Carbapenemase genes in clinical and environmental isolates of *Acinetobacter* spp. from Quito, Ecuador

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ABSTRACT

Carbapenem-resistant *Acinetobacter* spp. is associated with nosocomial infections in intensive care unit patients, resulting in high mortality. Although *Acinetobacter* spp. represent a serious public health problem worldwide, there are a few studies related to the presence of carbapenemases in health care facilities and other environmental settings in Ecuador. The main aim of this study was to characterize the carbapenem-resistant *Acinetobacter* spp. isolates obtained from four hospitals (52) and from five rivers (27) close to Quito. We used the disc diffusion and EDTA sinergy tests to determine the antimicrobial susceptibility and the production of metallo β-lactamases, respectively. We carried out a multiplex PCR of *gyrB* gene and the sequencing of partial *rpoB* gene to bacterial species identification. We performed molecular screening of nine carbapenem-resistant genes (bla_{SPM}, bla_{SIM}, bla_{GIM}, bla_{GES}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, and $bla_{OXA-143}$) by multiplex PCR, followed by identification using sequencing of bla_{OXA} genes. Our findings showed that carbapenem-resistant *A. baumannii* were the main species found in health care facilities and rivers. Most of the clinical isolates came from respiratory tract samples and harbored bla_{OXA-23}, $bla_{OXA-366}$, bla_{OXA-72}, bla_{OXA-65}, bla_{OXA-70}, and $bla_{OXA-143-like}$ genes. The river isolates harbored only the bla_{OXA-51} and probably $bla_{OXA-259}$ genes. We concluded that the most predominant type of carbapenem genes among isolates were both bla_{OXA-23} and bla_{OXA-65} among *A. baumannii* clinical isolates.

INTRODUCTION

Acinetobacter species are aerobic, Gram-negative pleomorphic, and non-lactose fermenting bacilli (*Murray, Rosenthal & Pfaller, 2021*) and they are ubiquitous in nature, including human skin (*Almasaudi, 2016*). In the past two decades, multidrug-resistant *Acinetobacter* spp. have become an important cause of health care-associated infections

(HAIs), contributing to increase mortality rates in intensive care units (ICUs) (*Castanheira, Mendes & Gales, 2023*). Of the all *Acinetobacter* species, *A. baumannii* is predominant in clinical settings (*Evans, Hamouda & Amyes, 2013*; *Silva et al., 2022*) although non-*baumannii* species are also considered clinically important (*Al Atrouni et al., 2016a*). The *World Health Organization (2017)* elevated *A. baumannii* to the top of the list of critical pathogens. In Ecuador, *A. baumannii* was the main bacterium associated with HAIs in the ICU in 2010 and 2011 (*Jiménez, 2013*).

Acinetobacter species have high carbapenem resistance by the acquisition of genetic determinants of resistance through horizontal gene transfer (*Castanheira, Mendes & Gales, 2023*). The environment plays a key role in the spread of resistance to clinically relevant antibiotics (*Singer et al., 2016*); the spread of antibiotic resistance genes in river systems is a potential threat to public health (*Wang et al., 2018*). Environmental strains harbor antibiotic resistance mechanisms, serving as reservoirs for resistant elements and some of them could cause HAIs (*Finley et al., 2013*; *Al Atrouni et al., 2016a*). Horizontal gene transfer plays an important role in the transmission antibiotic resistance between non-pathogens and human pathogens (*Wang et al., 2018*). Worldwide, *A. baumannii* has increased the carbapenem resistance in around 90% in the last decade (*Rosenthal et al., 2021*). In Latin America, *Acinetobacter* spp. resistance to imipenem and meropenem varied from 8% to 89% (*Pan American Health Organization, 2021*). In 2010, Ecuador reported that 54% of *A. baumannii* strains were resistant to meropenem and 51% to imipenem (*Labarca et al., 2016*); however, the percentage of carbapenem resistant increased to 70% according to the Antimicrobial Resistance Surveillance Network of Ecuador (*Satán et al., 2023*).

The main mechanism of carbapenem resistance in *Acinetobacter* species is the production of serin-β-lactamases and metallo-β-lactamases enzymes (*Castanheira, Mendes & Gales, 2023*). In South America, the most common enzymes are oxacillinases (OXA) encoded by bla_{OXA-23}, bla_{OXA-72}, bla_{OXA-51}, bla_{OXA-58}, $bla_{OXA-235}$, and $bla_{OXA-143}$ genes (*Escandón-Vargas et al., 2017*; *García-Betancur et al., 2021*). There is scarce information about carbapenem-resistant strains from clinical and environmental settings in Ecuador. In the country, studies reported the presence of bla_{NDM-1}, bla_{OXA-23}, and bla_{OXA-72} genes in *A. baumannii* clinical isolates (*Nuñez et al., 2016*; *Villacís et al., 2019*). Thus, the main aim of this study was to determine the presence of carbapenem-resistant clinical and river *Acinetobacter* spp.

