Antimicrobial Resistance and Molecular Characterization of Extended-Spectrum β -Lactamases of *Escherichia coli* and *Klebsiella* spp. Isolates from Urinary Tract Infections in Southern Brazil

Juliano Lacava Pereira,¹ Lisiane Martins Volcão,¹ Gabriel Baracy Klafke,²
Roseli Stone Vieira,³ Carla Vitola Gonçalves,² Ivy Bastos Ramis,¹
Pedro Eduardo Almeida da Silva,¹ and Andrea von Groll¹

The objective of this study was to evaluate the frequency of different extended-spectrum β -lactamases (ESBL) as well as to associate these ESBL with antimicrobial (ATM) resistance in *Escherichia coli* and *Klebsiella* spp. isolates from outpatients and inpatients with urinary tract infections. The study included 435 consecutive nonduplicate clinical isolates, including 362 *E. coli* isolates, 62 *Klebsiella pneumoniae* isolates, and 11 *K. oxytoca* isolates. Isolates were obtained from patients who were treated in a University Hospital between August 2012 and July 2013. Three multiplex PCR were performed to identify the ESBL groups. A total of 48 (11%) ESBL-producing isolates were found. The risk for the ESBL presence was significantly higher in males (26.4%) than females (8%), from hospital-acquired infections (29.1%) than community-acquired infections (7.0%) and in *Klebsiella* spp. (27.4%) than in *E. coli* (7.7%). ESBL-producing isolates presented a significantly higher percentage of resistance in 21 of the 23 ATMs analyzed. The CTX-M-1 group was the most predominant ESBL identified. The *bla*_{CTX-M-1-group} gene was found in 56% of the total ESBL producers from community and in 42.4% from hospital origins; it was followed in frequency by the *bla*_{CTX-M-8/25-group}, also found in both environments. *Klebsiella* spp. presented the largest variety of β -lactamase enzyme combinations and a higher level of resistance to cefotaxime. These findings contribute to better knowledge of the epidemiology of ESBL enzymes and are alarming for the reduced therapeutic options available for the risk groups identified in the studied populations.

Keywords: ESBL, antimicrobial resistance, *Escherichia coli*, multidrug resistance, *Klebsiella pneumoniae*

Introduction

MULTIDRUG-RESISTANT BACTERIA and the subsequent limitation of effective antimicrobial (ATM) therapies are a worldwide major health concern. The overuse and misuse of ATMs is identified as a significant driver in the emergence of ATM resistance.¹ *Escherichia coli* and *Klebsiella pneumoniae* are the most common causes of urinary tract infections (UTIs) for both community-acquired and nosocomial infections.² The World Health Organization has classified these extended-spectrum β -lactamase (ESBL)-producing species as a group of bacteria that has a major impact on public health around the world.³

ESBL are enzymes capable of, through hydrolysis, conferring bacterial resistance to penicillins, cephalosporins

(first, second, and third generation), and aztreonam, but not cephamycins or carbapenems, and which can be inhibited by clavulanic acid and other β -lactamase inhibitors.⁴ The major ESBL types are TEM, SHV, CTX-M, and OXA. SHV and TEM ESBL types are derived from SHV-1 and TEM-1 β -lactamases, respectively.⁵ The CTX-M family is formed by five main groups differing by $\geq 10\%$ in amino acid sequence identity: CTX-M-1-group, CTX-M-2-group, CTX-M-8-group, CTX-M-9-group, and CTX-M-25-group.⁶ The OXA-ESBL type has been found more frequently in *Pseudomonas aeruginosa*, but with its genes distributed on plasmids; OXA are currently found in several members of the Enterobacteriaceae family too.^{7,8}

Despite worldwide distribution of ESBL, their epidemiology varies according to the geographic region. The enzymes

¹Faculdade de Medicina, Medical Microbiology Research Center (NUPEMM), Universidade Federal do Rio Grande—FURG, Rio Grande, Brazil.

²Faculdade de Medicina, Universidade Federal do Rio Grande—FURG, Rio Grande, Brazil.

