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High Prevalence of *bla*$_{CTX-M}$ Extended Spectrum Beta-Lactamase Genes in *Klebsiella pneumoniae* Isolates from a Tertiary Care Hospital: First report of *bla*$_{SHV-12}$, *bla*$_{SHV-31}$, *bla*$_{SHV-38}$, and *bla*$_{CTX-M-15}$ in Brazil

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The aim of this study was to investigate the presence and prevalence of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ and *bla*$_{GES}$-like genes, responsible for extended spectrum beta-lactamases (ESBLs) production in clinical isolates of *Klebsiella pneumoniae* collected from a Brazilian tertiary care hospital. Sixty-five ESBL producing *K. pneumoniae* isolates, collected between 2005 and 2007, were screened by polymerase chain reaction (PCR). Identification of *bla* genes was achieved by sequencing. Genotyping of ESBL producing *K. pneumoniae* was performed by the enterobacterial repetitive intergenic consensus-PCR with cluster analysis by the Dice coefficient. The presence of genes encoding ESBLs was confirmed in 59/65 (90.8%) isolates, comprising 20 *bla*$_{CTX-M-2}$, 14 *bla*$_{CTX-M-59}$, 12 *bla*$_{CTX-M-15}$, 9 *bla*$_{SHV-12}$, 1 *bla*$_{SHV-2}$, 1 *bla*$_{SHV-2a}$, 1 *bla*$_{SHV-5}$, and 1 *bla*$_{SHV-31}$ genes. The ESBL genes *bla*$_{SHV-12}$, *bla*$_{SHV-31}$, and *bla*$_{CTX-M-15}$, and the chromosome-encoded SHV-type beta-lactamase capable of hydrolyzing imipenem were detected in Brazil for the first time. The analysis of the enterobacterial repetitive intergenic consensus-PCR band patterns revealed a high rate of multiclonal *bla*$_{CTX-M}$ carrying *K. pneumoniae* isolates (70.8%), suggesting that dissemination of encoding plasmids is likely to be the major cause of the high prevalence of these genes among the *K. pneumoniae* isolates considered in this study.

Introduction

Extended spectrum beta-lactamases (ESBL) producing bacteria are a leading cause of hospital-acquired infections worldwide.[52] Antibiotic resistance due to ESBLs production is an increasing problem, which has contributed to treatment failure with third-generation cephalosporins, increased mortality rates, and significant cost implications for healthcare systems.[44,45,47,61] Moreover, a multidrug-resistant profile is a frequent characteristic of ESBL producing microorganisms, because ESBLs are often encoded by genes located on large plasmids that also carry genes for resistance to other antimicrobial agents.[52] ESBLs were initially identified as variants of the common SHV-1 or TEM-1 beta-lactamase, often differing from the parent enzymes by only one or two amino acids. However, these early variants have been largely replaced by the CTX-M family of ESBLs,[7] and in the last years, the CTX-M enzymes have become the most prevalent ESBLs.[29,34,50] Organisms producing CTX-M have increasingly appeared in the hospital and community settings in European, African, Asian, South, and North American countries.[5,7,9,28,62] It has been shown that the broad dissemination of the CTX-M encoding genes is facilitated by its location in class 1 integrons bearing IS*CR1* insertion sequences or by the IS*Ecp*1 insertion sequences, often located in resistance cassettes carried by conjugative plasmids.[28] The CTX-M type ESBLs are originated from the chromosomal beta-lactamases of several species of the genus *Kluyvera* and can be divided, based on their amino acid identities, into the following five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25.[5,9] Also, ESBLs types such as PER, VEB, and GES, that

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are not closely related to any of these three established families have been increasingly isolated worlswide,[41] including in Brazil.[20]

Klebsiella pneumoniae is one of the major ESBL producers worldwide.[18,50] Despite the frequency of ESBL-producing *K. pneumoniae* being higher in Brazilian hospitals than in many European or United States hospitals,[31] few studies have been conducted to generate epidemiological data about ESBL-producing bacteria and ESBL genotypes. Thus, the main objective of this study was to investigate the prevalence and diversity of genes encoding ESBLs in clinical isolates of *K. pneumoniae* collected from patients admitted to a tertiary care hospital in Brazil.

Materials and Methods

Bacterial collection and susceptibility testing

A total of 65 *K. pneumoniae* isolates resistant to oxyimino-cephalosporins, collected over a period of 23 months (December 2005 to October 2007) in a teaching hospital in the northeast of São Paulo State, Brazil, were the subject of this study.

Bacterial identification and initial susceptibility testing were performed using the Microscan System (MicroScan WalkAway system; Dade Behring). Additionally, the minimal inhibitory concentrations (MICs) for the antibiotics aztreonam, cefotaxime, ceftazidime, ceftriaxone, cefepime, cefoxitin, imipenem and the associations ceftazidime/clavulanic acid, cefotaxime/clavulanic acid were determined using agar dilution method with Mueller–Hinton agar (Difco), and production of ESBL was phenotypically confirmed considering a $\geq$3 twofold decrease in MIC for ceftazidime and/or cefotaxime in combination with clavulanic acid versus its MIC when tested alone.[14] Antibiotics were purchased from Sigma-Aldrich. The control strains used for this study were *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603).