MATERIALS AND METHODS

Clinical isolates

The National Reference Laboratory for Antimicrobial Resistance provided us a total of 52 *Acinetobacter* spp. clinical isolates recovered through the surveillance network from January to December 2015. As part of the standard procedure, the Reference Laboratory get back the *Acinetobacter* isolates from four different hospitals: Hospital Pediátrico Baca Ortiz (H1, $n = 42$), Hospital Pablo Arturo Suárez (H2, $n = 5$), Hospital del Sur Enrique Garcés (H3, $n = 3$), and Hospital San Francisco de Quito (H4, $n = 2$) in Quito (Fig. 1).
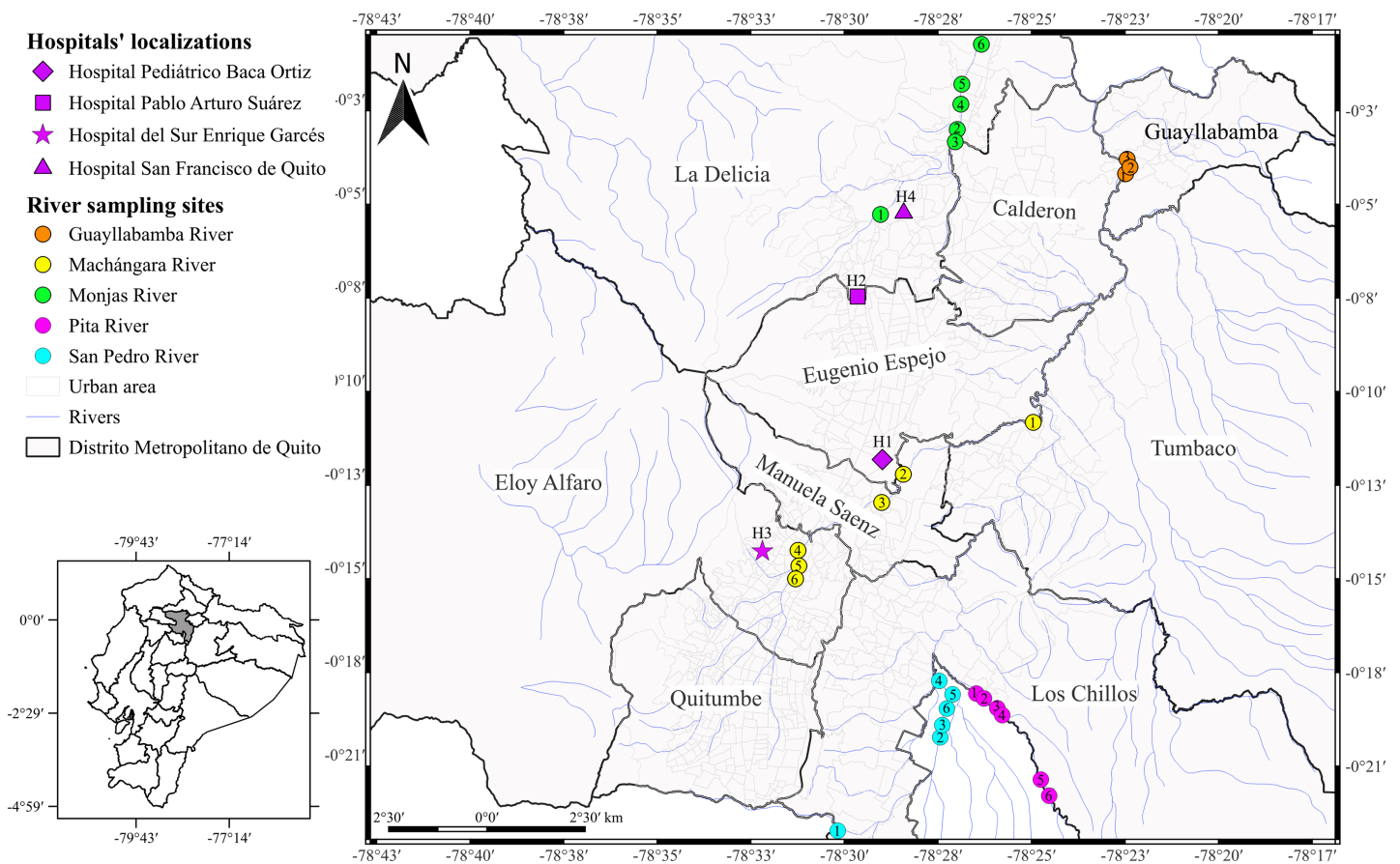
Figure 1 Map of the city of Quito showing sampling locations. *Acinetobacter* spp. clinical isolates recovered through the surveillance network from four hospitals in Quito. The hospitals' localizations are represented by diamond (H1), square (H2), star (H3), and triangle (H4). The river sampling sites are represented by the circles, which indicates the GPS points where we collected the water sample. Rosa de los Ángeles Bayas-Rea made this map.

The Reference Laboratory isolated *Acinetobacter* spp. from the following clinical samples: pleural fluid, cerebrospinal fluid, blood, urine, catheters, secretions, wounds, skin, abscesses, sputum, tracheal aspirates, and unidentified samples recovered from eleven units (ICU, general surgery, traumatology, burn unit, external consultation, neonatal unit, infectious diseases, pediatric unit, internal medicine, emergency, cardiology, and unidentified units). The data from each isolate are detailed in Table S1. The Reference Laboratory previously identified all isolates as *A. baumannii* based on VITEK 2 compact system® (Biomerieux, Marcy-l'Étoile, France).

We inoculated the frozen clinical isolates in sheep blood and MacConkey agars and incubated at 37 °C for 24 h. We used Gram staining, oxidase test, and catalase test to re-confirmed the identity of the isolates. We performed DNA extraction by boiling according to the protocol described by *Cuaical et al. (2012)*; briefly, we boiled an overnight culture diluted in sterile water (500 µl) at 100 °C for 15 min, following centrifugation at 10,000 g for 2 min. We quantified the supernatant by spectrophotometry using the Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C for subsequent amplification. We confirmed species identification by the amplification of

Table 1 Primers used in this study for the identification of *Acinetobacter* spp. and detection of carbapenem-resistant genes.

