 5 µg) (Oxoid Ltd., Hampshire, United Kingdom). Intermediate susceptibility to each antibiotic was considered to be resistance. Quality control was carried out using standard strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853).

According to the definitions proposed by Magiorakos *et al.* (2012), resistance to three or more antibiotic classes were defined as multidrug resistant *P. aeruginosa* (MDRPA).

Polymerase Chain Reaction (PCR) to Detect *bla* Genes

The extended-spectrum β-lactamases were identified by polymerase chain reaction with consensus primers targeting bla_{TEM-1}, $bla_{CTX-M-9}$, bla_{PER-1}, bla_{OXA-10}, bla_{IMP-1}, bla_{VIM-2} and bla_{AMPc} genes shown in Table 1.

Amplification was carried out in 50-µl volumes with 5-10 ng (genomic DNA) reaction buffer, 1 U of *Taq* polymerase (Bioline, London, United Kingdom), 200 µM each deoxynucleoside triphosphate, 1.5 or 2,5 mM $MgCl_2$, 10 pmol of each primer, and 4 µl of DNA as the template. PCR conditions generally were as follows: a hot start at 94°C for 5 min; 35 cycles of 30 s at 94°C, 45 s at either 52°C (bla_{TEM-1}, bla_{PER-1} and bla_{OXA-10}), 45 s at 51°C (bla_{CXT-M9} and bla_{AMPc}), 1 min at 51°C (bla_{VIM-2}, bla_{IMP-1} and bla_{KPC-2}), and 60 s at 72°C; and a final step of 10 min at 72°C.

Five micro-liter of PCR products were analyzed in 1.1% agarose (Sigma, USA) and the results were observed under UV light.

Single-strand conformation polymorphism (SSCP)- PCR for the analysis of *P. aureginosa* isolates

To define the SSCP profiles, the PCR amplification products of the 240 bp fragment of rDNA16 of *P. aeruginosa* was subjected to a denaturing process by treating 5 µL of denaturing buffer (5 mM EDTA, 0.05% bromephenol blue, 0.05% xylencianol and 95% formamide) with 5 µL of DNA amplified at 95°C for 10 minutes and perform electrophoresis on gels of 8.0% polyacrylamide in TBE buffer (0.09 M Tris-borate, 2 mM EDTA, pH 8.0).

Statistical methods

Differences in frequencies of antibiotypes and β-lactamases -genotypes among groups was evaluated using Chi-squared tests ($\chi 2$) on contingency tables with a significance level of $P<0.05$. Univariate analysis of

Table 1. Primers used in study

Primer	Oligonucleotide sequence (5'→3')	Size (pb)	References
Bla_{KPC-2}	F-5'GGACACACCCATCCGTTA-3' R-5'GCGGGCGTTATCACTGTATTG-3'	956	Correa *et al.*, 2015
$bla_{CTX-M-9}$	F-5'GTGACAAAGAGAGTGCAACGG-3' R-5'TGCAACTTCATGTTATGCCG-3'	856	Shacheraghi *et al.*, 2010
bla_{PER-1}	F-5'ATGAATGTCATTATAAAAGCT-3' R-5'TTAATTTGG GCTTAGGG-3'	927	Nordman & Naas, 1994
bla_{OXA-10}	F-5'TGAGCACCATAAGGCAACCA-3' R-5'TTGGGCTAAATGGAAGCGTTT-3'	311	Kuo *et al.*, 2010
bla_{IMP-1}	F-5'ATGAGCAAGTTATCCTTATTC-3' R-5'GCTGCAACGACTTGTTAG-3'	741	Khosravi *et al.*, 2008
bla_{VIM-2}	R-5'ATGATTCTCGCCGCTGAAGCC-3' F-5'AAAGTTATGCCGCACTCACC-3'	865	Poirel *et al.*, 2002
Bla_{ampC}	F-5'GGTATGGCTGTGGGTGTTA-3' R-5'TCCGAAACGGTTAGTTGAG-3'	882	Coudron *et al.*, 2000

variance was performed for inference on differences in average numbers of multi-drug resistance between β-lactamases genotypes. Analyses were performed in IBM SPSS Statistics version 23.0 (Inc, Chicago).

