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## Brief Communication

# Detection and molecular characterization of $\beta$ -lactamase genes in clinical isolates of Gram-negative bacteria in Southern Ecuador



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### ABSTRACT

This work performed a phenotypic and genotypic characterization of 79 clinical isolates of *Enterobacteriaceae* and *Pseudomonadaceae* collected in hospitals of Southern Ecuador in 2013. Our results showed a high incidence of  $\beta$ -lactamases and ESBLs with *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> as the prevalent genes, respectively. By direct sequencing of PCR amplicons, the different  $\beta$ -lactamases and variants of the genes were also distinguished. Our results revealed a predominance of TEM-1  $\beta$ -lactamase and the presence of different CTX-M variants with a prevalence of CTX-M-15. Two infrequent CTX-M variants in South America were also identified. To the best of our knowledge, this is one of the first studies describing the genetic characteristics of  $\beta$ -lactamases in Ecuador.

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Since the discovery of antibiotics, many pathogens which had been considered defeated have continued to develop resistance to these drugs. The emergence of resistant microorganisms to antibiotics is a problem that has extended globally.<sup>1,2</sup> This phenomenon is widespread in hospitals mainly because of the large and often inappropriate use of antimicrobials. However, due to the bacteria's ability to exchange genetic information, environmental and nosocomial pathogens have developed the same resistance mechanisms, since most resistance genes have their probable origin in environmental microorganisms.<sup>3</sup> Resistant pathogens to most antibiotics

have already been described, but in particular it is of great impact the resistance to  $\beta$ -lactams. Resistance to these antimicrobial agents is due to the production of  $\beta$ -lactamases, and the genes encoding these enzymes have been found worldwide mainly in Gram-negative bacteria, prevalently in *Enterobacteriaceae* and *Pseudomonas* spp.<sup>4,5</sup>

Among  $\beta$ -lactamases, extended-spectrum  $\beta$ -lactamases (ESBLs), AmpC-type  $\beta$ -lactamases, and carbapenemases are of great concern. In particular ESBLs are the most abundant in *Enterobacteriaceae*, with more than 600 natural variants (<http://www.lahey.org/Studies/>), and with a prevalence of

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**Table 1 – Resistance rate for the clinical isolates.**

Antimicrobial agent-disk concentration	Number (%) of resistant isolates		
	$\beta$ -Lactamase/ESBL (n = 73)	AmpC (n = 6)	Total (n = 79)
AMK-30 $\mu$ g	9 (11%)	0 (0%)	9 (11%)
AMC-20/10 $\mu$ g	19 (25)	2 (2%)	21 (27%)
AMP-10 $\mu$ g	50 (63%)	1 (1%)	51 (65%)
SAM-10/10 $\mu$ g	47 (59%)	1 (1%)	48 (61%)
ATM-30 $\mu$ g	30 (38)	1 (1%)	31 (39%)
FEP-30 $\mu$ g	13 (16%)	0 (0%)	13 (16%)
CTX-30 $\mu$ g	58 (73%)	0 (0%)	58 (73%)
FOX-30 $\mu$ g	17 (22%)	2 (2%)	19 (24%)
CAZ-30 $\mu$ g	18 (18%)	0 (0%)	18 (23%)
CIP-5 $\mu$ g	52 (66%)	0 (0%)	52 (66%)
IMP-10 $\mu$ g	7 (9%)	0 (0%)	7 (9%)
MEM-10 $\mu$ g	0 (0%)	0 (0%)	0 (0%)
NET-30 $\mu$ g	51 (65%)	1 (1%)	52 (66%)
PTZ-100/10 $\mu$ g	4 (5%)	0 (0%)	4 (5%)

Abbreviations: AMK, amikacin; AMC, amoxicillin/clavulanic acid; AMP, ampicillin; SAM, ampicillin/sulbactam; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; FOX, ceftazidime; CAZ, ceftazidime; CIP, ciprofloxacin; IMP, imipenem; MEM, meropenem; NET, netilmicin; PTZ, piperacillin/tazobactam.