Target gene	Sequence primer	Size (bp)	References
gyrB	Sp2F: GTTCTTGATCCGAAATTCTCG	490	*Higgins et al. (2007)*
	Sp4R: AACGGAGCTTGTCAGGGTTA		
	Sp4F: CACGCCGTAAGAGTGCATTA	294	
	Sp4R: AACGGAGCTTGTCAGGGTTA		
	Sp1F: GACAACAGTTATAAGGTTTCAGGTG	428	*Higgins et al. (2010a)*
	Sp1R: CCGCTATCTGTATCCGCAGTA		
	Sp3F: GATAACAGCTATAAAGTTTCAGGTGGT	194	
	Sp3R: CAAAAACGTACAGTTGTACCACTGC		
rpoB	Ac696F: TAYCGYAAAGAYTTGAAAGAAG	350	*La Scola et al. (2006)*
	Ac1093R: CMACACCYTTGTTMCCRTG		
	Ac1055F: GTGATAARATGGCBGGTCGT	450	
	Ac1598R: CGBGCRTGCATYTTGTCRT		
bla$_{SIM}$	F: TACAAGGGATTCGGCATCG	570	*Ellington et al. (2007)*
	R: TAAGGCCTGTTCCCATGTG		
bla$_{GES}$	F: ATGCGCTTCATTCACGCAC	863	*Opazo et al. (2012)*
	R: AACTCATCCTGAGCACGGAC		
bla$_{SPM}$	F: AAAATCTGGGTACGCAAACG	271	*Ellington et al. (2007)*
	R: ACATTATCCGCTGGAACAGG		
Bla$_{GIM}$	F: TCGACACACCTTGGTCTGAA	477	*Ellington et al. (2007)*
	R: AACTTCCAACTTTGCCATGC		
bla$_{OXA-23}$	F: GATCGGATTGGAGAACCAGA	501	*Woodford et al. (2006)*
	R: ATTTCTGACCGCATTTCCAT		
bla$_{OXA-24}$	F: GGTTAGTTGGCCCCCTTAAA	246	*Woodford et al. (2006)*
	R: AGTTGAGCGAAAAGGGGATT		
bla$_{OXA-51}$	F: TAATGCTTTGATCGGCCTTG	353	*Woodford et al. (2006)*
	R: TGGATTGCACTTCATCTTGG		
bla$_{OXA-58}$	F: AAGTATTGGGGCTTGTGCTG	599	*Woodford et al. (2006)*
	R: CCCCTCTGCGCTCTACATAC		
bla$_{OXA-143}$	F: TTCTGTCAGTGCATGCTCATC	728	*Opazo-Capurro (2014)*
	R: CAGGCATTCCTTGCTTCATT		
bla$_{OXA-51}$	F: CTTATAAGTCATATGAACATTAAAGC	975	*Heritier, Poirel & Nordmann (2006)*
	R: CTCTATAAAAAGGGATCCGGGCTA		
bla$_{OXA-24}$	F: ATGAAAAAATTTATACTTCCTA TATTCAGC	825	*Jeon et al. (2005)*
	R: TTAAATGATTCCAAGATTTTCTAGC		
bla$_{OXA-23}$	F: GATGTGTCATAGTATTCGTCG	1,058	*Afzal-Shah, Woodford & Livermore (2001)*
	R: TCACAACAACTAAAAGCACTG		

Note:
The primer name, the sequences of primer forward and reverse, the expected size, and reference where the sequences were obtained.

partial RNA polymerase β (*rpoB*) gene and DNA gyrase subunit B (*gyrB*) gene according to *La Scola et al. (2006)* and *Higgins et al. (2007, 2010a)*, respectively. The primers selected are detailed in Table 1.

River water collection and bacterial identification

We collected the water samples from five principal rivers: Monjas (MO, $n = 6$), Machángara (MA, $n = 6$), San Pedro (S, $n = 6$), Pita (P, $n = 6$), and Guayllabamba (G, $n = 3$) in March and April 2016 (Fig. 1 and Table S1). We chose those rivers for two reasons: (i) the four rivers are the main ones that run through Quito, all of them flow into the Guayllabamba River, and (ii) sewage is released into the city's rivers without prior treatment. In addition, we chose the number of water collection points based on the accessibility and proximity to the urban center. We collected 100 ml of water samples at each river point and transported to the laboratory with ice packs according to *Zhang et al. (2013)* with few modifications. Subsequently, we centrifuged the water samples at 1,000 g for 10 min at room temperature, resuspended the pellets with 5 ml of fluid thioglycollate medium, and incubated at 37 °C overnight. We inoculated thirty microliters of cultured sample onto CHROMagar *Acinetobacter* plates with antimicrobial selective supplement (CR 202; CHROMagar Company, Paris, France) and incubated at 37 °C overnight. We selected all colonies with red pigmentation and identified through morphological and conventional biochemical tests used for the clinical isolates. We identified the positive potential isolates using VITEK-2 compact system® (Biomerieux, Marcy-I'Étoile, France). We confirmed the species identification by the amplification of DNA gyrase subunit B (*gyrB*) gene according to *Higgins et al. (2007, 2010a)*.

Antimicrobial susceptibility test

We use the disc diffusion method and the EDTA sinergy test to determinate the antimicrobial susceptibility and the production of metallo β-lactamases of clinical and river *Acinetobacter* spp. isolates (*Clinical Laboratory Standards Institute, 2022*). We spread a concentration corresponding to 0.5 of the McFarland scale of each isolate on Muller Hinton agar plates and incubated at 37 °C overnight. We included the following eleven antimicrobial susceptibility test discs (OXOID, Waltham, MA, USA): ampicillin/sulbactam (20 μg), ceftazidime (30 μg), ciprofloxacin (10 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), amikacin (30 μg), cefepime (30 μg), trimethoprim/sulfamethoxazole (25 μg), piperacillin/tazobactam (110 μg), and tobramycin (30 μg). We interpreted the resistance based on the susceptibility breakpoints of the *Clinical Laboratory Standards Institute (2022)*. To assess the synergistic inhibition, we included an ethylenediaminetetraacetic (EDTA) disc (5 mg) with two carbapenem discs, imipenem (10 mg) and meropenem (10 mg), located at 2 cm from the center of the EDTA disc.

Molecular identification of carbapenems-resistant genes

We performed two different multiplex PCRs to screen the presence of carbapenems-resistant genes including six genes encoding enzymes type serine β-lactamases (bla_{GES}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, and $bla_{OXA-143}$) and three genes encoding metallo-β-lactamases (bla_{SPM}, bla_{SIM}, and bla_{GIM}) using specific primers

Table 2 Single and multiplex PCR reaction conditions.

Carbapenemase class	Gene	Thermal cycling conditions					Reference
		Initial denaturation	35 cycles			Final extention	
			Denaturation	Annealing	Extention		
Multiplex PCR 1	bla_{SPM} bla_{GES} bla_{SIM} bla_{GIM}	94 °C for 5 min	94 °C for 30 s	52.8 °C for 40 s	72 °C for 50 s	72 °C for 10 min	*Ellington et al. (2007)*
Multiplex PCR 2	bla_{OXA-23} bla_{OXA-24} bla_{OXA-51} bla_{OXA-58} $bla_{OXA-143}$	94 °C for 5 min	94 °C for 30 s	53 °C for 30 s	72 °C for 30	72 °C for 10 min	*Woodford et al. (2006)*
Sequencing	bla_{OXA-51}	94 °C for 2 min	94 °C for 1 min	52 °C for 30 s	72 °C for 1 min	72 °C for 10 min	*Heritier, Poirel & Nordmann (2006)*
	bla_{OXA-23}	94 °C for 5 min	94 °C for 30 s	52 °C for 40 s	72 °C for 50 s	72 °C for 10 min	*Afzal-Shah, Woodford & Livermore (2001)*
	bla_{OXA-24}	94 °C for 5 min	94 °C for 30 s	50 °C for 45 s	72 °C for 1 min	72 °C for 10 min	*Jeon et al. (2005)*
	$bla_{OXA-143}$	94 °C for 5 min	94 °C for 30 s	53 °C for 30 s	72 °C for 30	72 °C for 10 min	*Woodford et al. (2006)*