RESULTS

A total of 172 environmental isolates of *P. aeruginosa* were obtained from the sites: Wastewater treatment plant "Cañaveralejo PTAR-C" (n=14 during the dry season; n=20 during the wet season), rainwater channel (n= 8 during the dry season; n=9 during the wet season) near the wastewater treatment plant, Canal Intersector (CVC) Sur (n=29 during the dry season; n=17 during the wet season), water treatment plant "Puerto Mallarino" (n=13 during the dry season; n= 10 during the wet season) and Melendez River (n=20 during the dry season; n=32 during the wet season) and 48 clinical isolates during study time.

Patterns of susceptibility of the isolates clinical and environmental
Molecular identification of the *P. aeruginosa* was carried out by amplifying a specific fragment of DNAr 16S gene. A total 220 *P. aeruginosa* isolates were tested for drug susceptibility with a panel of eight antimicrobials, widely used in Colombian hospitals as antipseudomonal agents. The isolates analyzed showed different levels of resistance to ceftazidime, cefoxitin, cefotaxime, piperacillin/tazobactam and meropenem. All isolates of *P. aeruginosa* showed resistance to non-β-lactam agents, including trimetroprim/sulfamethoxazole, fluoroquinolones such as ciprofloxacin and aminoglycosides, such as gentamicin.

About 50% of the 172 enviromental isolates were resistant to at least one of the antibiotics tested. Differences were found between the environmental isolates of *P. aeruginosa* resistant obtained in the dry and wet season. Isolates with resistance to cefoxitin, cefotaxime, piperacillin/ tazobactam and trimetroprim/Sulfame-thoxazole were significantly more prevalent during the dry season (91.7% each, p<0.05).

Cefoxitin-resistant isolates (93.2%, p<0.05) continued to be prevalent during the wet season.

Among the clinical isolates, a significant prevalence of cefoxitin was detected (65%, p< 0.05).

Table 2 shows the number of isolates from *P. aeruginosa* with resistance to antibiotics in environmental and clinical samples. From this susceptibility analysis, five profiles or antibiotypes were determined among the *P. aeruginosa* isolates. Table 3 shows the antibiotic sensitivity and resistance profile for each antibiotype. We classified seventeen of these 220 isolates as wild-type (antibiotype 1), fifteen as resistant (antibiotype 2) and 188 as MDRPA (antibiotypes 3, 4 and 5).

Wild-type and resistant strains were present in the clinical isolates and only in the waters of the rainwater channel and the Melendez River, whereas MDRPA strains were found in 66.7% and 90.7% of the clinical and environmental isolates, respectively.

Additionally, it was observed that twenty one (12.2%) of the enviromental isolates were classified as antibiotype 3 which include seven (41.2%) isolates obtained from the rainwater channel, ten (21.7%) isolated from "Canal Intersector (CVC) Sur" and four (17.4%) isolated from the water treatment plant "Puerto Mallarino". Three isolates of *P. aueruginosa* obtained from pus from deep wounds shared this antibiotype.

The environmental isolates included in the antibiotype 4 were the most abundant (51.7%) and these were found in all the sites sampled, except in the rain water channel, obtaining a greater number of them in the Melendez River (63.5%). Some clinical isolates also presented this antibiotype (27.1%), and were found in seven urine samples and six sputum samples.

The antibiotype 5 grouped the isolates with resistance to all the antibiotics evaluated in this study. This antibiotype was the most representative among the clinical isolates (33.3%) and was found from sputum, deep wounds, urine and blood samples. In the environmental isolates, the largest number was determined in the wastewater treatment plant "Cañaveralejo PTAR-C" (50%).