Detection and identification of bla *genes by polymerase chain reaction and gene sequencing*

Polymerase chain reaction (PCR) amplification and sequencing was performed for the ESBL producing *K. pneumoniae* with specific primers to search and identify bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{GES} genes. Primers and protocols previously described were used to amplify bla_{SHV},[58] bla_{TEM},[13] and $bla_{CTX\text{-}M}$,[21] and bla_{GES}.[54] The amplicon sizes for bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ genes were 861, 1,088, 544, and 864 bp, respectively (Table 1). Also, isolates presenting MIC for imipenem $\geq$1 μg/ml were submitted to PCR for bla_{KPC} detection.[65]

To determine the complete sequence of bla_{SHV}, a combination of previously described primers[58] was used. For bla_{TEM} and $bla_{CTX\text{-}M}$ full sequencing, primers were specifically designed, using DS Gene 2.0 Software (Accelrys). Before sequencing, PCR products containing $bla_{CTX\text{-}M}$ were submitted to restriction fragment length polymorphism analysis, using the restriction endonucleases PstI e PvuII,[21] to subtype according to the five CTX-M groups.[5] Restriction fragment length polymorphism subtyping was further confirmed by PCR using group specific primers.[32] This procedure allowed the design of $bla_{CTX\text{-}M}$ group specific sequencing primers to obtain a precise identification of the genes by amplification and sequencing of the whole open reading frame. The $bla_{CTX\text{-}M}$ templates for sequencing were amplified using primers and protocols previously described.[32,59,60]

Reactions for bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ sequencing were performed using the BigDye terminator kit (Applied Biosystems) and the following cycle parameters: 25 amplification cycles of 30 min at 96°C (denaturation), 15 s at 50°C (annealing), 4 min at 60°C (chain elongation) with final elongation at 5°C. Products were purified in ethanol according to methodology described elsewhere[60] and subjected to direct sequencing with the ABI PRISM 377 automated sequencer (Applied Biosystems). The products were aligned with Accelrys Gene 2.0 (Accelrys Software Inc. 2006). Database similarity searches were run with BLAST at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Molecular typing by enterobacterial repetitive intergenic consensus-PCR

The epidemiological relationships among *K. pneumoniae* isolates were analyzed by enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) using the primer ERIC2 and protocol previously described.[11] Cycling conditions were as follows: initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 8 min. The final elongation step was extended to 16 min at 72°C. The PCR products were visualized by UV transllumination after electrophoresis in 1.5% agarose (Invitrogen) gel and ethidium bromide staining. BioNumerics software (Applied Maths) was used for dendrogram construction and clustering, based on the band-based Dice's similarity coefficient and using the unweighted pair group method using arithmetic averages. Band position tolerance was of 2.0% and optimization of 0.5%. Isolates were considered to belong to the same cluster when similarity coefficient was $\geq$90%.[6]

Results

Bacterial collection and susceptibility testing

Clinical samples were collected from patients admitted to general medical wards or intensive care units (Figs. 1 and 2), except by one urine sample from an outpatient clinic. Bacterial isolates were originated from patients with respiratory tract infections, urinary tract infections, bloodstream infections, wound and soft tissues infections, and catheter tip.

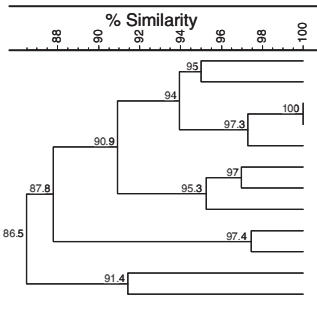
Table 2 shows the agar dilution MIC values of aztreonam, ceftazidime, cefotaxime, ceftriaxone, and the association ceftazidime/clavulanic acid and cefotaxime/clavulanic for *K. pneumoniae* isolates considered in this study. The isolates presented high MIC values of the oxyimino-cephalosporins. The only exception was isolate HB58, susceptible to ceftazidime, cefotaxime, and ceftriaxone. This isolate was included in this study, because Microscan results reported it as an ESBL producer (data not shown).