Note:
The different thermal cycling conditions for various molecular analyses in this study.

previously described (*Ellington et al., 2007*; *Woodford et al., 2006*) (Table 1). We achieved a PCR amplification in a final volume of 25 µl containing 1X of Master Mix Green GoTaq® (12.5 µl) (Promega, Madison, WI, USA), 10 µM of forward and reverse primers (0.5 µl), and 50 ng DNA (1 µl). To each PCR assay, we put positive, internal, and negative controls. Both multiplex PCR reaction conditions are detailed in Table 2. We identified all PCR products through electrophoresis in a 2% agarose gel stained with GelstarTM Nucleic Acid Gel Stain 10000 X staining (Lonza, Rockland, ME, USA) and visualized using Gel Doc XR system (Bio-Rad, Hercules, CA, USA).

We performed a conventional PCR to amplified the entire sequence of the following genes bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, and $bla_{OXA-143}$ genes using primers previously reported (*Heritier, Poirel & Nordmann, 2006*; *Afzal-Shah, Woodford & Livermore, 2001*; *Jeon et al., 2005*; *Woodford et al., 2006*) (Table 1), following the conditions detailed in Table 2. We cleaned the PCR products using exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT®). We send both DNA strands of all positive bla_{OXA} genes for sequencing to Macrogen (Seoul, Korea). We aligned manually both forward and reverse sequences for editing and cleaning to obtain a single consensus sequence with the Clustal W program implemented in the MEGA 7 software (*Kumar, Stecher & Tamura, 2016*). We performed the identification of sequences obtained using a basic local alignment search tool (BLAST) (*Altschul et al., 1997*). We determined the number of sequences of bla_{OXA-51} and bla_{OXA-23} genes with DnaSP v.5.10 software (*Rozas et al., 2010*).
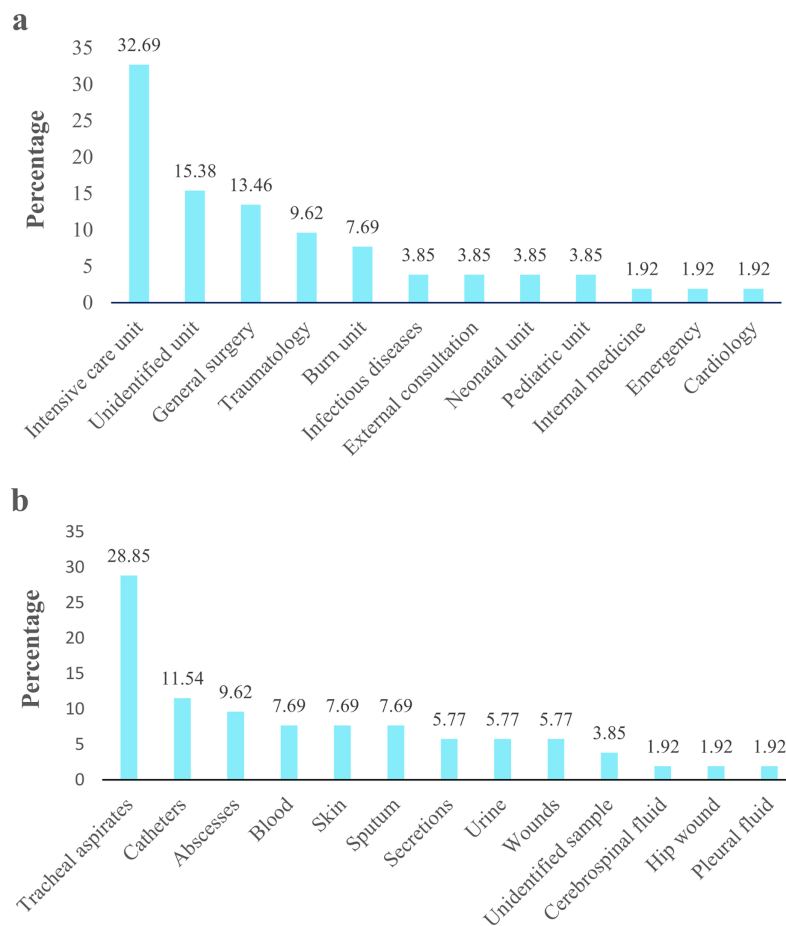
Figure 2 *Acinetobacter* spp. clinical isolates obtained from four hospitals in Quito. The percentage of *Acinetobacter* spp. clinical isolates. (A) Isolates obtained from different hospital service units. (B) Isolates recovered from clinical specimens.

RESULTS

Identification of *Acinetobacter* spp.

