Predominance of CTX-M–type extended-spectrum β -lactamase genes among enterobacterial isolates from outpatients in Brazil

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Abstract

Two hundred fifty-seven nalidixic acid-resistant enterobacterial isolates were collected in a Brazilian community from January 2000 to May 2005 to determine the prevalence of plasmid-encoded extended-spectrum β -lactamases. The *bla*_{CTX-M} genetic environment was determined by polymerase chain reaction and sequencing. Eleven isolates (4.2%) harbored a *bla*_{CTX-M-2} gene, 3 isolates *bla*_{CTX-M-9}, 2 isolates *bla*_{CTX-M-8}, and 6 isolates *bla*_{SHV-5}. Two novel *bla*_{CTX-M-2} variants, namely, *bla*_{CTX-M-74} and *bla*_{CTX-M-75}, were identified.

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Since the late 1990s, Enterobacteriaceae producing extended-spectrum β -lactamases (ESBLs) and, in particular, CTX-M enzymes have emerged in hospital and community settings as important sources of urinary tract infections (UTIs). In Brazil, ESBLs have been predominantly detected in *Klebsiella* spp. recovered from hospital settings (Cassetari et al., 2009; Minarini et al., 2008; Peirano et al., 2009), effluents of hospital sewages (Prado et al., 2008), exported chicken meats (Warren et al., 2008), and community-onset infections (Minarini et al., 2007). Isolates harboring the *bla*_{SHV-5}, *bla*_{SHV-11}, *bla*_{CTX-M-2}, and *bla*_{CTX-M-9} genes are the most frequently found (Minarini et al., 2007; Peirano et al., 2009). However, the distribution of ESBL producers among community-onset pathogens in South America remains unknown.

Our study was aimed to evaluate the prevalence of ESBLs among community-onset and quinolone-resistant enterobacterial isolates ($n = 257$) in a city in Southeast Brazil (Juiz de

Fora, State of Minas Gerais) during a 5 1/2-year period from January 2000 to May 2005. ESBL producers were searched among nalidixic acid-resistant isolates since linkage between resistance to quinolone/fluoroquinolone and ESBLs has been often reported (Minarini et al., 2007; Warren et al., 2008).

The antibiotic susceptibility of the enterobacterial isolates was determined by a disk-diffusion technique on Mueller–Hinton agar plates with β -lactam (cefotaxime, ceftazidime, and cefotaxime) and non- β -lactam (acid nalidixic, ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, trimethoprim–sulfamethoxazole, tetracycline, and gentamicin) antibiotic-containing disks according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). ESBL production was confirmed by the combination disk methods using Oxoid disks. Twenty-four (9.3%) ESBL-producing Enterobacteriaceae strains were detected in a collection of nalidixic acid-resistant enterobacterial isolates recovered from 21 patients. Species distribution for those 24 ESBL-producing isolates was as follows: 9 *Escherichia coli*, 6 *Klebsiella pneumoniae*, 5 *Enterobacter cloacae*, 2 *Providencia stuartii*, 1 *Morganella morganii*, and 1 *Citrobacter freundii*. ESBL-positive isolates were all from UTIs, except one recovered from a wound.

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Fluoroquinolone-resistant isolates harboring an ESBL phenotype were detected in some Brazilian medical centers (22.9%) in 2002 and 2003, indicating that these isolates are disseminated across Brazil (Pereira et al., 2007). In our study, isolates were tested for TEM-, SHV-, and CTX-M-type encoding genes, but the most commonly identified ESBLs were CTX-M-type enzymes ($n = 18$), followed by SHV type ($n = 6$). Primers used for polymerase chain reaction (PCR) amplification of β -lactam resistance genes and for analysis of their genetic environments are reported in Table 1. Sequencing of *bla*ESBL allowed to identify 18 CTX-M-producing strains distributed as follows: CTX-M-2-like ($n = 13$, 72%), CTX-M-8 ($n = 2$, 11%), and CTX-M-9 ($n = 3$, 17%). CTX-M-2-like determinants included *bla*CTX-M-2 and the 2 novel variants *bla*CTX-M-74 and *bla*CTX-M-75 (GenBank accession numbers GQ149243 and GQ149244, respectively). CTX-M-74 was from *E. cloacae* JF216 isolated in 2004 and differed from CTX-M-2 by a single amino acid alteration (Pro to Thr at position 170). CTX-M-75 was from *P. stuartii* JF29 isolated in 2002 and differed from CTX-M-2 by a single amino acid substitution (Pro to Ser at position 18). The *bla*SHV-5 gene was detected in the 6 remaining and *bla*CTX-M-negative isolates (25%). The high proportion of CTX-M producers found in this study (75%) is somewhat lower than that reported from other Latin American countries such as Bolivia and Peru (Pallecchi et al., 2007). However, this proportion is higher as compared with North America (Lewis et al., 2007; Pitout et al., 2007). CTX-M-2-producing strains were more prevalent in 2004 ($n = 7$) than in 2000 to 2003 (1 isolate per year), which could be related to a significant increase in the number of Enterobacteriaceae isolates producing ESBL in the community.