Detection and identification of bla *genes*

The $bla_{TEM\text{-}1}$ gene was detected in 43 isolates (66.1%). The bla_{SHV}-like gene was detected in all isolates, and 57 were identified by sequencing analysis: 19 $bla_{SHV\text{-}1}$, 20 $bla_{SHV\text{-}11}$, 9 $bla_{SHV\text{-}12}$, 2 $bla_{SHV\text{-}38}$, 1 $bla_{SHV\text{-}2}$, 1 $bla_{SHV\text{-}2a}$, 1 $bla_{SHV\text{-}5}$, 1 $bla_{SHV\text{-}25}$, 1 $bla_{SHV\text{-}31}$, and 1 $bla_{SHV\text{-}62}$. Only 13 isolates (20%)

Table 1. Primers Used During This Study

Primer name	*Sequences (5′-3′)*	*Annealing temperature (°C)*	*References*
	Amplification of bla_{SHV}		
KP-1	GGG TTA TTC TTA TTT GTC GC		
KP-2	GGT TAT GCG TTA TAT TCG CC	56°C	38
KP-3	TTA GCG TTG CCA GTG CTC		
	Amplification of bla_{TEM}		
TEM F	ATAAAATTCTTGAAGACGAAA	50°C	9
TEM R	GACAGTTACCAATGCTTAATCA		
	Amplification of bla_{CTX-M}		
CTX-M F	TTT GCG ATG TGC AGT ACC AGT AA	51°C	15
CTX-M R	CGA TAT CGT TGG TGG TGC CAT A		
	Amplification of $bla_{CTX-M-1}$ group		
CTX-MGroupI.F3	GACGATGTCACTGGCTGA GC	55°C	22
	Amplification of $bla_{CTX-M-2}$ group		
CTX-MGroupI.R2	AGC CGC CGA CGC TAA TAC A	55°C	22
	Sequencing of $bla_{CTX-M-1}$ group		
CTX-M GroupII.TOHO 1.2F	GCG ACC TGC TTA ACT ACA ATC	56°C	22
CTX-M GroupII.TOHO 1.1R	CGG TAG TAT TGC CCT TAA GCC		
	Sequencing of $bla_{CTX-M-2}$ group		
M2F	ATG ATG ACT CAG AGC ATT CG	56°C	39
M2R	TGG GTT ACG ATT TTC GCC GC		
	Sequencing of bla_{SHV}		
KP-4	GAA CAG CTG GAG CGA AAG AT		
KP-5	CAG ATC GGC GAC AAC GTC AC		
KP-6	CTG CAG TGG ATG GTG GAC GA		
KP-7	CCT GCT TGG CCC GAA TAA CA		
KP-8	GGG CCA AGC AGG GCG ACA AT	50°C	38
KP-9	TCG TCC ACC ATC CAC TGC AG		
KP-10	GTG ACG TTG TCG CCG ATC TG		
KP-11	ATC TTT CGC TCC AGC TGT TC		
KP-12	TAA TTT GCT CAA GCG GCT GC		
	Sequencing of bla_{TEM}		
TEM F 223	TCAACATTTTCGTGTCGC		
TEM R 253	AAAGGGAATAAGGGCGACAC		
TEM F 401	CGTTTTCCAATGATGAGCAC		
TEM R 396	TCGGGGCGAAAACTCTCAAG	50°C	This study
TEM F 592	CATGAGTGATAACACTGCTGC		
TEM R 615	TTGGCAGCAGTGTTATCACTC		
TEM F 799	ACTACTTACTCTAGCTTCCCG		
TEM R 830	TTAATTGTTGCCGGGAAGC		
	Sequencing of $bla_{CTX-M-1}$group		
M1seq65 F	GGT TAA AAA ATC ACT GCG TCA G		
M1seq272 F	GAT GTG CAG CAC CAG TAA AG		
M1seq480 F	AAG CTG ATT TCT CAC GTT GG		
M1seq650 F	TGG GTA AAG CAT TGG GTG AC	50°C	This study
M1seq825 F	AAA GAT CGT GCG CCG CTG ATT C		
M1seq772 R	TTA TCC CCC ACA ACC CAG GAA G		
M1seq400 R	TCC CAT CGA CGT GCT TTT C		
M1seq232 R	TCT GCT GTG TTA ATC AAT GCC		
	Sequencing of $bla_{CTX-M-2}$ group		
CTX-M 2 107 F	AGC TGG AAG CCC TGG AGA AAA G		
CTX-M 2 127 R	TTT TCT CCA GGG CTT CCA GC		
CTX-M 2 298 F	ATC AAG AAG AGC GAC CTG G		
CTX-M 2 301 R	TGA TTT CAA CGC GCT GAT TTA G	50°C	This study
CTX-M 2 510 F	CAC GCT CAA TAC CGC CAT TC		
CTX-M 2 528 R	AAT GGC GGT ATT GAG CGT GG		
CTX-M 2 698 F	TAG TGG GCG ATA AAA CCG GCA G		
CTX-M 2 700 R	CTA CCC ATG ATT TCG GCA GAC		
	ERIC Sequences		
ERIC-2	AAG TAA GTG ACT GGG GTG AGC G	52°C	7



