Identification of SHV-type and CTX-M-12 Extended-Spectrum β-Lactamases (ESBLs) in Multiresistant *Enterobacteriaceae* from Colombian Caribbean Hospitals

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Summary

The production of extended-spectrum beta-lactamases (ESBLs) in *Enterobacteriaceae* is the most prevalent resistance mechanism to third-generation cephalosporins. The aim of this study was to identify the ESBLs produced in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates from two hospitals of the Colombian Caribbean Region. A total of 30 clinical isolates of *K. pneumoniae* (21) and *E. coli* (9) ESBL-producers were collected in two hospitals from January, 2001 to June, 2003. Isoelectric point values were indicative of SHV-, and CTX-M-type β-lactamases. PCR amplification and sequencing of SHV genes revealed that SHV-12 was the most prevalent ESBL followed by SHV-5, SHV-2a, the novel SHV-86 and CTX-M-12. There was a geographic distribution of two particular PFGE subtypes in these two distant hospitals. Clonal and horizontal dissemination of resistance was observed.

***Key words:* *Escherichia coli, Klebsiella pneumoniae*, ESBL, SHV-12, SHV-86, CTX-M-12.**

INTRODUCTION

β-lactamases are enzymes that inactivate β-lactam antibiotics by performing ring-opening hydrolysis, rendering the drug unable to properly inhibit its target, the penicillin-binding proteins. In recent years there has been an increase in the spread of extended-spectrum β-lactamases (ESBL), enzymes that confer resistance to most β-lactam antibiotics, including penicillins, [illegible] of ESBLs is the most prevalent mechanism responsible for resistance against b-lactam antibiotics among clinical isolates. Among the TEM, SHV and CTX-M β-lactamase families, the ESBLs most commonly identified are derived by mutation from genes for the narrower spectrum TEM-1, TEM-2 or SHV-1 β-lactamases [1]. At present 181 and 133 members of TEM and SHV families are known, respectively. The CTX-M enzymes include 96 different types, (http://lahey.org/studies) and these enzymes are currently distributed worldwide [2].

In general the ESBLs are clavulanate-susceptible and confer resistance to penicillins, aztreonam and cephalosporins. They are detected most commonly in *Klebsiella pneumoniae* and *Escherichia coli* [4]. SHV-type ESBLs confer high-level resistance to ceftazidime but not to cefotaxime and cefazolin. In contrast to SHV-type ESBLs, most of the CTX-M enzymes are more active against cefotaxime and ceftriaxone than against ceftazidime. Thus, most of the CTX-M producers display significantly higher levels of resistance to cefotaxime than to ceftazidime [3].

In this study, 30 clinical isolates of *K. pneumoniae* and *E. coli* obtained in 2001-2003 from nosocomial infections from two Colombian Hospitals were examined. The isolates harbored transferable SHV-type and CTX-M-type ESBLs on a few clonal groups and plasmid profile.

METHODS

Hospital settings

The study was carried out in two tertiary-care hospitals from two different cities from the Colombian Caribbean region: Hospital San Jerónimo in Montería (HSJ-Mt) with 212 beds and Clínica General del Norte in Barranquilla (CGN-Bq) with 300 beds. These hospitals are 350 Km (220 miles) miles away. The hospitalization areas studied were: internal medicine (IM), intensive care unit (ICU), emergency (Urg), newborn (NB) retired (Re) and external consult (Ex C).

Bacterial strains

A total of 30 enterobacterial ESBL-producing clinical isolates were recovered from January, 2001 to June, 2003. All isolates were obtained from nosocomial infections and each one corresponded to one patient. Clinical specimens were obtained from bronchial secretion (27%), wound (23%), blood (16.7%), catheter (13.3%), other sites (13.3%) and urine (6.7%). The isolates were identified using the MicroScan NC32 system (Dade Behring, Ca, USA).

Susceptibility testing and ESBL identification

Susceptibility testing was performed by the disk diffusion method according to CLSI recommendations [5], using cefotaxime, ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, trimethoprim-sulfamethoxazole, ciprofloxacin and levofloxacin discs (Oxoid, Basingstoke, UK). ESBL production was performed by the disk diffusion method with cefotaxime and ceftazidime alone and in combination with clavulanic acid as recommended by the CLSI [5]. The minimum

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inhibitory concentrations (MICs) of amikacin, cefotaxime, cefotaxime-clavulanic acid, ceftazidime, ceftazidime-clavulanic acid, piperacillin, piperacillin-tazobactam, imipenem and ciprofloxacin were determined by agar dilution on Mueller-Hinton following the CLSI recommendations [5]. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as control strains.

