Diversity of genotypes in CTX-M-producing *Klebsiella pneumoniae* isolated in different hospitals in Brazil

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ABSTRACT

Objective: The present study was undertaken to characterize CTX-M ESBL-producing *Klebsiella pneumoniae* collected from hospitals in different cities of Brazil. **Material and Methods:** Eighty-five *K. pneumoniae* strains isolated from hospitalized patients in six different hospitals of three cities of Brazil were analyzed. ESBL production was confirmed by the standard double-disk synergy test and the Etest®. The MIC_{50} and MIC_{90} for ESBL-producing isolates were determined by the Etest® method. The antimicrobial susceptibilities of bacterial isolates were determined using the agar diffusion method according to the CLSI. Screening for $bla_{TEM,}$ $bla_{SHV,}$ bla_{CTX-M} genes and class 1 integron was performed by PCR amplification. To determine the genomic diversity of CTX-M-producers, isolates were analyzed by macrorestriction profile analysis following PFGE. **Results and Discussion:** Seventy-one *K. pneumoniae* isolates were ESBL-producing. PCR and sequencing experiments detected 38 CTX-M-producing *K. pneumoniae* belonged to groups CTX-M 1, CTX-M 2, CTX-M 8 and CTX-M 9. The association of different types ESBL (CTX-M, SHV and TEM) was frequent. All *K. pneumoniae* isolates carried class 1 integron. PFGE analysis revealed thirty-one clonal types among CTX-M-producing isolates. The data presented herein illustrate the diversity of genotypes of CTX-M producing *K. pneumoniae* among Brazilians hospitals.

Keywords: *Klebsiella pneumoniae*; β-lactamases; genotype.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen that has emerged as an important cause of hospital-acquired infections, especially in hospitalized immunocompromised patients with severe underlying diseases or admitted to neonatal intensive-care units.[1] The worldwide spread of plasmid-encoded extended-spectrum β-lactamases (ESBLs)-producing *Klebsiella* strains which are resistant to the bactericidal activity of all cephalosporins is also considered a great threat.[2] Various types of ESBL have been described worldwide.[3] In particular, CTX-M-type ESBLs have become the prevailing non-TEM, non-SHV ESBL among Enterobacteriaceae and is recognized as a rapidly growing family of ESBLs that preferentially hydrolyze cefotaxime rather than ceftazidime.[2,4,5]

In recent studies, high rates of CTX-M enzymes among ESBL-producing *K. pneumoniae* isolates have been reported from South America, Asia and Europe.[5-8] In some of those settings, rates of CTX-M-production as high as 58.5% in *K. pneumoniae* have been reported,[7] and South America appears as an important source of CTX-M type ESBL.[9,10]

In the present study we describe the molecular characterization of CTX-M-producing *K. pneumoniae* clinical strains isolated from six different hospitals in three cities of Brazil.

MATERIAL AND METHODS

Bacterial isolates

We analyzed 85 non-replicate *K. pneumoniae* strains isolated from hospitalized patients in six different hospitals of three cities of Brazil (Niterói, Rio de Janeiro, and São Paulo), from May 2003 to September 2006. The isolates were recovered from urine (n = 25), blood (n = 25),

pulmonary secretion (n = 5), catheter (n = 5), and other sites (n = 25). Preliminary identification of the isolates was accomplished using the Vitek® (bioMérieux) automated system and established biochemical procedures.

Antimicrobial susceptibility testing

ESBL production was confirmed by the standard double-disk synergy test and the Etest® (ceftazidime/ceftazidime + clavulanic acid) (AB Biodisk, Solna, Sweden). The MIC_{50} (minimum concentration capable to inhibit 50% of the isolates) and MIC_{90} (minimum concentration capable to inhibit 90% of the isolates) values of five antimicrobial agents (aztreonam, cefepime, ceftazidime, cefotaxime, and imipinem) for ESBL-producing isolates were determined by the Etest® method. All *K. pneumoniae* isolates were also tested against the following antimicrobial agents: gentamicin (CN); amikacin (AK); norfloxacin (NOR); ciprofloxacin (CIP); aztreonam (ATM); cefepime (FEP); imipinem (IPM); and trimethoprim-sulphametoxazole (SXT) by the disk diffusion method, and the results were interpreted based on the CLSI guidelines.[11] Quality control was carried out using standard strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27953), *Staphylococcus aureus* (ATCC 27953), and *Klebsiella pneumoniae* (ATCC 700803).