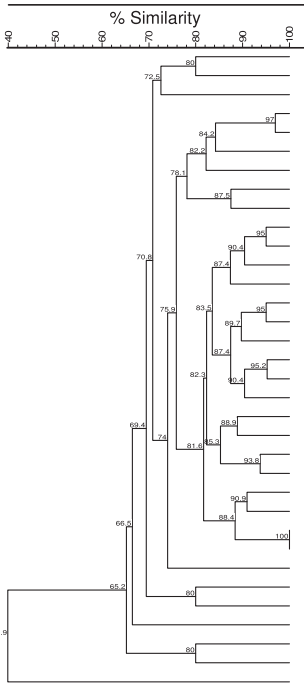
ERIC-2 profile	Cluster	Strain	Ward	Clinical sample	Date	bla_{ESBL}
	E_1I	HB91	P-ICU	Catheter tip	04/09/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1I	HB96	SICU	Urine	13/10/07	$bla_{CTX-M-15}$, bla_{SHV-11}
	E_1I	HB76	E-ICU	Trachael aspirate	25/05/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1I	HB80	C-ICU	Blood	21/09/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1I	HB84	N-D	Urine	07/10/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1I	HB89	E-ICU	Blood	24/08/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1I	HB92	N-D	Urine	11/10/07	$bla_{CTX-M-15}$, bla_{SHV-5}, bla_{TEM-1}
	E_1I	HB100	G-ICU	Catheter tip	22/10/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1II	HB65	G-ICU	CSF	06/05/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1II	HB74	C-ICU	Urine	15/05/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}
	E_1III	HB54	G-D	Blood	06/04/07	$bla_{CTX-M-15}$, bla_{SHV*}
	E_1III	HB82	G-ICU	Blood	25/09/07	$bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1}

FIG. 1. Dendrogram with Dice coefficients of enterobacterial repetitive intergenic consensus-polymerase chain reaction patterns of $bla_{CTX-M-15}$ harboring *Klebsiella pneumoniae* included in this study. G-D, gasthroenterology department; N-D, nephrology department; G-ICU, geral intensive care unit; P-ICU, pediatric intensive care unit; SICU, semi-intensive care unit; E-ICU, emergence room intensive care unit; C-ICU, cardiology intensive care unit.

carried bla_{SHV} type that code for an ESBL (bla_{SHV-2}, bla_{SHV-2a}, bla_{SHV-5}, bla_{SHV-12}, and bla_{SHV-31}). For isolate HB54, we did not obtain the full sequence of bla_{SHV}, making gene identification impossible. The bla_{CTX-M} genes were detected in 46 isolates (79.3%) representing the most prevalent ESBL coding gene in this study. No bla_{GES} or bla_{KPC} were detected. The sequencing analysis revealed the presence of three different bla_{CTX-M} genotypes; bla_{CTX-15}, belonging to bla_{CTX-M} group 1, detected in 12 isolates, and $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$, belonging to bla_{CTX-M} group 2, detected in 20 and 14 isolates, respectively. A high percentage (43.4%) of *K. pneumoniae* harboring $bla_{CTX-M-2}$, $bla_{CTX-M-59}$, and $bla_{CTX-M-15}$ isolated from urine samples was observed (Figs. 1 and 2). We did not detect ESBL encoding genes in isolates HB31, HB35, HB 48, HB49, HB68, and HB71 presenting high MIC values for third generation cephalosporins and/or a positive phenotypic test for ESBL production.

Molecular typing by ERIC-PCR

Since bla_{CTX-M} was the most prevalent ESBL type detected in this study, molecular typing by ERIC-PCR was performed to determine the genetic relatedness between isolates harboring $bla_{CTX-M-15}$ and also between isolates harboring the related $bla_{CTX-M-2}$ and $bla_{CTX-M-29}$ genes. The bla_{SHV} genes detected in some isolates harboring bla_{CTX-M} were considered in parallel. Four different clusters of $bla_{CTX-M-15}$ harboring *K. pneumoniae*, designated E_1I to E_1IV (Fig. 1) were identified. Among the strains harboring group 2 bla_{CTX-M} genes ($bla_{CTX-M-2}$ and $bla_{CTX-M-59}$), a wide diversity of genotypes distributed among