The genetic environment of the *bla*CTX-M genes was characterized by PCR and sequencing (Lartigue et al.,

2007). *ISEcp1*-like insertion sequences is known to play a role in the mobilization of the *bla*CTX-M genes by a transcriptional mechanism by recognizing a variety of DNA sequences as right inverted repeat. *ISEcp1* was identified upstream of all the *bla*CTX-M-9 genes, its right boundary being located 74 bp upstream of the β -lactamase gene. *ISCR1* that includes the *orf513* transposase gene has been always identified in association with the *bla*CTX-M-2-like genes, including the new alleles *bla*CTX-M-74 and *bla*CTX-M-75. The distance between the *ISCR1* element and the *bla*CTX-M-2 start codon varied; in 8 isolates (*E. coli* JF25, *E. coli* JF26, *E. coli* JF239, *E. coli* JF251, *E. coli* JF286, *P. stuartii* JF29, *K. pneumoniae* JF230, and *M. organii* 89), the distance between the right extremity of *ISCR1* and the *bla*CTX-M-2 gene was 266 bp. In the 4 *E. cloacae* isolates (isolates JF182, JF183, JF207, and JF216) and in the single *P. stuartii* isolate (JF244A), the *bla*CTX-M-2 gene was separated from *ISCR1* by 260 bp. Whereas a *P*_{ORI} promoter was identified in the region separating *ISCR1* from the *bla*CTX-M-2 gene as previously described by (Rodriguez-Martinez et al. 2006), a *P*_c promoter showing a variation in the –35 region (TTTTAC instead of TTATAC) was found in *K. pneumoniae* JF230. Downstream of the *bla*CTX-M-2-like genes, a sequence showing 99% identity with the downstream-located DNA sequence of the *bla*KLUA-1 gene from *Kluyvera ascorbata* was evidenced. This sequence was followed by a second copy of the 3'-CS belonging to a typical class 1 integron, as reported previously (Valverde et al., 2006). Overall, the genetic environment linked to the *bla*CTX-M genes was variable, showing that the dissemination of those genes in those isolates was not related to a single structure.

Plasmid DNA of enterobacterial isolates was extracted using the Kieser technique (Kieser, 1984). Conjugal transfer

Table 1

Sequences of primers used for detection of ESBL genes and their genetic environment (Lartigue et al., 2007)

Primer	Sequence	Location	Accession no. (start–stop)
CTX-MA1	5'-SCSATGTGCAGYACCAGTAA-3'	<i>bla</i> CTX-M gene	AF488377 (270–813)
CTX-MA2	5'-CCGCRATATGRTTGGTGGTG-3'	<i>bla</i> CTX-M gene, reverse primer	
CTXM2A	5'-GCCGCTCAATGTTAACGG-3'	CTX-M-2 group	EF592571 (6150–7003)
CTXM2B	5'-GAAACCGTGCGTTACGAT-3'	CTX-M-2 group, reverse primer	
CTXM9A	5'-CTGATGTAACACGGATTGAC-3'	CTX-M-9 group	AF252622 (1704–2599)
TOHO2b rev	5'-TTACAGCCCTTCGGCGAT-3'	CTX-M-9 group, reverse primer	
TEM α	5'-GAGTATTCAACATTTCCTGT-3'	<i>bla</i> TEM gene	EU418920 (648–1554)
TEM β	5'-TAATCAGTGAGGCACCTATCT-3'	<i>bla</i> TEM gene, reverse primer	
SHVA	5'-ATGCGTTATTCGCCTGTGT-3'	<i>bla</i> SHV gene	EU418908 (1–861)
SHVB	5'-TTAGCGTTGCCAGTGCTCG-3'	<i>bla</i> SHV gene, reverse primer	
CTX825F	5'-CGCTTTGCCATGTGCAGCACC-3'	<i>bla</i> CTX-M-8 gene	AF189721 (475–781)
CTX825R	5'-GCTCAGTACGATCGAGCC-3'	<i>bla</i> CTX-M-8 gene, reverse primer	
ISEcpPROM+	5'-TGCTCTGTGGATAACTTGC-3'	<i>ISEcp1</i>	AF252622 (1520–2599)
IS903bint	5'-GCTTTTTGACTTTCCACTCGC-3	IS903B transposase	
M2A3r	5'-ACCTTACTGGTACTGCACAT-3'	<i>bla</i> CTX-M-2 gene, reverse primer	EF592571 (5728–6360)
M2A5f	5'-CCAAATCGAACCTTATTAGAGC-3'	Orf513	
M2A4f	5'-CACAGTTGGTGACGTGGCTTAA-3'	<i>bla</i> CTX-M-2 gene, internal primer	EF592571 (6753–7611)
qacED1r	5'-CCTCCATGATACCGAGTGAGAA-3'	Orf3	
IS903bint	5'-GCTTTTTGACTTTCCACTCGC-3	IS903B transposase	