Characterization of β-lactamase-encoding genes

Screening for resistance genes was performed by PCR amplification using previously reported conditions and primer sets for detection of bla_{TEM}, bla_{SHV},[12] $bla_{CTX\text{-}M}$[13] and class 1 integron.[14] PCR products were sequenced with the ABI PRISM Dye Terminator Cycle Sequecing Ready Reaction Kit on a 3730 DNA Sequence Analyzer (Applied Biosystem). Obtained sequences were aligned and compared with those in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Pulsed-field gel electrophoresis (PFGE) typing

Clonal relationships were studied by pulsed field gel electrophoresis (PFGE) of SpeI-digested genomic DNA with a CHEF DRII apparatus (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).[15] Band patterns were compared visually and interpreted according to the criteria established by Tenover et al.,[16] and analyzed with BioNumerics v.4.0 software (Applied Maths, Sint-Martins-Latem, Belgium). Isolate clustering were performed by the unweighted pair group method using arithmetic averages (UPGMA) in combination with Dice similarity coefficient.

RESULTS

Of 85 *K. pneumoniae* isolates, 71 (84%) were ESBL-producing bacteria as determined by the double-disk synergy test and the Etest®. In 71 ESBL-producing isolates, the MIC_{50}/MIC_{90} values (μg/mL) of aztreonam, cefepime, ceftazidime, cefotaxime and imipinem were 16/256, 6/16, 32/256, 32/256 and 0.125/0.19, respectively. In this study, a multiresistant pattern was observed in ESBL-producing *K. pneumoniae* isolates and we detected the co-resistance of gentamicin (70%), amikacin (36%), norfloxacin (46%), ciprofloxacin (49%), aztreonam (68%), cefepime (41%), and trimethoprim-sulphametoxazole (71%).

Sixty-five (92%) of the isolates were positive for bla_{TEM} and 32 (45%) were positive for bla_{SHV}. All ESBL-producing isolates had class 1 integron. The genetic analysis of these isolates by PCR revealed that 38 of 71 (54%), from four different hospitals (Niterói and Rio de Janeiro cities), were positive for the CTX-M gene. The remaining 33 isolates (46%) were negative for this gene and these CTX-M gene-negative isolates showed other ESBL type (SHV and TEM) (Table 1). The association of different types of ESBL was frequent.

Considering the CTX-M-producers, 53% of these co-produced TEM and SHV, and 47% co-produced only TEM. It was possible to identify 4 CTX-M clusters. The genotyping of 38 CTX-M gene-positive isolates showed that 1 (3%), 23 (61%), 7 (18%) and 4 (11%) isolates belonged to groups CTX-M 1, CTX-M 2, CTX-M 8 and CTX-M 9, respectively. The remaining three isolates were not classified into these groups.

The PFGE analysis of CTX-M-producing *K. pneumoniae* isolates showed 31 clonal types, A – EE, considering the genetic relatedness strain (defined as Dice coefficients of < 85%) (Figure 1). Based on these data, a diversity of clones within the hospitals was found. However, two hospitals (HU1 ad HU5) presented the same clonal group of CTX-M-2, characterized as genotype O. All CTX-M-producers genotypes were characterized as multidrug-resistant (Table 2).