ERIC-2 profile	Cluster	Strain	Ward	Clinical sample	Date	bla_{ESBL}
	I	HB15	ER	Urine	07/02/06	bla_{CTX-M2}, bla_{SHV-11}
	II	HB97	N-D	Urine	15/10/07	bla_{CTX-M2}, bla_{SHV-1}, bla_{TEM-1}
	III	HB02	ER	Urine	05/12/05	$bla_{CTX-M59}$, bla_{SHV-1}, bla_{TEM-1}
	IV	HB94	N-D	Urine	11/10/07	bla_{CTX-M2}, bla_{SHV-1}
	IV	HB99	G-D	Urine	15/10/07	$bla_{CTX-M59}$, bla_{SHV-1}, bla_{TEM-1}
	V	HB04	G-ICU.	Urine	05/12/05	$bla_{CTX-M59}$, bla_{SHV-12}, bla_{TEM-1}
	VI	HB58	CS-D	Urine	07/04/07	$bla_{CTX-M59}$, bla_{SHV-11}, bla_{TEM-1}
	VII	HB40	CS-D	Urine	13/10/06	bla_{CTX-M2}, bla_{SHV-11}
	VIII	HB50	OP-C	Urine	22/03/07	bla_{CTX-M2}, bla_{SHV-11}
	IX	HB24	P-ICU	Blood	19/06/06	$bla_{CTX-M59}$, bla_{SHV-12}, bla_{TEM-1}
	IX	HB29	SICU	Endotracheal secretion	08/08/06	$bla_{CTX-M59}$, bla_{SHV-1}, bla_{TEM-1}
	IX	HB28	G-ICU	Urine	19/07/06	bla_{CTX-M2}, bla_{SHV-1}
	X	HB12	G-ICU	Urine	15/02/06	$bla_{CTX-M59}$, bla_{SHV-1}, bla_{TEM-1}
	XI	HB01	CS-D	Urine	04/12/05	$bla_{CTX-M-2}$, bla_{SHV-11}, bla_{TEM-1}
	XI	HB83	N-ICU	Urine	05/10/07	$bla_{CTX-M59}$, bla_{SHV-11}, bla_{TEM-1}
	XII	HB14	ER	Urine	07/02/06	bla_{CTX-M2}, bla_{SHV-11}, bla_{TEM-1}
	XIII	HB77	P-ICU	Blood	02/10/07	bla_{CTX-M2}, bla_{SHV-38}, bla_{TEM-1}
	XIII	HB78	P-ICU	Blood	26/09/07	bla_{CTX-M2}, bla_{SHV-38}, bla_{TEM-1}
	XIII	HB66	CS-D	Peritoneal fluid	07/05/07	bla_{CTX-M2}, bla_{SHV-11}, bla_{TEM-1}
	XIV	HB19	P-ICU	Pleural fluid	23/03/06	$bla_{CTX-M59}$, bla_{SHV-12}, bla_{TEM-1}
	XV	HB20	P-ICU	Urine	28/03/06	$bla_{CTX-M59}$, bla_{SHV-11}, bla_{TEM-1}
	XVI	HB17	CS-D	Wound secretion	27/02/06	$bla_{CTX-M59}$, bla_{SHV-11}, bla_{TEM-1}
	XVI	HB21	CS-D	Biliar fluid	10/04/06	bla_{CTX-M2}, bla_{SHV-12}
	XVII	HB13	N-ICU	Blood	17/02/06	bla_{CTX-M2}, bla_{SHV-11}
	XVII	HB33	E-ICU	Pleural fluid	28/08/06	bla_{CTX-M2}, bla_{SHV-11}
	XVIII	HB16	E-ICU	Endotracheal secretion	21/02/06	bla_{CTX-M2}, bla_{SHV-11}
	XVIII	HB18	G-ICU	Endotracheal secretion	18/02/06	bla_{CTX-M2}, bla_{SHV-11}
	XIX	HB90	P-ICU	Pleural fluid	24/08/07	bla_{CTX-M2}, bla_{SHV-1},
	XX	HB64	G-ICU	Endotracheal secretion	25/04/07	bla_{CTX-M2}, bla_{SHV-11}
	XXI	HB67	ID-D	Inguinal swab	07/05/07	$bla_{CTX-M59}$, bla_{SHV-11}, bla_{TEM-1}
	XXII	HB47	E-ICU	Peritoneal fluid	20/03/07	bla_{CTX-M2}, bla_{SHV-11}
	XXIII	HB03	N-Dep	Blood	01/12/05	$bla_{CTX-M59}$, bla_{SHV-12}
	XXIV	HB62	G-ICU	Blood	25/04/07	bla_{CTX-M2}, bla_{SHV-11}
	XXV	HB32	C-ICU	Urine	20/08/06	$bla_{CTX-M59}$, bla_{SHV-12}, bla_{TEM-1}

FIG. 2. Dendrogram with Dice coefficients of enterobacterial repetitive intergenic consensus-polymerase chain reaction patterns of $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$ harboring *K. pneumoniae* included in this study. ER, emergence room; OP-C, outpatient clinic, CS-D cardiac surgery department; ID-D, infectious diseases department; N-ICU, neonatal intensive care unit.

25 clusters, designated E_2I to E_2 XXV was observed (Fig. 2). Clusters included only one to few isolates, and isolates within each of these clusters had similarity coefficient ranging from 90.9% to 100%.

Discussion

The high MIC values observed for aztreonam, ceftazidime, cefotaxime, ceftriaxone, and the associations ceftazidime/clavulanic acid, cefotaxime/clavulanic acid are typical of ESBL producers.[14,44] Almost all isolates carried at least one ESBL genetic determinant belonging to the bla_{CTX-M} or bla_{SHV} families. The bla_{TEM-1} gene, carried by 43 isolates codes for a narrow spectrum beta-lactamase.[27]

The detection of bla_{SHV} type in all isolates confirms its ubiquity in *K. pneumoniae*.[2] However, in this study, SHV type ESBLs were not the most important cause of cephalosporin resistance among the isolates and presented low prevalence (13/58). The ESBL encoding genes bla_{SHV-12} and bla_{SHV-31}, and bla_{SHV-38}, a chromosome-encoded SHV-type β-lactamase capable of hydrolyzing imipenem[55] were detected and are described for the first time in Brazil.