Acinetobacter spp. clinical isolates originated mainly proceeded from pediatric population (77%; 40/52), followed by the adult population (19.2%; 10/52). Isolates derived principally from patients admitted in UCI 32.69% (17/52), followed by unidentified units 15.38% (8/52) and general surgery 13.46% (7/52) (Fig. 2A). According to the clinical specimens, the isolates came from tracheal aspirates 28.85% (15/52), followed by catheters 11.54% (6/52) and abscesses 9.62% (5/52) (Fig. 2B). The majority of clinical isolates were identified as *A. baumannii* (96.15% 50/52) and *A. pittii* (3.85%; 2/52) according *rpoB* gene. Only the identity of *A. pittii* (3.85%; 2/52) was confirmed with both markers, *rpoB* and *gyrB* genes. *A. baumannii* was found in all type of clinical specimen from all hospital units while *A. pittii* was found only in tracheal aspirates from UCI. The biochemical and molecular identification of each isolate is detailed in Tables S2 and S3. The nucleotide and amino sequences of partial *rpoB* gene are detailed in Informations S1 and S2.
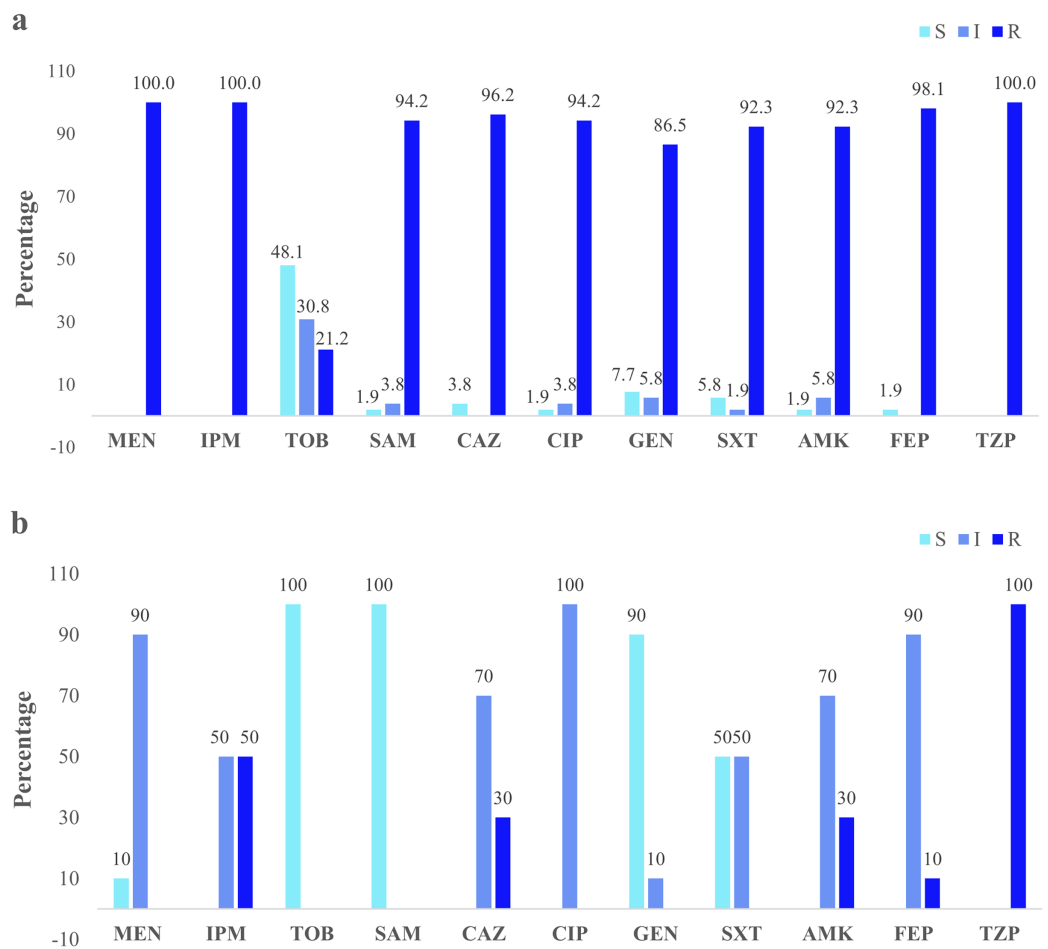
Figure 3 Clinical and environmental isolates susceptibility profile. The percentages of clinical isolates that were resistant, intermediate, or sensitive to each antibiotic. (A) Clinical isolates susceptibility profile, (B) river isolates susceptibility profile. Acronyms: CIP, ciprofloxacin; GEN, gentamycin; SXT, trimethoprim/sulfamethoxazole; AMK, amikacin; FEP, cefepime; MEN, meropenem; IMP, imipenem; TOB, tobramycin; SAM, ampicillin/sulbactam; CAZ, ceftazidime; TZP, piperacillin/tazobactam; R, resistant; I, intermediate; S, sensitive.

We submitted all *rpoB* sequences to GenBank with assigned accession numbers OP796738–OP796781.

A total of 27 water samples were collected from five rivers. Twenty-two samples presented suggestive *Acinetobacter* isolates, four samples (RMO3, RS5, RP4, and RG3) showed no growth, and one sample (RMO4) had isolates not related to *Acinetobacter* spp. We recovered twenty-six suggestive isolates from San Pedro (2), Machángara (4), Pita (1), and Monjas (3) rivers, of which only 38.5% (10/26) were identified as *Acinetobacter* spp. based on VITEK 2 compact® (Biomerieux, Marcy-I'Étoile, France) and as *A. baumannii* based on *gyrB* gene amplification. The remaining corresponded to other genera (*Pseudomonas aeruginosa*: 34.6%, *Stenotrophomonas maltophila*: 19.3%, *Burkkolderia cepacia*: 3.8%, and *Moraxella* group: 3.8%). The biochemical and molecular identification of each isolate is detailed in Tables S2 and S3.

Phenotypic antimicrobial susceptibility

Clinical isolates presented the higher resistance (100%) to the three antibiotics: imipenem, meropenem, and piperacillin/tazobactam. Forty-eight percent were susceptible to tobramycin (Fig. 3A). The river isolates displayed an antimicrobial profile that varied between resistant and intermediate to eight antibiotics tested. Nevertheless, the main resistance was to piperacillin/tazobactam (100%). All of them were susceptible to tobramycin and ampicillin/sulbactam (100%) (Fig. 3B). According to the synergistic inhibition, both river and clinical isolates did not present metallo-β-lactamases enzymes. The antimicrobial susceptibility profile of the clinical and river isolates is detailed in Table S2.

Molecular identification of carbapenem-resistance genes

According the screening of carbapenem-resistance genes, clinical isolates presented the following genes: bla_{SIM}, bla_{SPM}, bla_{GES}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, and $bla_{OXA-143}$; whereas, seven river isolates showed bla_{OXA-51} gene (Table S3). The bla_{SIM} gene, co-harbored with the bla_{OXA-72} and $bla_{OXA-143}$ genes, was the least frequently detected (1.9%; 1/52; 15-1175) in *A. pittii*. The bla_{SPM} gene, co-harbored with the bla_{OXA-51} and bla_{OXA-23} genes, was detected in 13.46% (7/52; 15-0122, 15-0591, 15-690, 15-780, 15-795, 15-853, 15-1,138) in *A. baumannii*. The bla_{GES} gene, co-harbored with the bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-72}, $bla_{OXA-143}$, and bla_{SIM} genes was present in 11.53% (6/52) in *A. baumannii* (15-0669, 15-690, 15-853, 15-900) and *A. pittii* (15-691, 15-1,175). The bla_{OXA-23} gene, co-harbored with the bla_{OXA-51} gene, was found in 98.07% (51/52) present in *A. baumannii*. The bla_{OXA-24} gene, co-harbored with both bla_{OXA-23} and bla_{OXA-51} genes, was present in 9.6% (5/52) in *A. baumannii* (15-0659, 15-0985, 15-1064, and 15-1367) and in *A. pittii* (15-0691). The $bla_{OXA-143}$ gene was the least frequently detected (1.9%; 1/52; 15-1,175); this gene was co-harbored with the bla_{OXA-24} gene in *A. pittii*.