Table 1. Frequency of β-lactamases genes detected in isolates (n = 71)

β-lactamases genes	Number of isolates (%)
$bla_{CTX\text{-}M}$	3 (4)
$bla_{CTX\text{-}M}$; bla_{TEM}	18 (26)
$bla_{CTX\text{-}M}$; bla_{TEM}; bla_{SHV}	20 (29)
$bla_{CTX\text{-}M}$; bla_{SHV}	0 (0)
bla_{TEM}	18 (26)
bla_{TEM}; bla_{SHV}	9 (13)
bla_{SHV}	3 (4)
Total	71 (100)

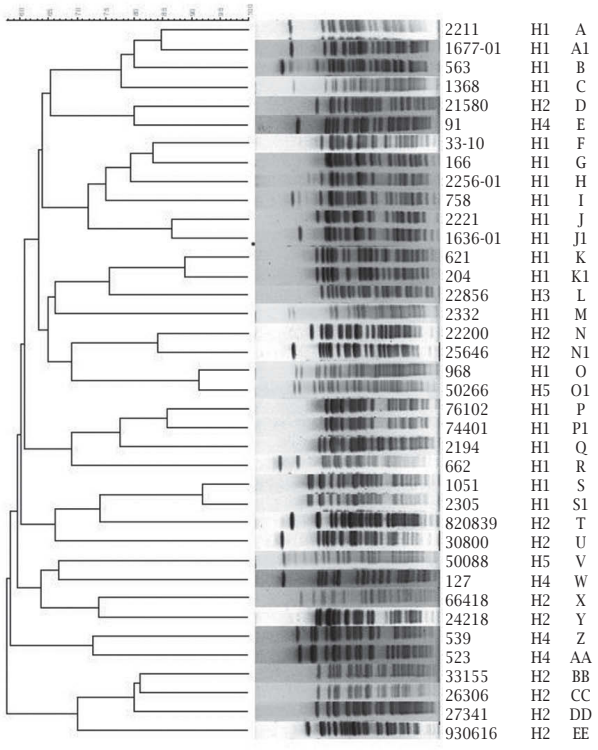

Figure 1: PFGE of SpeI-digested DNA of 38 CTX-M-producing *Klebsiella pneumoniae* isolates.

DISCUSSION

The prevalence of *K. pneumoniae* producing ESBL varies between different countries and regions. The clinical relevance of multidrug resistance among ESBL-producing *Klebsiella* spp. is of great concern due to the limited therapeutic options and increased risk of treatment failure in patients infected with such strains.[17] In Brazil, there has been some reports of multi-drug resistant hospital-acquired *K. Pneumoniae*.[18,19] In our study, 71 (84%) were ESBL positive by the double-disk synergy test and the Etest® and this value is considered high. This highlights the importance of investigating resistance mechanisms in hospitals.

Among the antimicrobials evaluated for minimum inhibitory concentration, the carbapenemics presented the best potency (MIC_{50}), with imipenem (0.125 µg/mL). Carbapenems such as imipenem and meropenem are recommended as therapy of choice for severe infections caused by CTX-M- and other types ESBL-producing bacteria. Most CTX-M positive isolates exhibited resistance to non-β-lactam antibiotics displaying co-resistance for gentamicin, amikacin, norfloxacin, ciprofloxacin, aztreonam, cefepime, and trimethoprim-sulphametoxazole.

In clinical strains, CTX-M-coding genes have been commonly located on plasmids and these plasmids can also carry genes for resistance to multiple other antibiotics, including aminoglycosides, chloramphenicol, sulfonamide, trimethoprim, and tetracycline. Resistance of ESBL-producing isolates to other classes of antimicrobial agents shown in this report has been confirmed by other studies.[5,20] Among Enterobacteriaceae, these resistance pattern was commonly associated with a few types of integrons.

In this study, all isolates that carried the gene $bla_{CTX\text{-}M}$ also contained class 1 integrons. Insertion sequences (IS) might be involved in the mobilization of $bla_{CTX\text{-}M}$ genes.[21] The $bla_{CTX\text{-}M}$ genes have also been associated with ISCR1, which is often found downstream of complex class 1 integrons.[22]

CTX-M-2, CTX-M-8 and CTX-M-9 groups were the most frequently detected CTX-M-type enzymes among ESBL-producing Enterobacteriaceae isolates from South American countries.[4,9,23] In the present study, CTX-M 2 (61%) was the predominant cluster. CTX-M-2 was first characterized and appears to be dominant in Argentina.[20] Studies carried out in Europe have shown that the occurrence of the $bla_{CTX\text{-}M\text{-}2}$ group is rare. Recent studies in Southeast Brazil have demonstrated the presence of $bla_{CTX\text{-}M\text{-}2}$ gene in *K. Pneumoniae*.[24,25] Dissemination of CTX-M-9 and CTX-M-8 clusters has also been reported in Brazil.[4,26,27]