³Hospital Universitário Dr. Miguel Riet Correa, Universidade Federal do Rio Grande—FURG, Rio Grande, Brazil.

of the CTX-M group currently overlap with other ESBL and have expanded beyond hospital borders.⁹ Due to plasmid dissemination, it is now a reality in outpatients^{10,11} and is strongly associated with other classes of ATM resistance.¹² Besides, the presence of different β -lactamases has influenced the minimum inhibitory concentration of cephalosporins and consequent breakpoints to determine susceptibility profile by microdilution methods.¹³ The objective of this study was to evaluate the frequency of different ESBL as well as to associate the presence of ESBL with resistance to ATMs in *E. coli* and *Klebsiella* spp. isolates from outpatients and inpatients with UTI in a hospital in Southern Brazil.

Methodology

Study setting

This is a retrospective, observational and analytical study that included 435 consecutive nonduplicate clinical isolates, with 362 isolates being identified as *E. coli* and 73 as *Klebsiella* spp. (62 *K. pneumoniae* and 11 *K. oxytoca*), obtained from patients with UTI treated at the University Hospital of the Universidade Federal do Rio Grande (HUFURG), Southern Brazil, from August 2012 to July 2013.

To identify patient features that were associated with ESBL-producing strains, medical records were reviewed, including age, gender, and origin of infection (community or hospital acquired). Hospital-acquired infection was defined as a UTI occurring within the first 72 hours after hospital admission or up to 72 hours after the patient was discharged from the hospital.¹⁴ Bacterial-related data were obtained with the EpiCenter software, version 6.20A (Phoenix System; BDTM), of the clinical laboratory register and included bacteria species, ESBL production, and the isolates' susceptibility profiles to ATMs.

The study was conducted based on Resolution 466/12 of the National Health Council and was approved by the Research Ethics Committee of the Health Area of the HUFURG under number 177/2013.

Bacterial collection

Ten microliters of urine samples was seeded on cystine lactose electrolyte deficient and MacConkey agar and incubated aerobically at 35°C for 18–24 hours. After Enterobacteriaceae isolation on MacConkey agar, the BD PhoenixTM automated microbiology system (Becton Dickinson Diagnostic Systems, Sparks, MD) was used for identification of bacterial species, ATM susceptibility profile, and phenotypic identification of ESBL producers according to the manufacturer's instructions. The clinical isolates were stored in Luria Bertani broth with 15% glycerol at –70°C in the Medical Microbiology Research Center, FURG (NUPEMM-FURG) bacterial collection.

Determination of minimal inhibitory concentration to cefotaxime

The minimal inhibitory concentration (MIC) of cefotaxime (CTX) of ESBL producers was determined by Broth Microdilution Method according to the CLSI M07-A9.¹⁵ Concentrations tested ranged between 0.5 and 512 mg/L. *E. coli* 25922 was used as control in the susceptibility test.

Molecular detection of ESBL

Total DNA extraction of all ESBL-producing *E. coli* and *Klebsiella* spp. isolates was obtained by heat treatment.¹⁶ The products were subjected to three distinct multiplex PCR as described previously.¹⁷ Reaction 1 is for the detection of ESBL of *bla*_{TEM-group}, *bla*_{SHV-group}, and *bla*_{OXA-group}, reaction 2 is for the detection of *bla*_{CTX-M} from groups 1, 2, and 9, and reaction 3 is for the identification of *bla*_{CTX-M-8/25-group}. The oligonucleotides used in all reactions are in Table 1. Hot-StarTaq[®] (Qiagen) was used for reaction 1 and Platinum[®] Taq DNA Polymerase (Invitrogen, Life Technologies Brazil) for reactions 2 and 3. The positive controls used were *bla*_{TEM-1}, *bla*_{SHV-2}, and *bla*_{OXA-1} for reaction 1, *bla*_{CTX-M-16}, *bla*_{CTX-M-2}, and *bla*_{CTX-M-9} for reaction 2, and *bla*_{CTX-M-8} for reaction 3, all kindly provided by Dr. Jorge Luiz Mello Sampaio. *E. coli* ATCC 25922 DNA was used as negative control.