CTX-M family that became predominant over TEM and SHV during the first decade of the 21st century.<sup>6</sup>

As far as we know, there is a lack of detailed reports characterizing the  $\beta$ -lactamase spread in Ecuador.<sup>7,8</sup> In this work a phenotypic and molecular characterization of  $\beta$ -lactamases was performed in clinical pathogens isolated in two main public hospitals of the Loja province, Ecuador.

The clinical isolates of *Enterobacteriaceae* and *Pseudomonas aeruginosa* were obtained from patients of Ygnacio Monteros and IESS Hospitals. The samples were collected between May and November 2013, while the bacterial cultures were grown in agar-MacConkey Petri dishes, identified by traditional biochemical testing and validated by Microgen™ Gna + B-ID system.

Antimicrobial susceptibility testing was performed by Kirby-Bauer disk diffusion procedure according to CLSI guidelines.<sup>9</sup> The isolates were tested for susceptibility to amikacin (AMK), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), ampicillin/sulbactam (SAM), aztreonam (ATM), cefepime (FEP), cefotaxime (CTX), ceftazidime (CAZ), ciprofloxacin (CIP), imipenem (IMP), meropenem (MEM), netilmicin (NET), and piperacillin/tazobactam (PTZ). The investigation of phenotypic production of ESBL and AmpC-type  $\beta$ -lactamase was performed by techniques of double disk synergism (DDST) on Mueller Hinton agar and combination disk method. In particular for ESBL detection, DDST was performed on agar with AMC-20/10  $\mu$ g disk positioned 25 mm (center-to-center) away from disks containing CAZ-30  $\mu$ g, CTX-30  $\mu$ g, FEP-30  $\mu$ g and ATM-30  $\mu$ g, and the confirmation combination disk method with disks of CTX-30  $\mu$ g, cefotaxime/clavulanic acid (30/10  $\mu$ g), CAZ-30  $\mu$ g and ceftazidime/clavulanic acid (30/10  $\mu$ g)<sup>10</sup>; for AmpC-type  $\beta$ -lactamase detection, DDST (for constitutive AmpC) was performed with boronic acid containing disk (30  $\mu$ g) placed at a center-to-center distance to a CAZ-30  $\mu$ g and a CTX-30  $\mu$ g disk of 25 mm, while for combination disk method (for inducible AmpC), disks of CTX-30  $\mu$ g, CAZ-30  $\mu$ g and ATM-30  $\mu$ g were placed close to a IMP-10  $\mu$ g.<sup>11,12</sup>

Three multiplex PCR and one simplex PCR were performed to determine the presence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA-1</sub>-like and *bla*<sub>CTX-M</sub> genes in ESBL-producing isolates, and *bla*<sub>ACC</sub>, *bla*<sub>FOX</sub>, *bla*<sub>MOX</sub>, *bla*<sub>DHA</sub>, *bla*<sub>LAT</sub>, *bla*<sub>CMY</sub>, *bla*<sub>MIR</sub> and *bla*<sub>ACT</sub> genes in plasmid-mediated AmpC  $\beta$ -lactamase-producing isolates. PCR primers and procedures were performed as previously described.<sup>13</sup>

Purification and sequencing of PCR products were carried out by Macrogen Inc. (Korea) and the types of  $\beta$ -lactamase genes were identified by comparison with the sequences in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

During the study, a total of 79 isolates resistant to  $\beta$ -lactams were recovered. The most frequent isolated bacterial species were *Escherichia coli* (39) followed by *Klebsiella pneumoniae* (9), *Klebsiella oxytoca* (7) *Enterobacter aerogenes* (7), *Enterobacter cloacae* (5) *Proteus mirabilis* (3), *Pseudomonas aeruginosa* (3), *Citrobacter freundii* (3), *Proteus vulgaris* (2), and *Enterobacter koseri* (1).