Table 2. Distribution of antibiotic resistence and *bla* genes in clinical and environmental isolates of *P. aeruginosa*

Isolates	No. of isolates with Antibiotic resistence									No. of isolates with *bla* genes (%)			
	FOX n (%)	CTX n (%)	CAZ n (%)	TZP n (%)	MEM n (%)	STX n (%)	CIP n (%)	GEN n (%)	Bla_{KPC-2} n (%)	bla_{IMP-1} n (%)	bla_{VIM-2}n n (%)	$bla_{CTX-M-9}$ n (%)	bla_{ampC} n (%)
Enviromental													
Dry Season n=84	77* (91.7)	77* (91.7)	63 (75)	77* (91.7)	59 (70.2)	77* (91.7)	66 (78.6)	66 (78.6)	35 (41.7)	–	49* (58.3)	–	7 (8.3)
Wet Season n=88	82* (93.2)	38 (43.2)	41 (46.4)	47 (53.4)	66 (75)	41 (46.4)	53 (60.2)	28 (3121)	22 (25)	28* (31.2)	25 (28.4)	22* (25)	16 (18.2)
Clinic n=48	32* (66.7)	24 (50)	32 (67.7)	22 (45.8)	32 (66.7)	32 (66.7)	32 (66.7)	34 (70.8)	34* (70.8)	2 (4.2)	7* (14.6)	2 (4.2)	–
Total	191	139	136	146	157	150	151	42	91 (41.3)	30 (13.6)	81 (36.8)	24 (10.9)	23 (10.5)

* Significant difference in prevalence antibiotic resistence and *bla* gene *p<0.05*.
Trimetroprim/Sulfametoxazol (SXT), Gentamicin (GEN), Ciprofloxacin (CIP), Ceftazidime (CAZ), Cefoxitin (FOX), Cefotaxime (CTX), Meropenem (MEM), Piperacillin/Tazobactam (TZP) (Oxoid).

Table 3. Distribution of antibiotypes in clinical and environmental isolates of *P. aeruginosa*

Ant	Clinic n (%)	No. of isolates in Sampling site (%)						Profile	
		Enviromental						Resistance	Sensivity
		Total n (%)	A n (%)	B* n (%)	C n (%)	D n (%)	E* n (%)		
1	13 (27.1)	4 (2.3)	–	4 (23.5)	–	–	–	–	FOX, CTX, CAZ, TZP, MEM, STX, CIP, GEN
2	3 (6.3)	12 (7)	–	3 (17.6)	–	–	9 (17.3)	STX/CIP	CAZ, TZP, MEM, GEN, FOX, CTX
3	3 (6.3)	21 (12.2)	–	7 (41.2)	10 (21.7)	4 (17.4)	–	STX, CIP, GEN	FOX, CTX, TZP, CAZ, MEM
4	13 (27.1)	89 (51.7)	17 (50)	-	23 (50)	16 (69.6)	33 (63.5)	STX, CIP, GEN, FOX/CTX, CAZ, MEM	TZP
5	16 (33.3)	46 (26.7)	17 (50)	3 (17.6)	13 (28.3)	3 (13)	10 (19.2)	FOX, CTX, CAZ, TZP, MEM, STX, CIP, GEN	–
Total	48	172	34	17	46	23	52		

Ant: Antibiotype; * Significant difference in prevalence of antibiotypes, *p<0,05*.
Enviromental isolates: wastewater treatment plant "Cañaveralejo PTAR-C" (A), rainwater channel (B), "Canal Intersector (CVC) Sur" (C), water treatment plant "Puerto Mallarino" (D) and Melendez River (E).

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Identification of Antibiotic Resistance *bla* Genes

The screening for antibiotic resistance genes revealed the absence of bla_{OXA-10} and bla_{PER-1} genes as they were not detected in any of the *P. aeruginosa* isolates.

On the other hand, bla_{KPC-2} gene was detected in the 70.8% of clinical isolates and 41.7% of environment isolates during dry season, but this decreased to 25% during wet season. Similarly, bla_{VIM-2} gene was detected in the 58.3% of environment isolates during dry season and decreased to 28.4%, although in clinical isolates they were only detected in 14.6% of them. The $bla_{CTX-M-9}$ and bla_{IMP-1} genes were absent in the environmental isolates during the dry season and the bla_{ampC} gene in clinical isolates (Table 2).