Many of the CTX-M positive isolates harbored other β-lactam resistance enzymes and the association of types TEM, SHV and CTX-M (n = 20) was more frequent. Previous studies have shown that ESBL mediating plasmids may carry more than one β-lactamase gene and that they may be responsible for high-level β-lactamase resistance phenotypes.[28] In our study, the resulting PFGE gel of CTX-M-producing *K. pneumoniae* isolates showed genotypic diversity. However, two hospitals presented the same clonal group, characterized as genotype O, CTX-M-2 producers, suggesting intrahospital dissemination.

The high levels of CTX-M ESBL detected are worrisome and warrant special attention by both the clinician and the microbiology laboratory. While the former has to re-evaluate the antibiotic policies, the laboratory must be capable to readily identify these isolates. Given the degree to which these CTX-M-producing microorganisms have spread, they should be seen as a city public health issue instead of a problem of each hospital. Widespread use of antimicrobial therapy has often been held responsible for the occurrence of multiresistant *Klebsiella* strains in hospitals.[29] Our data corroborate the importance of antibiotic use restriction and implementation of preventive measures.

Table 2. Characteristics of CTX-M-producing **K. pneumoniae** *isolated (n = 38)*

Hospital (city)	Isolate nº	Specimen	Resistance and co-resistance of ESBL-producing isolates	ß-lactamase detected	Phylo-genetic group
	1368	Bronchial secretion	CN, KF, CIP, NOR, FEP, SXT, ATM, CTX, CAZ	TEM, SHV, CTX-M-2 group	C
	1051	Bronchial secretion	CN, CTX	TEM, CTX-M-2 group	S
	968	Sputum	CN, NOR, CIP, CTX, FEP	TEM, CTX-M-2 group	O
	563	Bronchial secretion	CN, NOR, CIP, CTX, FEP, ATM, SXT	TEM, CTX-M-2 group	B
	652	Skin	AK, CN, CTX, ATM	TEM, SHV, CTX-M-9 group	R
	758	Pulmonary secretion	AK, CN, CAZ, CTX, ATM, SXT	TEM, SHV, CTX-M-2 group	I
	744-01	Pulmonary secretion	AK, NOR, CIP, CTX, SXT	TEM, CTX-M-2 group	P1
	761-02	Nasal swab	AK, NOR, CIP, CAZ, CTX, ATM, SXT	TEM, CTX-M-2 group	P
H U_1	2332	Urine	CN, CTX, ATM	TEM, CTX-M-2 group	M
(Niterói)	1636-01	Pulmonary secretion	CN, CAZ, CTX, FEP, ATM	TEM, SHV, CTX-M-2 group	J1
	2211	Pulmonary secretion	CN, NOR, CIP, CAZ, CTX, ATM, SXT	TEM, SHV, CTX-M-2 group	A
	1677-01	Catheter	CN, NOR, CIP, CAZ, CTX, FEP, ATM SXT	TEM, SHV, CTX-M-2 group	A1
	33-10	Blood	AK, CN, CAZ, CTX, ATM, SXT	TEM, SHV, CTX-M-2 group	F
	2256-01	Pulmonary secretion	CN, CTX, AK, NOR, CIP, CAZ, FEP, ATM, SXT	TEM, SHV, CTX-M-2 group	H
	2305	Tracheal secretion	CN, CTX	TEM, SHV, CTX-M-2 group	S1
	204	Urine	AK, CN, CAZ, CTX, FEP, ATM	TEM, CTX-M-2 group	K1
	621	Urine	AK, CN, CAZ, CTX, FEP, ATM, SXT	TEM, CTX-M-2 group	K
	2194	Urine	CN, NOR, CIP, CAZ, ATM, SXT	TEM, SHV, CTX-M-9 group	Q
	166	Blood	CN, CAZ, CTX, ATM, SXT	TEM, CTX-M-ND	G
	2221	Blood	AK, CN, CAZ, CTX, FEP, ATM	TEM, SHV, CTX-M-2 group	J

cont.