Table 2
Characteristics of the ESBL-producing isolates, including isolation dates, patient sex, and age

Isolates	Isolation date (month/day/year)	Patient age (years)	Sex	ESBL type	MIC values (µg/mL)								Coresistance phenotype	Transfer in <i>E. coli</i> recipients	Coresistance transferred with <i>bla</i> _{CTX-M} genes	Plasmid size (kb)	PFGE type
					FOX	CAZ	CTX	NAL	CIP	NOR	OFL	LVX					
<i>E. cloacae</i> JF182	6/8/2004	90	M	CTX-M-2	>128	64	>128	>128	32	64	16	8	NAL, CIP, SXT, TET	Transf.	TET	154	A
<i>E. cloacae</i> JF183	6/11/2004	68	F	CTX-M-2	>128	32	>128	>128	64	>128	32	32	NAL, CIP, GEN, SXT, TET	Conj.	SXT	154	B
<i>E. cloacae</i> JF207	8/11/2004	82	F	CTX-M-2	>128	64	>128	>128	64	>128	64	64	NAL, CIP, SXT, TET	Transf.	SXT	48	C
<i>E. coli</i> JF239	10/23/2004	35	F	CTX-M-2	8	4	128	>128	128	128	32	32	NAL, CIP, GEN, SXT, TET	Transf.	TET	154	H
<i>E. coli</i> JF25	11/3/2001	64	M	CTX-M-2	16	16	>128	>128	16	32	16	8	NAL, CIP, SXT, TET	Transf.	TET	154	I
<i>E. coli</i> JF251	11/16/2004	50	F	CTX-M-2	4	4	>128	>128	8	16	16	8	NAL, CIP, GEN, SXT, TET	Transf.	GEN	154	J
<i>E. coli</i> JF26	4/2/2002	47	F	CTX-M-2	8	8	>128	>128	8	32	16	8	NAL, CIP, TET	Transf.	None	114	L
<i>E. coli</i> JF286	1/24/2005	65	F	CTX-M-2	32	8	128	>128	64	128	128	64	NAL, CIP, GEN, SXT, TET	Transf.	None	154	N
<i>K. pneumoniae</i> JF230	10/4/2004	73	M	CTX-M-2	4	16	128	>128	16	64	32	16	NAL, CIP, SXT	Transf.	SXT	114	S
<i>M. morgani</i> JF89	12/26/2003	49	F	CTX-M-2	>128	8	128	>128	64	64	128	64	NAL, CIP, GEN, SXT	Transf.	GEN	48	ND
<i>P. stuartii</i> JF244	10/30/2004	88	M	CTX-M-2	4	16	128	>128	64	128	64	128	NAL, CIP, GEN, SXT, TET	Transf.	GEN	154	ND
<i>E. coli</i> JF213	8/25/2004	55	F	CTX-M-8	4	<1	8	>128	4	8	8	4	NAL, CIP, SXT, TET	Transf.	None	114	F
<i>E. coli</i> JF74	11/27/2003	60	F	CTX-M-8	8	128	16	>128	0.12	0.5	0.5	1	NAL, SXT, TET	Transf.	None	114	F
<i>C. freundii</i> JF130	3/22/2004	88	F	CTX-M-9	128	>128	8	>128	32	64	64	32	NAL, CIP, SXT, TET	Transf.	TET	114	ND
<i>E. coli</i> JF301	2/1/2005	80	F	CTX-M-9	32	16	>128	>128	32	32	16	8	NAL, CIP, GEN	Transf.	None	114	O
<i>K. pneumoniae</i> JF17	4/10/2002	8	F	CTX-M-9	32	8	64	>128	4	8	8	2	NAL, CIP, GEN, SXT, TET	Transf.	TET	114	Q
<i>E. cloacae</i> JF216	9/2/2004	82	F	CTX-M-74	>128	>128	>128	>128	16	64	16	8	NAL, CIP, SXT, TET	Conj.	SXT, TET	48	D
<i>P. stuartii</i> JF29	2/11/2002	48	F	CTX-M-75	32	<1	32	>128	32	>128	16	16	NAL, CIP, GEN, SXT, TET	Transf.	GEN, TET	180	ND
<i>E. cloacae</i> JF277	1/11/2005	51	M	SHV-5	>128	128	32	32	0.5	1	1	0.5	NAL, GEN	Conj.	GEN	60	E
<i>E. coli</i> JF220	9/15/2004	2	F	SHV-5	32	32	8	16	<0.03	<0.03	0.06	<0.03	NAL, SXT	Conj.	SXT	48	G
<i>K. pneumoniae</i> JF10	11/6/2001	40	F	SHV-5	4	>128	16	>128	4	16	4	4	NAL, SXT, GEN	ND	ND	ND	P
<i>K. pneumoniae</i> JF6	7/14/2001	29	M	SHV-5	8	>128	16	>128	8	64	16	8	NAL, GEN	ND	ND	ND	R
<i>K. pneumoniae</i> JF7	8/28/2001	47	F	SHV-5	8	128	16	>128	8	32	8	8	NAL, GEN	ND	ND	ND	R
<i>K. pneumoniae</i> JF16	3/15/2002	37	M	SHV-5	8	>128	32	>128	4	16	4	4	NAL, GEN	ND	ND	ND	R