Genetic characterization of *P. aeruginosa* isolates

Figure 2 shows PCR-SSCP fingerprints generated for each of the 220 *P. aeruginosa* isolates used in the antimicrobial testing, we detected three conformational patterns. Forty six enviromental isolates of the "Canal Intersector (CVC) Sur" showed identical banding patterns (bands of 330 and 320 bp) and were represented by the conformational pattern PCR-SSCP fingerprint I. A second group (composed of the bands of 280 and 340 bp) represented by PCR-SSCP fingerprint II, included all clinical isolates and of the Melendez River isolates, nineteen isolates from the water treatment plant "Puerto Mallarino" and thirty four isolates from the wastewater treatment plant "Cañaveralejo PTAR-C".

Seventeen isolates from water treatment plant "Puerto Mallarino" constituted by two bands of 340 and 380 bp (PCR-SSCP fingerprint III) clustered with four environmental isolates from rainwater channel (Table 4).

DISCUSSION

In this study, we analyzed 72 isolates of *P. aeruginosa* obtained from different types of clinical samples and artificial and natural waters of the city of Cali.

When comparing our sensitivity data with those available in Colombia and Latin America, the resistance rates of our *P. aeruginosa* isolates against piperacillin/tazobactam, ceftazidime, meropenem, ciprofloxacin, and gentamicin were comparable with those reported in clinical isolates (Gales *et al.*, 2012; Vanegas *et al.*, 2014; Correa *et al.*, 2015; Salvador *et al.*, 2018). These classes of antibiotics are the
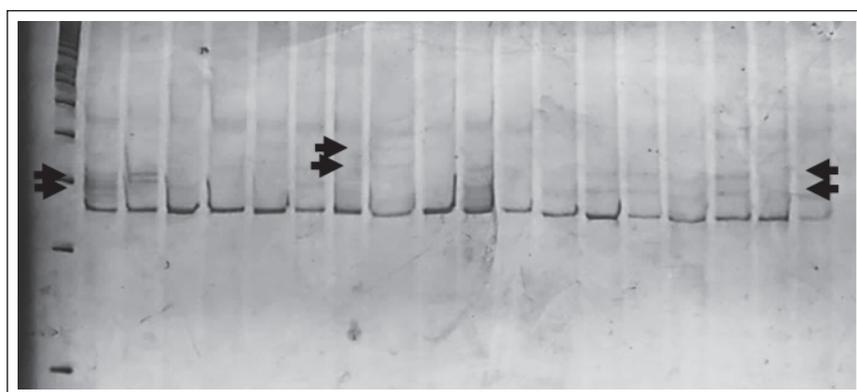

Figure 2. SSCP analysis of environmental and clinical isolates of *P. aeruginosa*. Lane 1: 100 bp molecular weight marker, lanes 2, 3: isolates of the "Canal Intersector (CVC) Sur"; lane 4: 5, 11,12 isolates of the Meléndez River; lanes 6, 7, 9: isolates of water treatment plant "Puerto Mallarino"; lane 8: isolates of rainwater channel; lane 10: isolates of the treatment plant "Cañaveralejo PTAR-C; lane 13-19: clinic isolates.

Table 4. Distribution and molecular characterization molecular of isolates.clinical and environmental isolates of *P. aeruginosa*

PCR-SSCP fingerprint (bp)	No. of isolates in Sampling site (%)							Resistance Genes				
	Clinic n (%)	Environmental						Bla_{KPC-2} n (%)	bla_{IMP-1} n (%)	bla_{VIM-2} n (%)	bla_{CTXM-9} n (%)	bla_{AmpC} n (%)
		Total n (%)	A n (%)	B* n (%)	C n (%)	D n (%)	E* n (%)					
I (330, 320)	–	46 (19.5)	–	–	46 (20.9)	–	–	15 (6.8)	3 (1.4)	15 (5.5)	3 (1.4)	–
II (280, 340)	20 (21.8)	105 (47.7)	34 (15.5)	–	–	19 (8.6)	52 (23.6)	67 (30.5)	18 (8.2)	57 (25.9)	6 (2.7)	–
III (340, 380)		35 (15.9)	–	17 (7.7)	–	4 (1.8)	–	9 (4.1)	9 (4.1)	9 (4.1)	15 (6.8)	23 (10.5)
Total	48	172	34	17	46	23	52	91 (41.4)	30 (13.6)	81 (36.8)	24 (10.9)	23 (10.5)