DNA viability of samples that did not present any *bla* gene amplification was confirmed by amplification of the chromosomal gene *gyrA*.¹⁸

Sequencing of the *bla*_{SHV} and *bla*_{TEM} genes

The differentiation of ESBL from penicillinases was carried out by the sequencing of *bla*_{SHV} and *bla*_{TEM} genes.

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED TO DETECT *BLA*_{ESBL} GENES

Multiplex reaction PCR	Name—prime	Sequence (5'–3')
Reaction 1	TEM—F	CATTTCCGTGTCGCCCT TATTC
	TEM—R	CGTTCATCCATAGTTGC CTGAC
	SHV—F	AGCCGCTTGAGCAAAT TAAAC
	SHV—R	ATCCCGCAGATAAATC ACCAC
	OXA—F	GGCACCAGATTCAACT TTCAAG
	OXA—R	GACCCCAAGTTTCCTGT AAGTG
	CTX-M-1—F	TTAGGAARTGTGCCGC TGGA ^a
	CTX-M-1—R	CGATATCGTTGGTGGT RCCAT ^a
Reaction 2	CTX-M-2—F	CGTTAACGGCACGAT GAC
	CTX-M-2—R	CGATATCGTTGGTGGTR CCAT ^a
	CTX-M-9—F	TCAAGCCTGCCGATCT GGT
	CTX-M-9—R	TGATTCTCGCCGCTG AAG
Reaction 3	CTX-M-8/25—F	AACRCRCAGACGCTC TAC ^a
	CTX-M-8/25—R	TCGAGCCGGAASGTG TYAT ^a

^aY = T or C; R = A or G; S = G or C.

ESBL, extended-spectrum β -lactamases; F, forward; R, reverse.

FIG. 1. Distribution of the clinical isolates according to gender, infection source, type of Enterobacteria, and frequency of ESBL. ESBL, extended-spectrum β -lactamases.

After purification of the amplicons,¹⁹ the sequencing was accomplished using the ABI 3500 Genetic Analyzer (Life Technologies Technology—Applied Biosystems). Nucleotide sequences were aligned with TEM-1 (KT314169.1 *K. pneumoniae* subsp. *pneumoniae* strain GF4K3 beta-lactamase TEM-1 gene) and SHV-1 (JX268740.1 *K. pneumoniae* strain AKH11/46 beta-lactamase SHV-1) sequences, using the program “Mega” software version 5.2.2. SHV and TEM types were defined by comparing the amino acid sequences by BLAST on the website <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. The SHV β -lactamases found were also classified according to a functional classification scheme by Bush *et al.*²⁰

Three *bla*_{OXA}-like genes, three *bla*_{CTX-M-1-group} genes, three *bla*_{CTX-M-2-group} genes, two *bla*_{CTX-M-8/25-group} genes, and one *bla*_{CTX-M-9-group} gene were sequenced to confirm that they were β -lactamases of their respective groups. The sequences of those genes were analyzed using “Blast” in <https://blast.ncbi.nlm.nih.gov/Blast.cgi>

Data analysis

Consistency analysis was performed through creating and categorizing variables and verifying frequencies in SPSS 17.0. A dependent variable was used as the presence of an ESBL producer, and the following independent variables were considered: demographic data (age and gender), infection source (hospital or community acquired), and bacterial species (*E. coli* or *Klebsiella* spp.). The χ^2 test at a 95% significance level was used to compare proportions. After obtaining the prevalence ratio and their respective confidence intervals (CIs, 95% CI), the variables with a p -value ≤ 0.20 were included in the multivariate analysis. Poisson regression with robust adjustment of variance was used in the following hierarchical levels: age and gender (first level), and infection source and bacterial species (second level). Variables with $p \leq 0.05$ were considered significant. The χ^2 test of linear association was used to evaluate the association of ATM resistance with the presence of ESBL, a $p \leq 0.05$ was considered statistically significant.