IEF and bioassay

Isoelectric focusing (IEF) was conducted according to the method described by Matthew *et al* [10]. Extracts from TEM-1, SHV-2, SHV-5 and TLA-1 producing strains were used as standards for pIs reference values of 5.4, 7.6, 8.2, and 9.0, respectively. After IEF b-lactamases and ESBLs were detected by spreading the nitrocefin-cefotaxime combination on the gel surface and bioassay [11].

PFGE and comparative analysis

Molecular epidemiologic typing was performed by pulsed-field gel electrophoresis (PFGE) [7]. The whole cell DNA from ESBL-producing *K. pneumoniae* and *E. coli* were obtained as previously described by Kaufmann et al [6]. Representative strains from each clone from both hospitals were compared by PFGE and analyzed using GelCompar II software (Applied Math, Kortrijk, Belgium). Similarity percentages were determined in a dendrogram derived from UPGMA and Dice coefficients (band position tolerance and optimization were set at 1.0% and 0.5%, respectively).

Mating experiments and plasmid isolation

Matings were performed as described by Miller [9] using a membrane filter, and *E. coli* J53-2 (F^-, *pro*$^-$, *met*$^-$, Rif ^{r}) as receptor strain. In all cases, transconjugants were selected on Luria agar supplemented with rifampin (100 µg/ml) in combination with cefotaxime (1 µg/ml) or ampicillin (50 µg/ml). Plasmid DNA was extracted from clinical isolates and respective transconjugants according to the method described by Kieser [8]. Plasmids R6K (40 kbp), RP4 (54 kbp), R1 (94 kbp), pMG229 (205 kbp) and pUD21 (275 kbp) were used as molecular size markers.

PCR amplification and DNA sequencing

To amplify TEM-related genes, PCR amplification with primers OT1 and OT2 was performed [14]. For SHV-specific PCR, primers SE5 and SB3 were used [12]. The primers CTXM-1F and CTXM-1R were used as a screen for group one of CTX-M β-lactamases [13]. Subsequently, for the amplification of complete genes, the following primers designed in this work were used: CTX-MF, (5'GCT GTT GTT AGG AAG TGT G3') and CTX-MR, (5'GGT GAC GAT TTT AGC CGC C3'). The PCR products of bla_{SHV} and bla_{CTX-M} were sequenced by chain termination methods with Big-Dye Terminator kit (Applied Biosystems Foster City, CA). The analysis was carried out on an ABIPRISMA-3100 (Applied Biosystems, Foster City, CA).

Sequence analysis

GenBank searches were performed online using the BlastN and BlastX programs. Multiple alignments with SHV-1 (Accession number GENBANK AF148850) and CTX-M-12 (Accession number GENBANK AF305837) genes were performed with the ClustalW program (http://clustalw.genome.jp/).

The nucleotide sequence of the novel β-lactamase SHV-86 was deposited in the GenBank database under accession number ABC58727.

RESULTS

Bacterial strains

A total of 30 enterobacterial ESBL-producing clinical isolates were recovered from the two hospitals and corresponded to 21 *K. pneumoniae* and 9 *E. coli*, 17 of which were obtained from HSJ-Mt and 13 from CGN-Bq hospitals. These clinical isolates were recovered mainly from ICUs (18/30, 60%) and internal medicine wards (4/30, 13%).

All 30 ESBL-producing clinical isolates were susceptible to imipenem. In general, resistance to ceftazidime was higher (75-88%) than cefotaxime (15-40%) in *K. pneumonia* and *E. coli* groups, respectively. This susceptibility was restored in the presence of clavulanic acid (*Table 1*). In all clinical isolates the resistance to piperacillin was high (95-100%), to amikacin was

TABLE 1 - *Antimicrobial susceptibilities of K. pneumoniae and E. coli ESBL-producing clinical isolates*