Enviromental isolates: wastewater treatment plant "Cañaveralejo PTAR-C" (A), rainwater channel (B), "Canal Intersector (CVC) Sur" (C), water treatment plant "Puerto Mallarino" (D) and Melendez River (E).

most used in *P. aeruginosa* isolates reflects the extensive and long-term use of these antimicrobial agents in our environment.

In our country, epidemiological data indicate a resistance to carbapenems in clinical isolates of *P. aeruginosa* greater than 50% (Gales *et al.*, 2012; Labarca *et al.*, 2016; Salvador *et al.*, 2018). In this study we found resistance to meropenem of 65% in clinical isolates and above 70% in those present in water systems, the rapid spread of carbapenemase-producing Gram-negative bacteria isolates has become a public health problem due to the few therapeutic options to treat the infections they cause.

Similarly, the epidemiological report of *Pseudomonas* spp. isolates obtained in river waters of the Republic of Congo and India and in sewage treatment plants in Switzerland indicated resistance to piperacillin and ceftazidime greater than 50%, all isolates from water treatment plants in Switzerland were susceptible to carbapenems, while 15 and 33% of the isolates from Republic of Congo and India, respectively, were resistant to carbapenems (Devarajan *et al.*, 2017).

According to these results, we can affirm that although the dynamics of resistance in *P. aureginosa* isolates varies between regions, there is a greater prevalence of resistant isolates in developing countries, such as Colombia.

Strains called wild-type (due to their sensitivity to all antibiotics evaluated in this study) were scarce. Instead, the prevalence of resistant strains was observed, especially MDRPA, which constituted 66.7% and 90.7% of clinical and environmental isolates, respectively.

Strains with the wild phenotype were preferentially isolated in clinical samples and only one isolate was detected in the rainwater channel (this channel also receives domestic water from the sector), while strains with MDRPA phenotype were more successful in colonizing all sampling areas in water systems, as well as the different clinical samples. However, in contrast to the results of the study conducted by Sivaraj *et al.* (2012), environmental isolates were highly resistance to the

antibiotic when compared to the clinical isolates.

The results of this study show that the problem of multi-resistance of *P. aeruginosa* is not only limited to clinical isolates, in natural environments such as water systems, the seasonal distribution of *P. aeruginosa* did not reflect differences during the dry and wet season, the recovery of the bacteria being viable throughout the year, without being affected by the temperature changes or levels of waters system.

Our results coincide with previous studies that show that the bacteria remain stable in the water and that water treatment is not effective in eliminating the MDR bacteria (Sivaraj *et al.*, 2012; Czekalski *et al.*, 2014; Devarajan *et al.*, 2016).

In the study conducted by Devarajan *et al.* (2017), multidrug resistant isolates were identified in all 3 sampling areas of India, Republic of Congo and Switzerland, concluding that water systems may play a role in preserving and dispersing MDRPA back to the human consumption via drinking water, fisheries or agricultural products using surface water for irrigation.

On the other hand, we found that all isolates belonging to antibiotics 3, 4 and 5 (considered MDRPA) had the highest number of *bla* genes encoding metallo-β-lactamases.

Several studies indicate that MDRPA carries *bla* genes encoding MBL, located in gene cassettes within integrons, transposons or plasmids, together with genes encoding resistance to other antibiotic families (Lambert, 2002; Rodríguez *et al.*, 2009). In our study, the isolates carrying bla_{KPC-2} and bla_{VIM-2} were 41.4% and 36.8%, respectively in accordance with epidemiological data indicating that in Colombia, KPC-2 and VIM-2 carbapenemases are the most frequently detected among the clinical isolates of *P. aeruginosa* in Colombian hospitals (Crespo *et al.*, 2004; Vanegas *et al.*, 2014; Correa *et al.*, 2015).

