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Spread of *bla*$_{\text{CTX-M-type}}$ and *bla*$_{\text{PER-2}}$ β-lactamase genes in clinical isolates from Bolivian hospitals

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***Objectives*: To assess the prevalence and types of genes encoding extended-spectrum β-lactamases (ESBLs) in clinical isolates of Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter* spp. from Bolivia.**

***Methods*: A total of 642 clinical isolates were collected consecutively during a 4 month period (September to December 2004). Resistance or reduced susceptibility to cefotaxime and/or ceftazidime and/or aztreonam was assessed using double disc synergy tests using clavulanic acid, cefotaxime, ceftazidime and aztreonam to identify putative ESBL-producing isolates. The ESBL determinants were characterized by colony blot hybridization, PCR and DNA sequencing.**

***Results*: Of the 642 isolates, 220 (34.3%) showed resistance or reduced susceptibility to cefotaxime and/or ceftazidime and/or aztreonam, and 150 (23.4%) were putative ESBL producers. A total of 106 ESBL-producing isolates contained the *bla*$_{\text{CTX-M-2}}$ gene, and 32 isolates had a novel allele, *bla*$_{\text{CTX-M-43}}$. *bla*$_{\text{CTX-M}}$ alleles were detected in all *P. aeruginosa* and *Acinetobacter* spp. studied. In contrast, only 12 ESBL-producing isolates had *bla*$_{\text{PER-2}}$, mainly Enterobacteriaceae, although it was also found in a strain of *P. aeruginosa*.**

***Conclusions*: This is the first study on ESBL-producing strains in Bolivia and it reveals a high prevalence of *bla*$_{\text{CTX-M}}$ genes. The PER-2 enzyme was less prevalent, but its gene was detected in several species, including *P. aeruginosa*, which is consistent with horizontal transfer.**

Keywords: CTX-M-43, CTX-M-2, PER-2, South America

Introduction

The production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) is one of the most important mechanisms of resistance against β-lactam antibiotics among clinical isolates of the Enterobacteriaceae and some non-fermenting bacilli. Many of these enzymes have evolved from TEM and SHV β-lactamases, but recently a large number of ESBLs unrelated to TEM and SHV, such as OXA, CTX-M and PER, have been described.

Infections caused by ESBL producers have become a serious problem in many parts of the world.[1,2] In Latin America, data suggest that 8.5% of *Escherichia coli* and 45% of *Klebsiella pneumoniae* have phenotypes consistent with ESBL production,[2] in particular CTX-M enzymes.[3] Currently, only one publication regarding the dissemination of CTX-M-type enzymes in Bolivia is available.[4]

PER enzymes are class A β-lactamases and they display a kinetic behaviour very similar to other ESBLs of the same class. The PER-1 enzyme was isolated for the first time in a *P. aeruginosa* strain in Turkey,[5] while the PER-2 enzyme was identified in Argentina in a *Salmonella enterica* strain.[6] To date the PER-2 enzyme has been found exclusively in South America.[3]

Materials and methods

Study design

During a 4 month period, from September to December 2004, 642 Gram-negative clinical isolates collected consecutively from four hospitals in the city of Santa Cruz de la Sierra, Bolivia, were analysed for resistance to β-lactam antibiotics. Participating hospitals were: Hospital Universitario Municipal 'San Juan de Dios'; Caja

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Table 1. Oligonucleotide primers used in this study

Genes	Amplicon size	T_m (°C)[a]	Primers	Sequences	Ref.
bla_{TEM}	861 bp	50	TEM/F	5′-ATGAGTATTCAACATTTCCG-3′	this study
			TEM/R	5′-TTACCAATGCTTAATCAGTGAG-3′	this study
bla_{SHV}	~900 bp	60	SHV/1	5′-GCCCGGGTTATTCTTATTTGTCGC-3′	this study
			SHV/2	5′-TCTTTCCGATGCCGCCGCCAGTCA-3′	this study
bla_{PER-1}	927 bp	48	PER-1_for	5′-ATGAATGTCATTATAAAAGCT-3′	this study
			PER-1_rev	5′-TTAATTTGGGCTTAGGG-3′	this study
bla_{PER-2}	927 bp	49	PER-2_for	5′-ATGAATGTCATCACAAAATG-3′	this study
			PER-2_rev	5′-TCAATCCGGACTCACT-3′	this study
$bla_{CTX-M-type}$	~600 bp	50	CTX-MU1	5′-ATGTGCAGYACCAGTAARGT-3′	4
			CTX-MU2	5′-TGGGTRAARTARGTSACCAGA-3′	4
$bla_{CTX-M-2-type}$	876 bp	56	CTXB_for	5′-ATGATGACTCAGAGCATTCGCCGC-3′	this study
			CTXB_rev	5′-TCAGAAACCGTGGGTTACGATTTT-3′	this study

[a]Annealing temperature used in the PCR experiments.