***Table 2. Characteristics of CTX-M-producing* K. pneumoniae *isolated (n = 38)* (Cont.)**

Hospital (city)	Isolate nº	Specimen	Resistance and co-resistance of ESBL-producing isolates	ß-lactamase detected	Phylo-genetic group
	24218	Blood	NOR, CIP, CTX, FEP	TEM, SHV, CTX-M-8 group	Y
	30800	Bronchial secretion	CN, CTX, FEP, ATM	TEM, CTX-M-8 group	U
	25646	Urine	AK, CN, NOR, CIP, CTX, SXT	TEM, SHV, CTX-M-8 group	N1
	65416	Urine	CN, NOR, CIP, CAZ, CTX, FEP, ATM, SXT	TEM, SHV, CTX-M-1 group	X
H U_2	21530	Urine	CN, CIP, CTX, FEP, ATM	TEM, CTX-M-2 group	D
(Rio de Janeiro)	26306	Blood	AK, CN, NOR, CIP, CTX, FEP, SXT	TEM, CTX-M-8 group	CC
	820839	Urine	CN, NOR, CIP, CYX, FEP, ATM, SXT	TEM, SHV, CTX-M-2 group	T
	930616	Bronchial secretion	CN, CTX, FEP, ATM	TEM, CTX-M-2 group	EE
	27341	Blood	AK, CN, NOR, CIP, CTX, FEP, SXT	TEM, SHV, CTX-M-8 group	DD
	33155	Bronchial secretion	AK, CN, NOR, CIP, CTX, FEP, SXT	TEM, CTX-M-ND	BB
	22200	Urine	AK, CN, NOR, CIP, CTX, SXT	TEM, SHV, CTX-M-2 group	N
H U_3 (Rio de Janeiro)	22856	Urine	AK, CN, NOR CIP, ATZ, CAZ, CTX, SXT	TEM, SHV, CTX-M-9 group	L
H U_4	91	Blood	NOR, CIP, CTX, FEP, SXT	TEM, CTX-M-8 group	E
(Rio de Janeiro)	127	Blood	CAZ, CTX, ATM, SXT	TEM, SHV, CTX-M-ND	W
	523	Blood	AK, CN, SXT	TEM, CTX-M-9 group	AA
	539	Blood	AK, CN, NOR, CAZ, SXT	TEM, CTX-M-9 group	Z
H U_5	50088	Blood	CN, CIP, FEP, ATM, SXT	TEM, SHV, CTX-M-2 group	V
(Rio de Janeiro)	50266	Urine	AK, CN, ATM	TEM, SHV, CTX-M-2 group	O1

AK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; CTX, ceftriaxone; FEP, cefepime; IPM, imipinem; NOR, norfloxacin; SXT, trimethoprim-sulphametoxazole; ND, not determined.

CONCLUSION

In conclusion, CTX-M enzymes have emerged in our hospitals. The intensive use of broad-spectrum cephalosporins such as cefotaxime could account for the emergence of the CTX-M plasmid-mediated enzymes among pathogens. Several groups of *bla* genes were detected in clinical samples in the studied hospitals, but the $bla_{\text{CTX-M 2}}$ was the predominant. The data presented herein illustrate the diversity of genotypes of CTX-M producing *K. pneumoniae* among hospitals and the intrahospital dissemination of these genotypes was uncommon. Thus, the high prevalence of CTX-M *K. pneumoniae* in our hospitals is probably not a consequence of the transmission of a common strain between patients. Diversity of genotypes was also described in other studies suggested that the increase of ESBL *K. pneumoniae* was mainly due to horizontal dissemination of gene transfer between isolates.[30] In contrast, it is important to note that currently there is a worldwide spread of ESBL and KPC-producing *K. pneumoniae*, which seems to occur due to the dissemination of specific clones ST258, ST11 and ST437 in Brazil.[31,32]

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