The detection of bla_{CTX-M} genes in 79.3% of the isolates indicate that CTX-M-type are the main ESBL enzymes produced in our isolates and support the recognition of CTX-M as the most prevalent type of ESBL in the world.[7–9,32,48,50,52] The high percentage of *K. pneumoniae* harboring $bla_{CTX-M-2}$, $bla_{CTX-M-59}$, and $bla_{CTX-M-15}$ isolated from urine samples is worrisome, as this bacteria is a well-described pathogen associated with urinary tract infections in hospitalized and outpatients,[38] and community-acquired urinary tract infections have been related to previous acquisition during hospital stay.[17]

The $bla_{CTX-M-15}$ gene was detected in 18.5% of isolates, and except by isolate HB92 that carried a bla_{SHV-5},[4] none of the $bla_{CTX-M-15}$ carriers presented other ESBL genes. The CTX-M-15 ESBL, first described in 2001[30] has been described from all continents except Antartica and recently emerged as the dominant type of CTX-M type ESBL in Gram-negative pathogens causing outbreaks in nosocomial as well as community settings.[15,22,51] To our knowledge, this is the first report of this ESBL gene in Brazil. The emergence of this CTX-M variant in the country is worrisome, because it presents strong activity against ceftazidime and cefepime[37,55] and spreads fast,[33] as it efficiently mobilizes among unrelated strains by different genetic mobile elements,[1] is flanked by insertion sequences that facilitate the hyperexpression[35,40] and, as recently reported, is able to integrate in the chromosome.[15]

The $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$ genes were detected in isolates from patients admitted to different hospital wards over the 23 months (Fig. 2). The $bla_{CTX-M-2}$ was the most common ESBL gene in this study, followed by $bla_{CTX-M-59}$, detected in 30%, 8% and 21%, 5% of the isolates, respectively. The $bla_{CTX-M-59}$, first described in 2008 during an outbreak of *K. pneumoniae* in a neonatal care unit in Brazil, is a novel variant of $bla_{CTX-M-2}$, leading to a His89Leu substitution.[24] The highest activity for both enzymes was cefotaxime. The $bla_{CTX-M-2}$ gene has been detected in several South American countries,[9,62] including Brazil,[19,39] and the previous detection of $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$ in a different hospital in Brazil suggests that these genes may be disseminated in the country. Actually, rapid spread of $bla_{CTX-M-2}$ was previously observed in Argentina, where CTX-M-2 was first described in 1992[3] and is now the most prevalent CTX-M type ESBL.[57]

Interestingly, we did not detect ESBL encoding genes in some isolates (HB31, HB35, HB48, HB49, HB68, and HB71) presenting high MIC values for third-generation cephalosporins and/or a positive phenotypic test for ESBL production. In these bacteria, other clinically relevant types of ESBLs such as VEB and PER,[23,41] or other resistance mechanisms such as decreased outer membrane permeability, hiperexpression of efflux pumps, and production of plasmid mediated AmpC beta-lactamases may be present.[27,48,49] Various isolates included in this study as well as isolates HB48, HB49, HB68, and HB71 presented MIC values for imipenem higher than 1 μg/ml (data not shown) and were investigated for the presence of the bla_{KPC} gene. KPC confer decreased susceptibility or resistance to virtually all beta-lactams and is the most important carbapenemase, particularly in *K. pneumoniae*.[16,25,42] Although KPC has already been reported in Brazil,[46,66] it was not detected in this study. Beyond other possible mechanisms, we suspect that an association between CTX-M production and porin loss may be responsible for the decrease in susceptibility to imipenem, as previously reported.[36,64]

The high coefficient of similarity among isolates harboring $bla_{CTX-M-15}$ within each cluster suggests a close genetic relationship. Also, the lowest similarity coefficient among the four clusters was 82.4%, indicating that they may be closely related.[51] Thus, we believe that the occurrence of $bla_{CTX-M-15}$ is due to dissemination of mobile genetic elements among genetically related isolates or clusters of *K. pneumoniae*, as plasmids encoding ESBLs are efficiently transferred among *Klebsiella* spp.[59] and dissemination of bla_{CTX-M} genes is facilitated by its location in class 1 integrons.[26] In fact, the predominance of bla_{CTX-M} genes in hospital settings due to the spreading of CTX-M encoding plasmids and mobile genetic elements has already been reported.[43,55,63] Clonal spread could be inferred only for strains HB76 and HB80, which presented 100% similarity. Although we did not determine any epidemiological relationship between these isolates, obtained from patients admitted to different hospital wards at different times, we consider that transmission through colonized healthcare workers may have been the cause, as this is a well-known route of transmission.[12]