	K. pneumoniae				*E. coli*			
	n= 21				n= 9			
Antibiotic	**MIC_{50}**	**MIC_{90}**	**Range (µg/ml)**	**Resistance %**	**MIC_{50}**	**MIC_{90}**	**Range (µg/ml)**	**Resistance %**
Cefotaxime	16	128	0.125 - 128	40	8	32	0.125 - 128	15
Cefotaxime/ clavulanic acid	0.125/4	0.25/4	0.125/4 - 128/4		0.125/4	0.125/4	0.125/4 - 128/4	
Ceftazidime	256	>256	0.125 -256	75	64	256	0.125 -256	88
Ceftazidime/ clavulanic acid	0.5/4	128/4	0.125/4 - 256/4		0.5/4	0.25/4	0.125/4 - 256/4	
Piperacillin	>256	>256	0.125 - 256	95	>256	>256	0.125 - 256	100
Piperacillin/ tazobactam	4/4	256/4	0.125 - 256	40	8/4	32/4	0.125 - 256	25
Imipenem	0.25	0.5	0.125 - 128	0	0.125	0.5	0.125 - 128	0
Amikacin	8	64	0.125 - 128	40	8	8	0.125 - 128	0
Ciprofoxacin	16	>16	0.125 - 128	65	16	>16	0.125 - 128	100

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40% only in the *K. pneumoniae* group. Finally, all *E. coli* isolates were resistance to ciprofloxacin, as compared to only 65% in the *K. pneumoniae* group (*Table 1*).

Genome fingerprinting

PFGE analysis of the *K. pneumoniae* ESBL-producing isolates showed four clusters and two unrelated strains (*Table 2*). The similarity percentage determined in the dendrogram (>80 coefficient) confirmed these clusters. In the *K. pneumoniae* fingerprinting, the clone A included three clinical isolates, two of which corresponded to HSJ-Mt hospital (4510 and 4512), isolated in 2002 and one surprisingly from CGN-Bq hospital (4513) isolated in 2003 (*Figure 1*). Concerning the *E. coli* ESBL-producers, one cluster (P) containing three identical isolates including two from HSJ-Mt hospital (4525 and 4531) obtained in 2002 and 2003, respectively, and one isolate from CGN-Bq hospital (4527) obtained in 2003 were identified. A high level of genetic heterogeneity was identified in the six other isolates of *E. coli* (*Table 1*, *Figure 1*).

Bacterial conjugation and plasmid profile

All ESBL-producing clinical isolates contained from 1 to 4 plasmids with sizes from 40 to 300 kbp, approximately. To determine if a plasmid-borne ESBL occurred in the clinical isolates, 21 clinical isolates (13 *K. pneumoniae* and 8 *E. coli*) were selected, considering the PFGE pattern, for bacterial conjugation experiments. Successful mating was detected in 20 of the 21 clinical isolates tested; of these, 12 corresponded to *K. pneumoniae* and 8 to *E. coli*. Nineteen of the 20 transconjugants obtained only one plasmid from the parental strain and the other transconjugant (4512) harbored two plasmids of 160 and 90 kpb (*Table 2*). All transconjugants were resistant to ampicillin, cefotaxime and ceftazidime, and in addition, 70% were resistant to kanamycin, 47% to chloramphenicol, 35% to gentamicin and 17% to tetracycline.

β-lactamase profile and ESBL identification

Thirty ESBL-producing clinical isolates and the 20 respective transconjugants were subjected to IEF in order to identify the isoelectric focusing β-lactamases. In *K. pneumoniae* clinical isolates, one predominant β-lactamase profile with pI values of 7.6 and 8.2 was identified in 15 isolates, six of them expressed an extra enzyme with pI value of 5.4 and one with pI value of 7.0. Only two isolates expressed a β-lactamase with pI value of 8.9. In the *E. coli* group, a major pattern was identified in six isolates with pI values of 5.4 and 8.2. and one isolate with pI of 5.4