Petrolera de Salud; Caja Nacional de Salud; and Hospital de Niños 'Mario Ortiz'. The isolates were from blood, sputum, urine, the respiratory tract, and urine and vascular catheters. To avoid duplicates only one isolate per species was selected from each patient, unless isolates showed different resistance profiles. Isolates from hospitalized and non-hospitalized patients were included in the study. Isolates were screened for reduced susceptibility or resistance to ceftazidime and/or cefotaxime and/or aztreonam, according to CLSI guidelines.[7]

Antimicrobial susceptibility tests

The double disc synergy test was performed on Mueller–Hinton agar (Oxoid, Milan, Italy) using discs containing ceftazidime, cefotaxime, aztreonam and amoxicillin/clavulanate (Oxoid, Milan, Italy), according to CLSI guidelines.[7]

To increase the reliability of the method, discs containing cefotaxime/clavulanate (30 + 10 μg) and ceftazidime/clavulanate (30 + 10 μg) were placed on the same plate.[7] A disc containing imipenem (10 μg) was also used to select carbapenem-resistant isolates. MICs of β-lactam antibiotics were determined using a microdilution broth procedure, as recommended by the CLSI,[7] using a bacterial inoculum containing 10^5 cfu/mL. β-Lactam compounds were from Sigma Chemical Co. (St Louis, MO, USA) except for clavulanic acid and ceftazidime (GlaxoSmithKline, Verona, Italy), piperacillin and tazobactam (Wyeth-Lederle, Catania, Italy) and cefepime (Bristol-Myers Squibb, Wallingford, CT, USA).

Molecular methods

In order to evaluate the presence of ESBL-encoding genes, colony blot hybridization was performed on nylon membranes according to manufacturer's instructions, using random primed ^{32}P-labelled DNA probes (Hybond-N+, Amersham-Pharmacia Biosciences). *In situ* lysis of bacterial colonies was carried out as described previously.[8] bla_{TEM-1}, bla_{SHV-1}, bla_{PER-1}, $bla_{CTX-M-2}$ and $bla_{CTX-M-3}$ used as probes in the hybridization experiments correspond to the entire sequence of the genes.

Hybridization-positive isolates were analysed using PCR in order to isolate the genes. The primers used for the amplification are shown in Table 1. All PCRs were performed as described previously.[8] The nature of the ESBL was determined by direct sequencing of the products from two independent PCRs, using an ABI Prism 310 (Applied Biosystems, Monza, Italy). The deduced amino acid sequences were compared with those of known TEM, CTX-M, SHV and PER variants.

Results

A total of 642 clinical isolates of Enterobacteriaceae (n = 538), *Pseudomonas aeruginosa* and *Acinetobacter* spp. (n = 104) were studied; 42.8% (Enterobacteriaceae and non-fermenting bacilli) were isolated from hospitalized patients, and 57.2% were from outpatients, although most of the latter reported previous hospitalization. Of these 642 isolates, 220 (34.3%) showed resistance or reduced susceptibility to ceftazidime and/or cefotaxime and/or aztreonam, and 150 (23.4%) isolates were identified as putative ESBL producers using double disc susceptibility tests. The most common putative ESBL-producing organisms were *P. aeruginosa* (38.7%) followed by *K. pneumoniae* (16.0%), *E. coli* (14.0%), *Enterobacter cloacae* (13.3%), *Acinetobacter* spp. (8.7%), *Enterobacter aerogenes* (4.7%), *Citrobacter freundii* (2.0%), *Klebsiella oxytoca* (2.0%) and *Morganella morganii* (0.7%). The MICs of amoxicillin, piperacillin, cefalotin, ceftazidime, cefotaxime, aztreonam and cefepime for all ESBL producers were above the resistance breakpoints, and β-lactamase inhibitors did not restore susceptibility (data not shown).

Genes encoding CTX-M-type ESBL were detected in 138 (92%) ESBL-producing isolates. Sequencing identified CTX-M-2 and a novel variant, designated CTX-M-43 (deposited as GenBank DQ102702) (Table 2). A $bla_{CTX-M-2}$ allele was identified in 106 isolates, representing all the species studied except for *K. oxytoca*, *M. morganii* and *Acinetobacter* spp.; CTX-M-43 was identified in 32 isolates of *P. aeruginosa*, *Acinetobacter* spp., *M. morganii* and *Enterobacter* spp. CTX-M-2 was found in isolates from all the hospitals involved in this study; CTX-M-43 was found in isolates from three of these centres (not found in Hospital de Niños). bla_{PER-2}, encoding PER-2 enzyme, was identified in 11 isolates of Enterobacteriaceae (*E. aerogenes*, *E. cloacae, E. coli* and *K. oxytoca*) and in one *P. aeruginosa*. Of the four hospitals sampled, it was detected in isolates from two hospitals only. bla_{TEM-1} and bla_{SHV-1} alleles were also detected (Table 2).