Results

Characterization of the patients and clinical isolates

Of the 435 clinical isolates, 72 (16.5%) were obtained from male and 363 (83.5%) from female patients with UTI (Fig. 1). Of the 72 isolates from males, 33 (45.8%) were hospital acquired and 39 (54.2%) were community acquired; of the 363 isolates from females, 46 (12.7%) were hospital acquired and 317 (87.3%) were community acquired. Figure 1 shows the distribution of bacteria according to infection source and gender. In males, *Klebsiella* spp. represented 45.5% of the hospital-acquired isolates and 30.8% of the community-acquired isolates, while in females, 19.6% were hospital acquired and 9.8% were community acquired.

Frequency of ESBL

A total of 48 (11%) isolates were identified as ESBL producers, 26 from outpatients and 22 from inpatients (Fig. 1). The risk for the ESBL presence was significantly higher in males (26.4%) than females (8%) ($p=0.047$), from hospital-acquired infections (29.1%) than community-acquired infections (7.0%) ($p=0.004$), and in *Klebsiella* spp. (27.4%) than in *E. coli* (7.7%) ($p=0.002$) (Table 2). In relation to age of the patients, the risk of ESBL was progressive according to population age, but there was no significant association between the three age ranges examined (Table 2).

Antimicrobial resistance associated with the presence of ESBL

ESBL-producing isolates presented a significantly higher percentage of resistance to 21 of the 23 agents tested (Fig. 2), including resistance to penicillins (AMP, TZP, and AMC), cephalosporins (CFZ, CXM, CAZ, CRO, and CTX e FEP), ATM, fluoroquinolones (CIP and LEV), aminoglycosides (AMI, GEN, and TOB), and carbapenems (ETP and MEM), FOX, NIT, SXT, and TET. However, the resistance rate to imipenem and ampicillin with sulbactam was not significantly in relation to non-ESBL-producing isolates (Fig. 2).

TABLE 2. FREQUENCY AND ADJUSTED ANALYSIS OF ASSOCIATED FACTORS TO EXTENDED-SPECTRUM β -LACTAMASES PRODUCERS

Categories	Total number	ESBL n (%)	Adjusted analysis PR (CI _{95%})	p-Value
Age				
≥61	173	25 (14.5)	1.0	
41–60	120	15 (12.5)	0.83 (0.39–1.74)	0.628
21–40	96	6 (6.2)	0.54 (0.20–1.46)	0.230
Gender				
Female	363	29 (8.0)	1.0	
Male	72	19 (26.4)	2.15 (1.01–4.58)	0.047*
Origin				
Community	356	25 (7.0)	1.0	
Hospital	79	23 (29.1)	2.93 (1.42–6.05)	0.004*
Bacteria				
<i>Escherichia coli</i>	362	28 (7.7)	1.0	
<i>Klebsiella</i> spp.	73	20 (27.4)	3.07 (1.50–6.25)	0.002*

*Statistically significant.

PR, prevalence ratio; CI, confidence interval.

FIG. 2. Resistance to antimicrobials in ESBL (black bar)- and non-ESBL (white bar)-producing isolates. *Indicates a significant difference between ESBL and non-ESBL with $p \leq 0.05$. AMP, ampicillin; SAM, ampicillin with sulbactam; TZP, piperacillin with tazobactam; AMC, amoxicillin with clavulanic acid; CFZ, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CXM, cefuroxime; FEP, cefepime; FOX, ceftiofur; ATM, aztreonam; ETP, ertapenem; MEM, meropenem; IMI, imipenem; GEN, gentamicin; AMI, amikacin; TOB, tobramycin; NIT, nitrofurantoin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin.