Carbapenem-resistance genes sequences analysis

We reported 47 bla_{OXA-51} sequences: 42 complete sequences of 813 bp and five partial sequences of 340 bp from *A. baumannii* clinical isolates. Of bla_{OXA-51} sequences, we identified two genes encoding OXA-65 ($n = 44$), OXA-70 ($n = 2$) and one unidentified (MF594746). Forty complete sequences (MF594725–MF594728, MF594730–MF594737, MF594735, MF594739, MF594741–MF594745, OP554146–OP554168) and four partial sequences (MF594729, MF594734, MF594736, and MF594737) shared 100% of nucleotide identity with bla_{OXA-65} of *A. baumannii* (GenBank accession number CP033869.1); whereas two entire sequences (MF594740 and MF594747) shared 100% nucleotide identity with bla_{OXA-70} of *A. baumannii* (GenBank accession number NG_049811.1).

We found 48 bla_{OXA-23} sequences: 42 complete sequences of 820 bp and six partial sequences of 471 bp from *A. baumannii* clinical isolates. Of bla_{OXA-23} sequences, we identified two genes encoding OXA-23 ($n = 38$) and OXA-366 ($n = 10$). Thirty-two entire sequences (MF594756–MF594759, MF594762–MF594767, MF594769, MF594772–MF594777, OP554169, OP554170, OP554172–OP554178,

										Polymorphic sites																			
	2	5	9	1	1	1	1	1	1	1	3	4	4	5	8	**9**	1	1	1	1	1	1	1	1	1	1	2	2	2	2
				1	2	3	5	7	8	9	9	4	5	1	0	**4**	0	1	2	2	3	4	4	6	6	9	0	0	2	3
																	3	5	0	3	8	4	9	0	6	0	4	6	5	0
Aba OXA-143 NG_049441.1	S	T	D	F	H	T	N	Q	H	E	K	S	T	T	T	**M**	D	E	T	D	N	D	V	V	F	F	D	T	A	I
Aba OXA-143 KX34928.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Aba OXA-253 NG_049548.1	.	.	.	S	D	I	D	.	Q	G	E	.	.	A	.	.	.	.	.	.	.	.	.	.	L	V	.	I	P	L
Aba OXA-253 KF824909.1	.	.	.	S	D	I	D	.	Q	G	E	.	.	A	.	.	.	.	.	.	.	.	.	.	L	V	.	I	P	L
Aba OXA-231 NG_049527.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
Aba OXA-231 JQ676953.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
Aba OXA-182 NG_049483.1	.	.	.	.	.	I	.	K	.	.	E	.	S	V	N	.	E	D	I	N	.	.	I	.	L	V	.	S	S	.
Aba OXA-182 JQ964242.1	.	.	.	.	.	I	.	K	.	.	E	.	S	V	N	.	E	D	I	N	.	.	I	.	L	V	.	S	S	.
Api OXA-255 NG_049550.1	T	N	K	S	D	I	D	.	.	.	E	R	I	V	.	.	.	.	.	.	S	N	.	A	L	V	.	.	P	L
Api OXA-499 NG_049775.1	.	N	N	S	D	I	D	.	.	.	E	R	I	V	.	.	.	.	.	.	.	N	.	A	L	V	.	.	P	L
Api OXA-499 KX828713.1	.	N	N	S	D	I	D	.	.	.	E	R	I	V	.	.	.	.	.	.	.	N	.	A	L	V	.	.	P	L
Api **OXA-143 This study**	.	N	N	S	D	I	D	.	.	.	E	R	I	V	.	**V**	.	.	.	.	.	N	.	A	L	V	.	.	P	L

Figure 4 Alignment of OXA-143 amino acid sequences. The polymorphic sites at the amino acid sequence level. The identity of the sequences was compared to the OXA-143 variant (GenBank protein number access WP_063861042.1 or its nucleic number access NG_049441.1). Numbers in the heading row indicate the base pair position of the polymorphic sites. Dots (.) show the identity with the Aba OXA-143. Acronyms: Aba, *Acinetobacter baumannii*; Api, *A. pittii*.

OP554180–OP554182, OP554185–OP554187, OP554189, OP554191–OP554193) and six partial sequences (MF594755, MF594760, MF594761, MF594768, MF594770, and MF594771) shared 100% of nucleotide identity with bla_{OXA-23} of *A. baumannii* (GenBank accession number CP110468.1). Ten complete sequences (MF594757, MF59474–MF59476, OP554171, OP554179, OP554183, OP554184, OP554188, and OP554190) shared 100% nucleotide identity with $bla_{OXA-366}$ of *A. baumannii* (GenBank accession number NG_049659.1).

We reported five $bla_{OXA-24/40}$ entire sequences of 817 bp (MF594778, MF594779, MF594781, MF594782, and MF594783) and one partial sequence of 204 bp (MF594780). Of $bla_{OXA-24/40}$ sequences, we identified one gene encoding OXA-72 present in three *A. baumannii* and two *A. pitti* clinical isolates that shared 100% of nucleotide identity with bla_{OXA-72} of *A. baumannii* (GenBank accession number MN495626) and also *A. pittii* (GenBank accession number MN481287.1).

Sequencing of the $bla_{OXA-143}$ PCR product obtained from *A. pitti* clinical isolates identified gene encoding OXA-499. Our $bla_{OXA-143}$ sequence (MF594724) shared 99% nucleotide and amino acid identity with $bla_{OXA-499}$ of *A. pittii* (GenBank accession numbers NG_049775.1 and ALM96709.1). The nucleotide sequence presented one specific change in nucleotide 280, first position of the codon (GTG) (ATG), resulting in a valine (V) to methionine (M) change in position 94 (Fig. 4).