All isolates showed antimicrobial resistance to one or more  $\beta$ -lactam antibiotics, and among them, 73 were confirmed to be positive for  $\beta$ -lactamase/ESBL and six for AmpC  $\beta$ -lactamase production (see Table 1 for details).

By PCR amplification, it was found that 31% of the samples showed the presence of one  $\beta$ -lactamase gene, whilst isolates often carried two (28%), three (20%), four (18%), and occasionally five (3%) different variants of  $\beta$ -lactamase enzymes (see Table 2 for details).

The genes encoding CTX-M were the most common and were found in 63 of the 73 ESBL-producing isolates. In particular the genes *bla*<sub>CTX-M</sub> belonging to phylogenetic groups 1, 2 and 9 were found in 48, 4 and 20 isolates, respectively. It was also detected that 52 of the ESBL-producing isolates carried *bla*<sub>TEM</sub>, 21 *bla*<sub>SHV</sub>, and 34 *bla*<sub>OXA-1</sub>-like genes. Finally, *bla*<sub>DHA</sub> was the most frequently observed plasmid-mediated AmpC  $\beta$ -lactamase gene (found in four isolates), while one isolate carried *bla*<sub>LAT</sub> and one *bla*<sub>MOX</sub>. To identify the specific *bla* genes detected in the PCR assays, amplicons DNA sequence analyses were performed and the results are summarized in Table 2.

**Table 2 – Summary of  $\beta$ -lactamase encoding genes detected in ESBL-producing bacteria from clinical isolates.**

Enzyme	Class	Family	Group	Variant	Spectrum	Combination	
$\beta$ -Lactamase (79)	$\beta$ -Lactamase (73)	CTX-M (72)	Group 1 (48)	CTX-M-1 (5)	Extended		
				CTX-M-3 (6)	Extended		
				CTX-M-12 (5)	Extended		
				CTX-M-15 (30)	Extended	BL/ESBL 3/2 (2)	
				CTX-M-55 (2)	Extended	BL/ESBL 3/1 (7)	
			Group 2 (4)	CTX-M-2 (4)	Extended	BL/ESBL 2/2 (8)	
				Group 9 (20)	CTX-M-14 (16)	Extended	BL/ESBL 2/1 (9)
			CTX-M-65 (4)		Extended	BL/ESBL 1/2 (4)	
			TEM (52)		TEM-1 (51)	Broad	BL/ESBL 1/1 (20)
				TEM-12 (1)	Extended	BL 3 (2)	
		SHV (21)		SHV-1 (5)	Broad	BL 2 (2)	
				SHV-2 (4)	Extended	BL 1 (3)	
				SHV-11 (10)	Broad	ESBL 1 (16)	
		AmpC (6)	OXA-1-like (34)	SHV-12 (2)	Extended	AmpC (6)	
				DHA (4)	Broad		
				LAT (1)	Broad		
				MOX (1)	*OXA-1-like (34)	Broad	
					DHA-1 (4)	Broad	
					CMY-75 (1)	Broad	
					MOX (1)	Broad	

The numbers in brackets represent the number of isolates (in enzyme and class columns) and of found genes (in family, group, variant and combination columns). The last column represents the different combinations of  $\beta$ -lactamase (BL), ESBL and AmpC genes found. \*In the case of bla<sub>OXA-1</sub>-like, it was not possible to distinguish the different variants by direct sequencing of PCR amplicons.<sup>13</sup>

Many studies on the resistance to  $\beta$ -lactams conducted in South America were performed using mainly a phenotypic approach. In Ecuador the few reports published on this topic lack almost totally of a genotypic study,<sup>14-17</sup> and, to the best of our knowledge, this is one of the first studies describing the genetic characteristics of  $\beta$ -lactamases in this country.<sup>8,9,17</sup>

Our data confirmed the prevalence in Loja province of *E. coli* and *Klebsiella* spp. as  $\beta$ -lactamase producers,<sup>6,18</sup> and a predominance of ESBL producers, showing the highest resistance levels mainly against cefotaxime, ciprofloxacin, netilmicin, and ampicillin.