TABLE 3. FREQUENCY OF EXTENDED-SPECTRUM β -LACTAMASES GENE FAMILIES FOUND IN *ESCHERICHIA COLI* AND *KLEBSIELLA* SPP. CLINICAL ISOLATES

Genes	E. coli n (%)		Klebsiella spp. n (%)		Total n (%)
	Community 17 (100)	Hospital 11 (100)	Community 8 (100)	Hospital 12 (100)	
<i>bla</i> _{CTX-M-1-group}	8 (47.1)	6 (54.5)	6 (75.0)	8 (66.7)	28 (58.3)
<i>bla</i> _{CTX-M-2-group}	1 (5.9)	1 (9.1)	0 (0.0)	0 (0.0)	2 (4.2)
<i>bla</i> _{CTX-M-8/25-group}	5 (29.4)	2 (18.2)	1 (12.5)	5 (41.6)	13 (27.1)
<i>bla</i> _{CTX-M-9-group}	1 (5.9)	0 (0.0)	1 (12.5)	0 (0.0)	2 (4.2)
<i>bla</i> _{SHV-2a}	1 (5.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.1)
<i>bla</i> _{SHV-11}	0 (0.0)	0 (0.0)	1 (12.5)	1 (8.3)	2 (4.2)
<i>bla</i> _{SHV-26}	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	1 (2.1)
<i>bla</i> _{ESBL} no detected	1 (5.9)	2 (18.2)	0(0.0)	0 (0.0)	3 (6.3)

Molecular characterization of ESBL

Table 3 shows the frequency of ESBL genes found in the 48 ESBL producers, of which the *bla*_{CTX-M-1-group} was the most predominant in *E. coli* as well as in *Klebsiella* spp. This gene was found in 56% of the total ESBL producers from community origin and in 42.4% from hospital origin; it was followed in frequency by the *bla*_{CTX-M-8/25-group}, also found in both environments.

Among the genes of the *bla*_{CTX-M} family, at lower frequency, two *E. coli* isolates presented the *bla*_{CTX-M-2-group} and one *E. coli* and one *Klebsiella* spp. isolate presented the *bla*_{CTX-M-9-group}. In relation to the *bla*_{SHV} family, after sequencing of the 14 *bla*_{SHV} genes, the *bla*_{SHV-2a} genotype was found in one *E. coli* isolate, the *bla*_{SHV-11} in two *Klebsiella* spp. isolates, and the *bla*_{SHV-26} in one *Klebsiella* spp. isolate. In two isolates of ESBL-producing *E. coli*, none of studied genes was found.

Among isolates that presented the *bla*_{SHV} gene, there were four with SHV enzymes presenting phenotype (2br or 2be) consisting of one SHV-2a, two SHV-11, and one SHV-26. Sample A27 showed a single glutamine to histidine change at amino acid 150, which did not allow for characterization of the SHV enzyme type (Table 4). The *bla*_{TEM} gene was detected in 13 isolates, being identified as encoding the TEM-1 penicillinase, and none was characterized as encoding ESBL.

Several clinical isolates presented multiple genes encoding ESBL. Table 5 shows the combination of genes found in the 48 ESBL-producing strains, including the non-ESBL genes *bla*_{OXA}, *bla*_{TEM-1}, and *bla*_{SHV-1}, which are penicillinases. The *bla*_{OXA} was always found in presence of the

*bla*_{CTX-M-1-group} (Table 5). In *E. coli*, this combination was found in all 14 clinical isolates. For *Klebsiella* spp., the combination of *bla*_{CTX-M-1-group} and *bla*_{OXA} was also found, as well as the combination with *bla*_{SHV-1} and/or *bla*_{TEM-1}. Table 5 shows that the majority of cases of *bla*_{SHV-1} β -lactamase found also presented the gene *bla*_{CTX-M-1-group}, except for one isolate that presented only the gene responsible for the TEM-1 β -lactamase.