Sequencing of the bla_{OXA-51} PCR products obtained from *A. baumannii* river isolates identified two genes encoding OXA-65 and OXA-259. Four partial sequences (MF594748–MF594751) from three rivers (Pita, Machángara, and San Pedro) shared

100% of nucleotide identity with bla_{OXA-65} of *A. baumannii* (GenBank accession number OL961415.1) and three partial sequences (MF594752–MF594754) from Machángara River shared 100% of nucleotide identity with $bla_{OXA-259}$ of *A. baumannii* (GenBank accession number CP053098.1).

The nucleotide and amino sequences of bla_{OXA} genes are detailed in the Informations S3 to S10. We submitted all sequences to GenBank with assigned accession numbers MF594724–MF594783, OP554146–OP554193.

DISCUSSION

Acinetobacter spp. in clinical and river isolates

Our findings indicated that *Acinetobacter baumannii* is the most common species found in clinical isolates from tracheal aspirates from ICU. Similarly, studies reported high rates of *baumannii* species in ICU samples elsewhere (Qin et al., 2021; Bakhshi et al., 2022). Previous Ecuadorian studies reported high percentages of *A. baumannii* from respiratory tract samples, bronchial secretions, or tracheal aspirates (Jiménez, 2013; Nuñez et al., 2016; Villacís et al., 2019). On the other hand, we found *A. baumannii* in the most polluted river environments, Machángara and Monjas rivers (Yungán, 2010; Roldós, 2015). Similar, some studies found these bacteria in river water as well as sewage water (Hrenovic et al., 2016; Kisková et al., 2023). Pathogens of clinical origin as *A. baumannii* can reach aquatic environments as a result of different human activities, such as sewage water discharge directly into the river without pretreatment (Silva et al., 2016). *Acinetobacter* spp. survive in wastewater contributing to the spread clinical antibiotic resistance genes in aquatic systems (Kisková et al., 2023; Tobin et al., 2024). Particularly, hospital sewage water is considered a major source of multidrug-resistant pathogenic bacteria and antibiotic residues (Zhang et al., 2013). Contrary, we did not isolate any *Acinetobacter* spp. from the Guayllabamba River probably due to the distance (2 km), the collection points were far away from the wastewater reservoirs of urban areas and the bacterial concentrations decreases and distribution changes (Zhang et al., 2009).

Carbapenem-resistant *Acinetobacter* spp.

In our study, the clinical and environmental *Acinetobacter* isolates showed different susceptibility profiles. Clinical isolates exhibited a high rate of resistance to imipenem and meropenem; while, river isolates displayed a low resistance only to imipenem. In general, clinical isolates have high resistance to both antibiotics (Cortivo et al., 2015). The percentage of resistance found in this study was higher compared with an Ecuadorian study (Jiménez, 2013). River isolates are usually more sensitive compared to clinical isolates. The difference in the patterns of resistance found can be due to various factors such as, biological conditions, virulence factors, epidemiology, or place of origin (Karmostaj, Najar & Salmanian, 2013). In general, it is expected that the antibiotic concentration is lower in aquatic environments than in clinical environments (Zhang et al., 2009; Zhang et al., 2013). In clinical environments, antibiotics (in particular broad-spectrum antibiotics) are supplied continuously, promoting selective pressure in clinical isolates. Following this, the transmission of genetic determinants among clinical

isolates could take place through horizontal gene transfer (*Zhang et al., 2009*; *Castanheira, Mendes & Gales, 2023*). Nonetheless, the possibility of clonal dissemination as a mean of increasing antibiotic resistance, should not be discarded. This situation generates a faster antibiotic resistance evolution in clinical isolates than in environmental isolates (*Zhang et al., 2013*). However, the susceptibility results of environmental samples should be treated with caution, as these profiles were based on clinical standards (CLSI) and a small sample size ($n = 10$).

Carbapenemases genes in *Acinetobacter* spp.

The high percentage of carbapenem-resistance found in clinical isolates may be increased due to the presence of bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-72}, and $bla_{OXA-143}$ genes. According to some studies, the association of bla_{OXA-23} and bla_{OXA-51} genes as the main cause for carbapenem resistance (*i.e.*, *Martínez & Mattar, 2012*; *Teixeira et al., 2013*; *Cortivo et al., 2015*). The bla_{OXA-72} gene increases its resistance activity in the presence of imipenem and ampicillin/sulbactam (*Kuo et al., 2013*). The $bla_{OXA-143}$ and bla_{OXA-72} genes in association with intrinsic mechanisms as the alteration of the of the outer membrane proteins promotes a high resistance to meropenem (*Mostachio et al., 2012*; *Sen & Joshi, 2015*). The low percentage of carbapenem-resistance found in environmental isolates may be explained by the absence of bla_{OXA} genes. It is necessary the presence of insertion elements to promote overexpression of the bla_{OXA-51} gene since this gene it generates a weak resistance (*Higgins, Zander & Seifert, 2013*; *Teixeira et al., 2013*; *Sen & Joshi, 2015*). The bla_{OXA-51} overexpression would be linked to the resistance to imipenem in *A. baumannii* river isolates (*Martínez & Mattar, 2012*; *Evans, Hamouda & Amyes, 2013*). It would be important to know about possible IS*Aba1* insertions upstream of the gene or alternative genes conferring resistance in the five isolates with resistance phenotype.

We found a high percentage of bla_{OXA-23} and bla_{OXA-51} genes in *A. baumannii* and *A. pittii* clinical isolates. The high percentage of bla_{OXA-23} gene found in *A. baumannii*, is related to its distribution worldwide since a single sequence was found in the study. The bla_{OXA-23} gene distribution has been associated with a clonal spread (*Higgins et al., 2010b*; *Labarca et al., 2016*). In the other hand, bla_{OXA-51} gene has been reported principally in *baumannii* species (*Lee et al., 2012*; *Teixeira et al., 2013*; *Périchon et al., 2014*). Since bla_{OXA-51} gene can be found in the chromosome (*Poirel, Naas & Nordmann, 2010*), some authors consider it as an intrinsic marker for *A. baumannii* identification (*Higgins et al., 2010b*; *Périchon et al., 2014*). However, the gene may be in transposons and plasmids (*Liu et al., 2015*; *Wang et al., 2015*; *Sennati et al., 2016*). Its dissemination among other *Acinetobacter* species from different environments is possible, suggesting that the dissemination may not be by clonal strains (See *Labarca et al., 2016*).