M = male; F = female; FOX = ceftoxitin; CAZ = ceftazidime; CTX = cefotaxime; NAL = acid nalidixic; CIP = ciprofloxacin; NOR = norfloxacin; OFL = ofloxacin; LVX = levofloxacin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; GEN = gentamicin; Transf. = transfer by electroporation to *E. coli* TOP10; Conj. = transfer by conjugation to *E. coli* J53 Azi^R; ND = not determined.

of CTX-M determinants was performed in liquid culture media using an azide-resistant *E. coli* J53 (Azi^R) as recipient strain, and transformation of nonconjugative *bla*_{CTX-M} harboring plasmids was carried out by electroporation into *E. coli* TOP10. Plasmid DNAs of these transformants were extracted using the Kieser technique, and their size was estimated by comparison with reference plasmid DNAs of *E. coli* 50192 (Girlich et al., 2007). Hybridization experiments were performed after a Southern transfer from an agarose gel containing plasmid DNA of *E. coli* TOP10 electroporants and original *bla*_{CTX-M}-positive strains. Plasmids harboring *bla*_{CTX-M} genes ranged from 48 to 180 kb in size. Of the 18 *bla*_{CTX-M}-positive isolates, plasmid conjugation was obtained for only 2 (*E. cloacae* JF216 and JF183), with plasmids of different sizes (48 and 154 kb, respectively). The 16 other *bla*_{CTX-M}-positive plasmids were not self-transferable, although high molecular weight plasmids were visualized in donor strains by hybridization (data not shown) (Table 2).

The spread of *bla*_{CTX-M}-positive *E. coli* and *K. pneumoniae* isolates was not related to the spread of a single clone as reported in several studies focusing on community-onset infections (Ho et al., 2007; Lartigue et al., 2007), therefore, suggesting the dissemination of *bla*_{CTX-M}-associated mobile genetic elements. Genotyping performed by pulsed-field gel electrophoresis (PFGE) (Gauton, 1997; Tenover et al., 1995) identified 16 PFGE types, of which 14 were unique and 2 (F and R types) included more than 1 isolate (Table 2). Nine pulsotypes were identified for *E. coli*, 5 for *E. cloacae*, and 4 for *K. pneumoniae*. The isolates harboring CTX-M-2- and CTX-M-9-type determinants had distinct PFGE profiles, whereas 3 of the 4 *K. pneumoniae* isolates harboring the *bla*_{SHV-5} gene and recovered from different patients corresponded to a single clone (type R).

Two *E. coli* isolates harboring the *bla*_{CTX-M-8} gene identified among Brazilian isolates in 1997 (Bonnet et al., 2000) were also identified with the same PFGE profile, although they had been isolated from different patients.

This study provides additional information regarding the molecular epidemiology of plasmid-encoded ESBL determinants among enterobacterial isolates circulating in Brazilian community settings. The prevalence (9.3%) of ESBL producers among quinolone-resistant enterobacterial isolates mostly recovered from UTIs is high, considering the large diversity of bacterial species, enzymes, and genotypes circulating in the community. The finding of 2 novel variants, *bla*_{CTX-M-74} and *bla*_{CTX-M-75}, differing by single nucleotide substitutions from the most prevalent *bla*_{CTX-M} gene (*bla*_{CTX-M-2}) detected in Brazil suggests a local evolution of those resistance determinants.

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