The isolates harboring the $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$ genes were included in a wide diversity of clusters (Fig. 2), and even when high similarity coefficients were observed, isolates presented different bla_{CTX-M} and even bla_{SHV} genes. This observation gives support to the belief that the occurrence of $bla_{CTX-M-2}$ and $bla_{CTX-M-59}$ in the hospital during the study is due to dissemination of encoding plasmids, as previously reported in a study including strains isolated from surveillance cultures obtained during an outbreak in a Brazilian hospital in 2004, when different clones of *K. pneumoniae* carrying the $bla_{CTX-M-59}$ gene were detected. The authors showed that in addition to spread of each clonal group, dissemination of identical or related plasmids harboring the CTX-M-type ESBL genes among different clonal groups was responsible for the persistence of ESBL producing *K. pneumoniae* overtime.[24] In this study, we could not determine whether the $bla_{CTX-M-59}$ variant originated from its parental $bla_{CTX-M-2}$ by mutation within the hospital setting or was introduced from an outside source, as its detection was concomitant with the detection of $bla_{CTX-M-2}$. The unique evidence of clonal dissemination was noted for isolates in

Table 2. Agar Dilution Minimal Inhibitory Concentrations of Antibiotics for *Klebsiella pneumoniae*

	MIC (μg/ml)							
Strain n°	*AZT (R: ≥32 S: ≤8)*	*CAZ (R: ≥32 S: ≤8)*	*CRO (R: ≥64 S: ≤8)*	*CTX (R: ≥64 S: ≤8)*	*CAZ/CLA*	*CTX/CLA*	bla *genotype*	*Accession No.*
HB-01	512	128	>512	512	2.0	128	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815243; GQ387358
HB-02	>512	512	>512	512	2.0	128	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}1}$	FJ815244; GQ380692
HB-03	>512	256	256	256	8.0	64	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}12}$	FJ815245; GQ389700
HB-04	>512	256	512	256	8.0	64	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}12}$	FJ815246; GU064382
HB-12	256	16	>512	>512	1.0	64	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}1}$	FJ815247; GQ389701
HB-13	512	128	>512	>512	0.5	32	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815248; GQ389702
HB-14	256	64	32	16	0.5	0.5	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815249; GQ389703
HB-15	64	32	4.0	2.0	0.5	0.5	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815250; GQ389704
HB-16	128	16	256	128	1.0	32	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815251; GQ389705
HB-17	256	16	>512	512	1.0	64	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}11}$	FJ815252; GQ389706
HB-18	128	16	>512	512	1.0	16	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815253; GQ389707
HB-19	256	128	128	256	2.0	1.0	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}12}$	FJ815254; GQ389708
HB-20	64	64	128	128	0.5	4.0	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}11}$	FJ815255; GQ407109
HB-21	>512	256	32	16	0.5	0.5	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}12}$	FJ815256; GQ407110
HB-24	>512	256	>512	512	8.0	128	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}12}$	FJ815257; GQ407111
HB-28	64	8.0	>512	512	1.0	32	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}1}$	FJ815258; GQ407112
HB-29	512	128	>512	256	2.0	64	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}1}$	FJ815259; GQ407113
HB-31	512	256	64	128	0.5	0.5	$bla_{SHV\text{-}25}$	GU064391
HB-32	64	32	>512	256	0.25	16	$bla_{CTX\text{-}M\text{-}59}$, $bla_{SHV\text{-}12}$	FJ815260; GQ407114
HB-33	256	32	>512	>512	0.25	128	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	J815261; GQ407115
HB-35	256	128	32	64	8.0	0.5	$bla_{SHV\text{-}11}$	GQ407117
HB-40	128	32	4.0	64	0.5	0.5	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815262; GU064383
HB-41	64	64	128	16	0.25	0.5	bla_{SHV}like	NA
HB-43	0.5	64	128	4.0	0.25	0.5	$bla_{SHV\text{-}1}$	GU083598
HB-47	256	32	>512	512	2.0	128	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815263; GU064384
HB-48	0.5	1.0	128	64	0.25	0.5	$bla_{SHV\text{-}1}$	GU083599
HB-49	512	512	64	16	0.25	2.0	$bla_{SHV\text{-}11}$	GU064392
HB-50	128	32	>512	512	1.0	32	$bla_{CTX\text{-}M\text{-}2}$, $bla_{SHV\text{-}11}$	FJ815264; GU064385
HB-52	>512	256	32	32	0.25	0.5	$bla_{SHV\text{-}12}$	GU064391
HB-53	256	64	128	64	0.5	0.5	bla_{SHV}like	NA
HB-54	512	256	>512	>512	0.5	4.0	$bla_{CTX\text{-}M\text{-}15}$, bla_{SHV}like	FJ815265;