It is widely documented that in South America ESBL-producing *Enterobacteriaceae* have one of the highest incidence in the world and CTX-Ms are the most broadly distributed.<sup>9</sup> In particular the type CTX-M-2 is the most frequent in the Southern Cone,<sup>19</sup> CTX-M-2-group, CTX-M-8, and CTX-M-9 in Brazil,<sup>20</sup> CTX-M-1-group in Colombia,<sup>21</sup> and CTX-M-2, CTX-M-14, CTX-M-15, CTX-M-24, and CTX-M-56 in Peru and Bolivia.<sup>22</sup>

In our study, it is documented that CTX-M enzymes are the dominant ESBLs in the Loja province, and among them the prevailing types are CTX-M-15 and CTX-M-14. These data are consistent with the literature<sup>21,22</sup> as these two variants are present and quite frequent in Ecuador neighboring countries. Interestingly, the types CTX-M-55 and CTX-M-65 were detected. Actually, these variants are common in East Asia,<sup>23,24</sup> and one possible hypothesis of their presence in Southern Ecuador can be related to the recent immigration from China and the trade relationship between Ecuador and China.<sup>25</sup> As expected, a strong presence of TEM-type  $\beta$ -lactamases was also found, in particular TEM-1  $\beta$ -lactamase. TEM-type presents a wide geographical distribution, and in Ecuador these data are not particularly surprising considering the incidence of TEM in neighboring Colombia<sup>26</sup> and the trade and migratory flows between these two countries, as well as the recent intense Ecuadorian immigration to and from Europe and North America where TEM is predominant such

as CTX-M-15. It is worth to mention the finding of one isolate harboring TEM-12. This ESBL variant has been already found in South America<sup>27</sup> and it is one of the most common in North America.<sup>28</sup> In our study, the presence of the non-ESBL OXA-1-type, SHV-1 and SHV-11, mainly found in *Klebsiella* (9 isolates) and *Proteus* (4 isolates), and the ESBL SHV-2 and SHV-12, found in *Enterobacter*, *Klebsiella* and *E. coli*, were also detected. The non-ESBL OXA-1-type and SHV-1 are among the most widespread in *Enterobacteriaceae*,<sup>29</sup> while to the best of our knowledge SHV-11 variant is emergent in South America. Regarding ESBLs, the variants found in this study are among the most common in Latin America, indeed SHV-2 with SHV-5 were the first ESBL SHV-types described in *Enterobacteriaceae*,<sup>30</sup> while SHV-12 is highly prevalent in Mexico, Colombia, and Brazil.<sup>31</sup> Concerning AmpC  $\beta$ -lactamase, there is not sufficient data to draw significant conclusions on its epidemiology, but it is interesting to note the presence of two CMY-75 genes found in *C. freundii*. This variant has been already detected in *C. freundii* in Spain (EMBL/GenBank/DBJ databases: AFK73434.1), but is emergent in South America. On the other hand DHA-1 has been previously recognized in South America, being quite predominant in Argentina.<sup>32</sup>

In conclusion, in this work the predominance of ESBLs in Southern Ecuador was demonstrated with a prevalence of CTX-M-type enzymes and we identified two emergent variants in South America. A high presence of TEM-type  $\beta$ -lactamases were also detected, being TEM-1  $\beta$ -lactamase the most frequent variant found. However, there are some limitations in this study, including the low number of isolates and the restricted geographical area of sampling. In order to minimize these problems and gather more indicative information on molecular epidemiology and genetic characteristics of  $\beta$ -lactamases within the entire territory of Ecuador, further isolates from different health institutions and regions of Ecuador have to be collected and characterized.

## Conflicts of interest

The authors declare no conflicts of interest.

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