Among the environmental isolates, mostly harboring bla_{VIM-2} and bla_{KPC-2} significantly present during the dry season (58.3% and 41.7%, respectively) and continued to be detected during the wet season (28.4% and 25%, respectively). As suggested

by Scotta *et al.* (2011), environmental bacteria represent a reservoir for the dissemination of clinically relevant β-lactamase genes.

In this study, we found that bla_{KPC-2} was detected mainly among clinical isolates (70.8%), in accordance with epidemiological data in Colombia, the clinical isolates of *P. aeruginosa* harbored bla_{KPC-2} are the main contributors of resistance in Colombian hospitals (Crespo *et al.*, 2004; Vanegas *et al.*, 2014; Correa *et al.*, 2015).

Some reports indicate that ST111 and ST235 of *P. aeruginosa* are associated with the dissemination bla_{VIM-2} and bla_{KPC-2} in Colombia (Correa *et al.*, 2015).

The presence of MDRPA isolates carrying *bla* genes in water systems may be related to uncontrolled elimination in the environment of the chemicals and antibiotics that are often used as β-lactams, which creates a selective pressure on these microorganisms that leads to multiple resistance to drugs with transfer of resistance genes from pathogenic to non-pathogenic strains or vice versa in the environment.

Using the PCR-SSCP analysis, our data shows that there is a great adaptability of *P. aeruginosa* isolates. There are isolates clearly adapted to the aquatic environment (PCR-SSCP fingerprint I and III). Environmental isolates from "Canal Intersector (CVC) Sur" were more closely related among themselves (PCR-SSCP fingerprint I). This channel collects domestic and industrial waste from the southeast of the city and the waters of the Cañaveralejo River.

However, the largest number of isolates were grouped in PCR-SSCP fingerprint II, clinical isolates and environmental isolates from the surface waters of the Melendez River, among others were similar. In addition, the isolates of this group had the highest number of *bla* genes, especially bla_{VIM-2} genes. A large number of environmental isolates originating from clinical isolates can remain viable throughout the year in water systems representing a risk, due to their propagation capacity, stability in different environments and resistance mediated by β-lactamases, including carbapenemases such as VIM-2 and KPC-2.

The results of the PCR-SSCP analyzes are consistent with the results of Finnan *et al.* which show that *P. aeruginosa* strains recovered from clinical or environmental sources have a highly conserved genome, that allows to inhabit the widest possible range of environments and confers the ability to cause human infections after relatively infrequent encounters (Finnan *et al.*, 2004).

On the other hand, it can be seen that isolates with the bla_{kpc-2}, $bla_{CTX-M-9}$, bla_{IMP-1} and bla_{VIM-2} genes are found in the three types PCR-SSCP fingerprint, a fact that suggests that the resistance phenotype to β-lactams is the result of several transmission events, suggesting a complex transmission dynamic between hospital and environmental isolates.

As suggested by Wolfgang *et al.* (2003), the genome of *P. aeruginosa* is a dynamic entity in which horizontal gene transfer and recombination, together with gene loss, are a driving force in genetic diversity and play an important role in the evolution of this species. Through this mechanism, a conserved genome can have variability in virulence phenotypes and resistance to antibiotics.

We show here that *P. aeruginosa* is ubiquitous in the city's artificial and natural water systems and that antibiotic-resistant (and, in particular, MDRPA) strains that are released from hospitals into the environment show great ecological efficacy when colonizing and remain for several periods in the city's water systems, especially strains that harboring bla_{VIM} and bla_{KPC-2}.

The appearance of MDRPA in natural and artificial waters is of great concern due to the possible transfer of resistance genes from non-pathogenic strains to pathogenic strains or vice versa in the environment.

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