HB-56	512	256	64	32	0.25	0.5	bla_{SHV-2a}	GU064394
HB-58	16	1.0	2.0	0.5	0.25	0.5	$bla_{TX-CM-59}$, bla_{SHV-11}	FJ815266; GU064386
HB-61	512	256	128	64	0.25	0.5	bla_{SHV-62}	GU064395
HB-62	256	32	>512	>512	1.0	64	$bla_{CTX-M-2}$, bla_{SHV-11}	FJ815267; GU064387
HB-63	2.0	16	0.5	32	0.5	0.5	bla_{SHV-31}	GU064396
HB-64	512	64	>512	>512	0.5	64	$bla_{CTX-M-2}$, bla_{SHV-11}	FJ815268; GU064388
HB-65	256	128	512	256	0.5	32	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815269; GU064389
HB-66	128	32	>512	>512	4.0	128	$bla_{CTX-M-2}$, bla_{SHV-11}	FJ815270; GU064390
HB-67	64	32	>512	512	1.0	32	$bla_{CTX-M-59}$, bla_{SHV-11}	FJ815271; GQ407118
HB-68	32	32	128	64	0.25	4.0	bla_{SHV-1}	GQ407119
HB-70	256	64	64	64	0.25	0.5	bla_{SHV-2}	GQ407120
HB-71	128	128	128	64	0.25	16	bla_{SHV-1}	GQ407121
HB-74	256	64	512	512	0.5	32	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815272; GQ407122;
HB-75	256	128	32	64	0.25	0.5	bla_{SHV}-like	NA
HB-76	256	128	512	512	0.5	32	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815273; GQ407123
HB-77	64	64	512	256	0.5	2.0	$bla_{CTX-M-2}$, bla_{SHV-38}	FJ815274; GQ407124
HB-78	128	64	>512	>512	0.5	16	$bla_{CTX-M-2}$, bla_{SHV-38}	FJ815275; GQ407125
HB-79	256	32	2.0	64	0.25	0.5	bla_{SHV-11}	GQ407126
HB-80	256	128	>512	512	0.5	32	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815276; GQ407127
HB-82	128	64	512	256	0.5	32	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815277; GQ407129
HB-83	512	128	>512	>512	4.0	32	$bla_{CTX-M-59}$, bla_{SHV-11}	FJ815278; GQ407130
HB-84	256	128	>512	>512	1.0	64	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815279; GQ407131
HB-86	256	256	64	16	8.0	32	bla_{SHV-1}	GQ407133
HB-87	512	256	256	128	0.5	1.0	bla_{SHV-12}	GQ407134
HB-88	>512	512	64	128	0.5	0.5	bla_{SHV-12}	GQ407135
HB-89	256	128	>512	256	1.0	NT	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815280; GQ407136
HB-90	256	64	>512	256	0.5	64	$bla_{CTX-M-2}$, bla_{SHV-1}	FJ815281; GQ407137
HB-91	512	128	>512	512	1.0	4.0	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815282; GQ407138
HB-92	512	128	>512	256	1.0	128	$bla_{CTX-M-15}$, bla_{SHV-5}	FJ815283; GQ407139
HB-94	512	64	>512	512	8.0	0.5	$bla_{CTX-M-2}$, bla_{SHV-1}	FJ815284; GQ407140
HB-96	>512	256	>512	>512	8.0	0.5	$bla_{CTX-M-15}$, bla_{SHV-11}	FJ815285; GQ407141
HB-97	256	32	>512	256	4.0	>128	$bla_{CTX-M-2}$, bla_{SHV-1}	FJ815286; GQ407142
HB-99	512	128	>512	512	8.0	128	$bla_{CTX-M-59}$, bla_{SHV-1}	FJ815287; GQ407144
HB-100	512	128	>512	256	1.0	128	$bla_{CTX-M-15}$, bla_{SHV-1}	FJ815288; GQ407145

AZT, Aztreonam; CAZ, Ceftazidime; CRO, Ceftriaxone; CTX, Cefotaxime; CAZ/CLA, Ceftazidime + Clavulaninc Acid; CTX/CLA, Ceftazidime+ Clavulanic Acid; NA: not applicable; MIC, minimal inhibitory concentration.

cluster E_2XVIII, where HB16 and HB18 presented 100% similarity and the same diversity of *bla* ($bla_{CTX\text{-}M\text{-}2}$ and $bla_{SHV\text{-}11}$) genes. Other ESBL encoding genes ($bla_{SHV\text{-}2}$ and $bla_{SHV\text{-}12}$) were detected among the $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}59}$ carrying isolates at lower frequencies. These genes are likely to be in constant mobilization among different resistance plasmids, as they are associated to insertion sequences IS*26*.[10,56] To ascertain the mechanisms of transmission and persistence of ESBL genes among Gram-negative bacteria in our institution, studies to identify and characterize mobile elements such as plasmids, insertion sequences, transposons, and integrons are of great importance and are under current investigation. In this study, we observed a high rate of $bla_{CTX\text{-}M}$ carriage among *K. pneumoniae* isolates, and the $bla_{CTX\text{-}M\text{-}15}$ gene was detected for the first time in Brazil. These results, associated with the reported detection of CTX-M enzymes in other Brazilian hospitals and in the community, are a public-health concern and reinforce the requirement for an increase in monitoring, transmission control measures and, policy for antibiotics prescription.

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Disclosure Statement

No competing financial interests exist.

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