Our findings detected two different bla_{OXA-51} variants OXA-65 and OXA-70 were circulating in the four hospitals; while, in rivers probably are OXA-65 and OXA-259, suggesting that the genes were under selective pressure. Similarly, previous studies OXA-65 was reported in *A. baumannii* clone ST79 from Guayaquil (*Rodríguez et al., 2016*). In Latin America, is one of the variants found in *A. baumannii* in Brazil, Argentina, Paraguay, Mexico, and Honduras (*Rodríguez et al., 2016*; *Nodari et al., 2020*;

Graña-Miraglia et al., 2020). There is not report about the presence of OXA-70 neither OXA-259 variant in South America. The information about OXA-70 variant is scare worldwide. OXA-70 has been reported in Hong Kong, Malaysia, Ghana, and Honduras (*Alattraqchi et al., 2020*; *Ayibieke et al., 2020*; *Brown & Amyes, 2005*; *Galac et al., 2020*). OXA-70 belong to the most diverse collection of class D carbapenemases, subgroup 3 that is the major mechanism of carbapenem resistance in *A. baumannii* (*Brown & Amyes, 2005*). OXA-259 variant has been reported in *A. baumannii* clinical isolates from China, Tanzania, and Thailand (*Jia et al., 2019*; *Moyo et al., 2021*; *Chukamnerd et al., 2022*) as well in bulk tank milk in Germani (*Sykes et al., 2023*). The variant OXA-259 is located on the chromosome of *A. baumannii* and constitute an intrinsic oxacillinase genes with low-level carbapenemase activity that naturally occur (*Chukamnerd et al., 2022*), due there is a not ISA$_{balike}$ elements (*Fedrigo et al., 2022*). To the best of our knowledge, this is the first report of the OXA-70 and OXA-259 in *A. baumannii* in Ecuador.

Our findings showed the presence of $bla_{OXA-366}$ gene in both species *A. baumannii* and *A. pittii* clinical isolates from Ecuador. The information about this variant is scare. GenBank previously reported a sequence of $bla_{OXA-366}$ in *A. baumannii* from USA. Whereas the OXA-366 variant has been reported also in *A. baumannii* clinical isolates in Iran (*Bahador et al., 2015*). To the best of our knowledge, this is the first report of the OXA-366 in *A. baumannii* and *A. pittii* in South America.

The bla_{OXA-72} gene co-harbored with bla_{OXA-23} and bla_{OXA-51} genes was identified in low levels in *A. baumannii* and *A. pittii*. This variant was documented previously in *A. baumannii* clinical isolates from Guayaquil (*Nuñez et al., 2016*; *Rodríguez et al., 2016*) and from neighboring countries Colombia, Peru, and Brazil (*Saavedra et al., 2014*; *Saavedra et al., 2017*; *Werneck et al., 2011*; *de Sá Cavalcanti et al., 2013*; *Levy-Blitchtein et al., 2018*; *Nodari et al., 2020*) and in *A. pittii* from Colombia (*Montealegre et al., 2012*), Brazil (*Brasiliense et al., 2019*), European and Asian countries (*Al Atrouni et al., 2016b*; *Bonnin et al., 2014*). The presence of this gene in two species may be due to the bla_{OXA-72} gene spreads through horizontal gene transfer mediated by plasmids (*Montealegre et al., 2012*; *Kuo et al., 2013*; *Nuñez et al., 2016*; *Rodríguez et al., 2016*). To the best of our knowledge, this is the first report of the bla_{OXA-72} gene in *A. pittii* in Ecuador.

This is also the first report of the $bla_{OXA-143}$ gene in *A. pittii* clinical isolates from Ecuador. In South America, this gene and its variants (OXA-231, OXA-253, and OXA-255) have been reported in *A. baumannii* clinical isolates in Brazil (*Higgins et al., 2009*; *Gionco et al., 2012*; *Girlich et al., 2014*), Perú (*Levy-Blitchtein et al., 2018*), and Colombia (*Saavedra et al., 2017*). The variant found in this study is very different from OXA-143 variants reported in *A. baumannii* from Brazil, Honduras, and Korea (*Higgins et al., 2009*; *Zander et al., 2014*; *Kim et al., 2010*). Interestingly, the sequence alignment showed a high similarity between our variant with two OXA-143 variants (OXA-499 and OXA-255) described to *A. pittii* in Korea (*D'Souza et al., 2017*) and United States (*Zander et al., 2014*). In particular, the difference between the variant reported in this study differs by a change in amino acid sequence with OXA-499 reported by *D'Souza et al. (2017)*. To the best of our knowledge, this is the first report of a variant similar to OXA-499 in *A. pittii* in South America.

CONCLUSIONS

Overall, our results showed that the most of the *Acinetobacter* spp. clinical isolates came from tracheal aspirates from ICU. Our findings showed that carbapenem-resistant *A. baumannii* were the main species found in the study. We reported the presence of bla_{OXA-65}, bla_{OXA-70}, bla_{OXA-72}, bla_{OXA-23}, $bla_{OXA-366}$, and bla_{OXA143}-carrying multidrug-resistant genes in *baumannii* and no *baumannii* species. We report a new possible variant of OXA-143 circling in different hospitals in Quito and the possible presence of OXA-259 in aquatic environment. The presence of *A. baumannii* in the rivers increases the concern, since the resistance can be disseminated, polluting water sources, and promoting community acquired outbreaks.

To our knowledge, this is the first study that has characterized the carbapenemases genes in *Acinetobacter* spp. carbapenem-resistant isolates from four hospitals and five rivers in Quito. No bla_{OXA-65}, bla_{OXA-70}, bla_{OXA-72}, bla_{OXA-23}, $bla_{OXA-366}$, or bla_{OXA143}-carrying multidrug-resistant *baumanii* and no *baumannii* species have been described in Quito so far.

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Nicole Sotomayor performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, water sample collection, and approved the final draft.
- José Eduardo Villacis conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Noela Burneo conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, water sample collection, and approved the final draft.
- Jorge Reyes conceived and designed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Sonia Zapata conceived and designed the experiments, authored or reviewed drafts of the article, funding granted by the USFQ necessary for the sequencing of the complete genes bla_{OXA-51}, bla_{OXA-23}, and bla_{OXA-24}, and approved the final draft.
- Rosa de los Ángeles Bayas-Rea conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The sequences are available at GenBank: MF594724–MF594783, OP796381. 1–OP796781.1, OP554146.1–OP554193.1.

The raw measurements are available in the Supplemental Files.

Supplemental Information

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