TABLE 2 - *General characteristics of Enterobacteriaceae ESBL-producers.*

Species	Isolate No.	Hospital Region	Year of isolation	Ward[a]	Sample origin	PFGE pattern	Plasmid pattern (kb)[b]	β-Lactamase pattern (pI)[b,c]	ESBL[d]	Transconjugants Phenotype
K. pneumoniae	4510	1	2002	Urg	Pleural	A	NI	(7.6), (8.2)	SHV-12	
	4513	2	2003	ICU	Slough	A	160, **90**	7.6, **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Cl, Gm
	4512	1	2002	ICU	Bronchial	A	>300, **200,130**	(7.6), **(8.2)**	**SHV-12**	AMP,CAZ, CTX, Km, Cl, Gm
	4503	1	2001	NB	Blood	B	170,160	(7.6), (8.2)	SHV-12	
	4501	1	2001	Surg	Wound	B	300, **100**	(7.6), **(8.2)**	**SHV-12**	AMP, CAZ, CTX
	4506	1	2002	Re	Catheter	B1	300	7.6, (8.2)		
	4508	1	2002	ICU	Bronchial	C	290, 230, 190, 130	7.6, (8.2)		
	4509	1	2002	ICU	Catheter	C	290, 230, 200, 100	(7.6), (8.2)	SHV-12	
	4516	2	2003	ICU	Bronchial	D	**230**, 170	**(8.2)**	**SHV-5**	AMP, CAZ, CTX, Km, Cl, Gm
	4517	2	2003	ICU	Wound	D	300,220,100	(8,2)		
	4507	1	2002	IM	Wound	E	300,240,170	(8,9)	CTX-M-12	
	4502	1	2001	Re	Bronchial	F	**210**, 100	**(8,2)**	**SHV-12**	AMP, CAZ, CTX, Km, Cl, Gm
	4505	2	2002	ICU	Catheter	G	**230**	(7.6), **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km, Gm
	4521	1	2003	ICU	Bronchial	H	190,**100**	5.4, 7.6, **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Cl
	4520	2	2003	ICU	Abscess	I	260,110	5.4, (7.6), (8.2)	SHV-5	
	4504	1	2001	NB	Blood	J	**210**	(5.4), (7.6), **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km, Tc, Cl
	4519	2	2003	ICU	Blood	K	200,130, 60,<40	5.4, 7.6, (8.2)	SHV-86[e]	
	4518	2	2003	ICU	Bronchial	L	270, 230, **<40**	7.0, **(7.6)**, (8.2)	**SHV-2a**	AMP, CAZ, CTX, Km
	4514	2	2003	ICU	Wound	M	270,140, 80	(8,9)	CTX-M-12	
	4511	1	2002	IM	Bronchial	N	200, **70**	5.4, 7.6, **(8.2)**	**SHV-12**	AMP, CAZ, CTX
	4515	2	2003	ICU	Catheter	O	**230**, 210	5.4, (7.6), **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km
E.coli	4527	2	2003	ICU	Peritoneal	P	250, 170, **<40**	5.4, **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km, Tc, Cl, Gm
	4531	1	2003	IM	Wound	P	300,200,<40	5.4, (8.2)		
	4525	1	2002	Re	Wound	P	260, 150, **50**	5.4, **(8.2)**	**SHV-12**	AMP, CAZ, CTX
	4522	1	2001	IM	Blood	Q	220,**<40**	5.4, 6.0, 6.9, **(7.8)**	**SHV-12**	AMP, CAZ, CTX, Km
	4529	2	2003	ICU	UTC	R	260, **60**	5.4, **(7.6)**	**SHV-2a**	AMP, CAZ, CTX
	4523	1	2001	ICU	Blood	S	180, **60**	5.4, 6.0, 6.9, **(7.8)**	**SHV-12**	AMP, CAZ, CTX
	4528	2	2003	ICU	UTC	T	NI	5.4, **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km, Tc, Cl, Gm
	4524	1	2002	Ex C	Catheter	U	260, **<40**	5.4, **(8.2)**	**SHV-12**	AMP, CAZ, CTX, Km
	4530	2	2003	ICU	Pleural	V	250, **200**, 60	5.4, 7.2, **(8.2)**	**SHV-5**	AMP, CAZ, CTX, Km

[a] IM Internal Medicine, ICU, Intensive Care Unit, Urg, emergency, NB, new born, Re, retired, Ex C, external consult.
[b] Values in **bold** indicate presence in the clinical isolate and the respective transconjugant. NI, not identified.
[c] ( ) Indicate an ESBL in the bioassay.
[d] ESBL identified in the transconjugants.
[e] Possible new SHV-86 ESBL with Gln35Leu, Ser238Gly and Arg240Glu amino acids changes compared to bla_{SHV-1}.