Table 2. Enterobacteriaceae and non-fermenting bacilli analysed using colony hybridization and DNA sequencing

Organisms	Selected versus CTX, CAZ, ATM[a]	$bla_{CTX\text{-}M\text{-}type}$				$bla_{PER\text{-}2}$				$bla_{CTX\text{-}M}$ and bla_{PER} negative[b]		
		$bla_{CTX\text{-}M}$	$bla_{CTX\text{-}M}$ $bla_{SHV\text{-}1}$	$bla_{CTX\text{-}M}$ $bla_{TEM\text{-}1}$	$bla_{CTX\text{-}M}$ $bla_{TEM\text{-}1}$ $bla_{SHV\text{-}1}$	$bla_{PER\text{-}2}$	$bla_{PER\text{-}2}$ $bla_{SHV\text{-}1}$	$bla_{PER\text{-}2}$ $bla_{TEM\text{-}1}$	$bla_{PER\text{-}2}$ $bla_{TEM\text{-}1}$ $bla_{SHV\text{-}1}$	$bla_{SHV\text{-}1}$	$bla_{TEM\text{-}1}$	$bla_{TEM\text{-}1}$ $bla_{SHV\text{-}1}$
C. freundii	3	1		2								
E. aerogenes	15	3	1	2				1			8	
E. cloacae	23	5	2	9		4					2	1
E. coli	44	3	3	10	2		2		1	2	12	9
K. oxytoca	4								3	1		
K. pneumoniae	37	5		13	6					3	6	4
M. morganii	2	1									1	
Acinetobacter spp.	23	7	1	5						2	6	2
P. aeruginosa	69	36	9	9	3			1		4	6	1
Total	220	61	16	50	11	4	2	2	4	12	41	17
		Total $bla_{CTX\text{-}M}$ positive			138	Total $bla_{PER\text{-}2}$ positive			12	Total		70

[a]Isolates presenting resistance or reduced susceptibility to cefotaxime (CTX) and/or ceftazidime (CAZ) and/or aztreonam (ATM).
[b]$bla_{CTX\text{-}M}$ and bla_{PER} negative isolates carrying bla_{TEM} and bla_{SVH} genes.

Molecular tests were performed on the 70 isolates that tested negative for the double disc synergy test in order to identify the nature of the determinants causing reduced susceptibility to cefotaxime and/or ceftazidime and/or aztreonam. In these strains the presence of only $bla_{TEM\text{-}1}$ and $bla_{SHV\text{-}1}$ alleles was identified; 24.3% (17 out of 70) showed the presence of both TEM-1 and SHV-1 β-lactamases.

No carbapenem resistance was observed in any of the isolates tested in this study.

Discussion

Although several surveys of ESBL-producing Enterobacteriaceae have been undertaken in South America,[2] in particular in Argentina[3] and Brazil,[9] low-income countries have not been studied extensively. In this article we have reported the first survey of ESBL-producing Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter* spp. in four hospitals in the city of Santa Cruz de la Sierra, Bolivia.

The first remarkable information is the absence of carbapenem-resistant organisms among the isolates collected during this study. Some *P. aeruginosa* isolates showed a reduced susceptibility to imipenem, but further analysis demonstrated the absence of carbapenemase activity (data not shown).

Only ESBLs belonging to CTX-M (92.0%) and PER types (8.0%) were identified in this study. The CTX-M-2 enzyme represented the most abundant CTX-M variant (77%), which is consistent with previous epidemiological studies in Latin America.[2] CTX-M-2 was widespread among almost all the species studied except *K. oxytoca*, *M. morganii* and *Acinetobacter* spp. It was the only CTX-M variant identified in *E. coli* and the only ESBL found in the paediatric Hospital de Niños 'Mario Ortiz'. These data are in accordance with previous data on the dissemination of CTX-M-2 among intestinal *E. coli* isolates collected from healthy children in Bolivia.[4]

A new variant CTX-M-43, which differs from Toho-1 by only a single mutation (D240G), was found in *E. aerogenes*, *E. cloacae*, *M. morganii*, *Acinetobacter* spp and *P. aeruginosa*.

The spread of PER-2 enzyme is limited to South America and in particular in Argentina, where it was first indentified.[6] In this study we confirm that PER-2 is present in diverse Enterobacteriaceae (11 out of 12 isolates), and we also detected it in a strain of *P. aeruginosa*.

To our knowledge this is the first identification of the $bla_{PER\text{-}2}$ allele in a non-fermenting bacillus, and this suggests horizontal transfer of this gene.

Even though this survey was performed in only four centres in the city of Santa Cruz, it is the first study carried out on clinical isolates in Bolivia and it provides some important information about the epidemiology of ESBL-producing organisms in this country. In summary, TEM and SHV variant ESBLs were not found, although we identified TEM-1 and SHV-1 β-lactamases, often in association with other ESBLs. Our data show that CTX-M-type and PER enzymes are the predominant ESBLs in Bolivia.

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Transparency declarations

None to declare.

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