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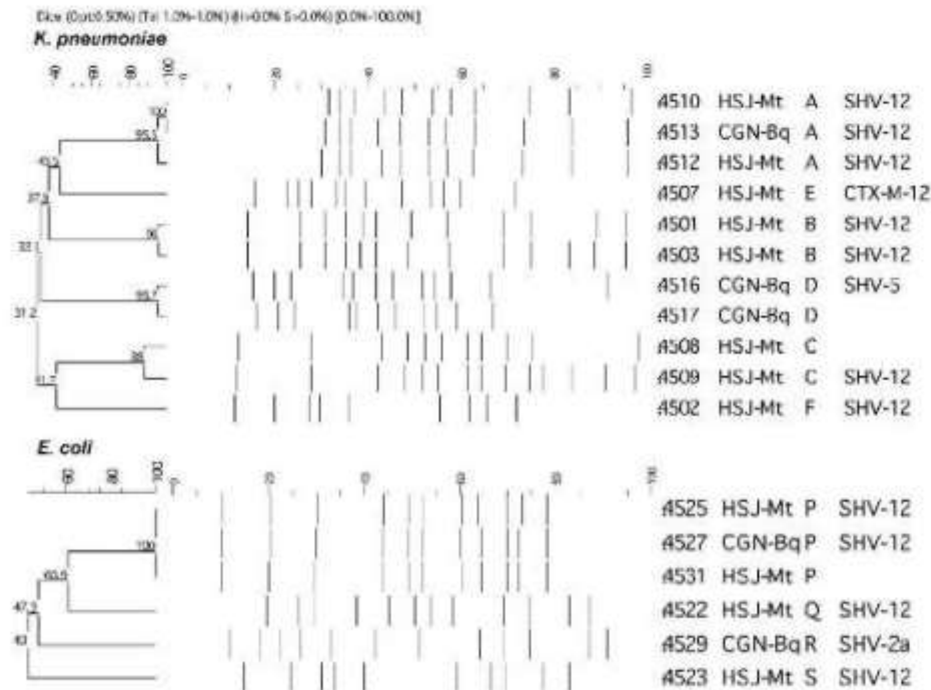

FIGURE 1 - Dendrogram of XbaI macrorestriction fragment of representative *K. pneumoniae* and *E. coli* ESBL-producing clinical isolates. Isolates showing a similarity coefficient of >80% were considered to be genetically related in this study. Isolate number, medical center, and clone designation are indicated.

and 7.6. With respect to transcongujants in both bacterial groups, the ESBL with pI value 8.2 was transferred preferably; but also the enzyme with a pI value of 7.6 was transferred. In both groups, the ESBLs with cefotaximase activity were identified by bioassay and corresponded to β-lactamases with pI values of 7.6, 8.2 and 8.9 (indicated in brackets) and the ESBLs identified in the respective transconjugant are indicated in bold (*Table 2*).

ESBL characterization

According to the clonal group and pI values of ESBLs obtained, PCR amplification and sequence analysis were conduced. Total DNA preparations from 25 different clinical isolates were processed; results indicated 23 amplified for SHV-type and two for CTX-M-type genes. The sequence analysis identified SHV-12 (17/23) as the most frequent enzyme in the bacterial isolates evaluated, followed by SHV-5 (3/23), SHV-2a (2/23) and a possibly new SHV-86 (1/23) β-lactamase gene. This last enzyme was identified in a *K. pneumoniae* clinical isolate with the L35Q, G238S and E240R amino acid substitutions. Two unrelated *K. pneumoniae* clinical isolates expressed the CTX-M-12 enzyme, corresponding to an ESBL with a pI value of 8.9. The β-lactamase with pI value of 5.4 corresponded to TEM-type β-lactamase, however the nucleotide sequence was not obtained. Distribution of the enzymes is indicated in *Table 2*.

Nucleotide sequence accession number

The nucleotide sequence of the new β-lactamase SHV-86 was deposited in the GenBank database under accession number ABC58727.

DISCUSSION

Many different types of β-lactamases are reported around the world. They differ in their susceptibility to β-lactamase inhibitors, expression, substrate specificity and genetic location. The variety of genetic vehicles associated with ESBL genes was recently investigated, highlighting those implicated in the spread of antibiotic resistance genes, and the factors that may trigger either their emergence or expression [15].

Infections due to ESBL-producing Enterobacteriaceae have become a major problem in hospital settings. SHV- and CTX-M-type enzymes are reported with increasing frequency and are prevalent in Colombia. *K. pneumoniae* SHV-5-producer has been found in several regions in Colombia including Bogota [16]. Also, enzymes of the CTX-M-type, particularly CTX-M-12, have

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been described in two cities of the Colombian Caribbean [17]. In this work the spread of SHV-12 in clinical isolates of enterobacteria ESBL-producers causing nosocomial infections in two Colombian Caribbean hospitals is described. In addition, to our knowledge, the SHV-2a and the new SHV-86 enzymes have not yet been identified in this region. We also identified the CTX-M-type, a more prevalent enzyme than TEM- and SHV-type ESBLs in the Colombian hospital settings [13,18].

The analysis performed by analytical IEF of the β-lactamases indicated a similarity of profiles in both *K. pneumoniae* and *E. coli* clinical isolates. Most of the *K. pneumoniae* isolates produced a β-lactamase with a pI of 7.6 which was likely to be a chromosomally encoded enzyme [19]. Nevertheless, the enzyme with cefotaximase activity with this pI value was not transferred by conjugation when co-existing with the ESBL with the pI value of 8.2. However this was not the case for *E. coli* which expressed only one ESBL. Isolates expressing an additional β-lactamase with a pI value of 5.4, 6.0, 6.9 and 7.0 and no cefotaximase activity, could correspond to TEM and SHV-type β-lactamases [16]. In general, transconjugants from both bacterial groups expressing the β-lactamases with pI values of 8.2 or 8.9, possess cefotaximase activity, according to the bioassay approach, indicating an ESBL enzyme.

Specific PCR amplifications followed by sequencing of these products revealed one identical sequence of the bla_{SHV-12} gene. In this work, we identified a β-lactamase variant similar to SHV-12, with one amino acid substitution in region E240R and named SHV-86. The combination of Leu-35 → Gln and Gly-238 → Ser and Gln-240 → Lys mutations characterizes SHV-12 with a pI value of 8.2 [20]. A limitation of this work is the lack of kinetic characterization of this enzyme, which confirms the role of the single mutation Glu-240 → Arg in cefotaxime and ceftazidime hydrolysis in comparison with SHV-1 and SHV-12 enzymes. Further kinetic parameters of this enzyme against several ß-lactam substrates will be performed in order to know the role of the E240R substitution in this enzyme.

These data including the pI values of β-lactamases and plasmid profiles show a complex picture of the spreading of ESBL-producing *K. pneumoniae* and *E. coli* in the two hospitals. The molecular analysis of the isolates by PFGE demonstrated heterogeneity between them even though both *K. pneumoniae* and *E. coli* clinical isolates were recovered in different years and from hospitals which were located in two distant cities, thus indicating the dissemination of these strains. Three different ESBLs were present in the population of strains at the time of the study; these SHV- and CTX-M-type ESBLs have been identified recently in Colombian hospital environments [21]. In general the clinical isolates expressing the SHV-12 or a related enzyme have different PFGE profiles (*Table 2*). In the clonally related isolates, their plasmid profile was different, indicating an independent evolution of the plasmids and maintaining the same pI β-lactamase profile.

The emergence and spread of CTX-M-type genes which we have found is in agreement with other reports and confirms the current relevance of CTX-M enzymes in pathogenic enterobacterial isolates [18]. Clinical microbiology laboratories should take into account this epidemiological change and accordingly adjust the screening and confirmatory procedures for ESBL identification, in both ceftazidime and cefotaxime, in the testing procedures [22].

The increase in ESBL-producing microorganisms is associated with the clonal dissemination of some epidemic stocks and plasmids conferring resistance to bacteria due to improper use of β-lactam agents in hospitals [23]. Carbapenems appear to be the most effective antimicrobial agents against these kinds of microorganisms. Thus, measures are required that allow the prudent use of these agents, especially cephalosporins, to reduce the dissemination of resistant stocks and the opportune detection of ESBL-producing enterobacteria in microbiology laboratories to reduce the morbidity-mortality rates in these institutions. The presence of multiple β-lactamases in the same isolate is disconcerting because it effectively limits β-lactam therapeutic options.

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