Of the 48 ESBL-producing isolates, the MIC for CTX was determined in 40 (23 *E. coli* and 17 *Klebsiella* spp.), while 8 isolates presented a loss of viability during the study. In *E. coli*, for most of the isolates presenting the *bla*_{CTX-M-1-group}, *bla*_{CTX-M-8/25-group}, or *bla*_{CTX-M-9-group} genes, as well as the three samples in which *bla*_{ESBL} genes were not detected (including *bla*_{TEM-1} alone), the MIC ranged from 4 to 64 mg/L. The two exceptions were the *bla*_{CTX-M-1-group} isolate that had an MIC of 256 mg/L and the *bla*_{CTX-M-2-group} isolate that had an MIC $\geq$ 512 mg/L (Table 5). *Klebsiella* spp. presented MIC ranging from 256 to 512 mg/L in isolates with the *bla*_{CTX-M-1-group} and 32 to 256 mg/L in isolates with the *bla*_{CTX-M-8/25-group} (Table 5). Figure 3 shows that *Klebsiella* spp. presented higher MIC to CTX than *E. coli*.

Discussion

In this study, the frequency of *E. coli* and *Klebsiella* spp. producing ESBL was 11%; however, 7.0% of community- and 29.1% of hospital-acquired infections were ESBL producers. were hospital-acquired infections. Regarding the community-acquired origin, the frequency in this study was similar to other studies in different regions of Brazil, with

TABLE 4. CHARACTERIZATION OF ISOLATES PRESENTING THE *BLA*_{SHV} GENE WITH MUTATION(S) IN RELATION TO THE PENICILLINASE *BLA*_{SHV-1} GENE

Sample	Bacteria	ESBL by Phoenix	<i>bla</i> _{SHV} type	Mutation (aa)	Phenotype ^a	Other genes (bla) found
A3	<i>Klebsiella</i> spp.	Yes	SHV-26	A183T	2br	CTX-M-8/25-group and TEM-1
A27	<i>Klebsiella</i> spp.	Yes	SHV	Q150H	—	CTX-M-9-group
A29	<i>Klebsiella</i> spp.	Yes	SHV-11	L31Q	2be	CTX-M-8/25-group and TEM-1
A43	<i>Klebsiella</i> spp.	Yes	SHV-11	L31Q	2be	CTX-M1-group and TEM-1
A109	<i>E. coli</i>	Yes	SHV-2a	L31Q and G234S	2be	TEM-1

^a2br= β -lactamase resistant to inhibitors and 2be=ESBL.

Mutation (aa): A, alanine; T, threonine; Q, glutamine; H, histidine; L, leucine; G, glycine; S, serine.

TABLE 5. PROFILE OF *BLA* GENES FOUND IN ESBL-PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA* spp. AND THE MIC TO CTX

Combination of genes (bla)	<i>E. coli</i> n (%)	Range MIC (mg/L)	<i>Klebsiella</i> spp. n (%)	Range MIC (mg/L)
CTX-M-1-group	—	—	1 (5%)	256
CTX-M-1-group + OXA-1-like	14 (50%)	8–32; 256 ^a	4 (20%)	256–512
CTX-M-1-group + OXA-1-like + SHV-1	—	—	2 (10%)	256–512
CTX-M-1-group + OXA-1-like + SHV-1 + TEM-1	—	—	4 (20%)	256–512
CTX-M-1-group + SHV-11 + TEM-1	—	—	1 (5%)	NP
CTX-M-1-group + SHV-1	—	—	1 (5%)	256
CTX-M-2-group	2 (7.1%)	>512	—	—
CTX-M-8/25-group	6 (21.4%)	4–32	—	—
CTX-M-8/25-group + TEM-1	1 (3.6%)	32	2 (10%)	256
CTX-M-8/25-group + SHV-1 + TEM-1	—	—	1 (5%)	NP
CTX-M-8/25-group + CTX-M-1-group + OXA-1-like + SHV-1	—	—	1 (5%)	64
CTX-M-8/25-group + SHV-11 + TEM-1	—	—	1 (5%)	32
CTX-M-8/25-group + SHV-26 + TEM-1	—	—	1 (5%)	256
CTX-M-9-group	1 (3.6%)	64	—	—
CTX-M-9-group + SHV ^b	—	—	1 (5%)	NP
TEM-1	1 (3.6%)	8	—	—
SHV-2a + TEM-1	1 (3.6%)	NP	—	—
Not detected	2 (7.2%)	64	—	—
Total	28 (100%)	—	20 (100%)	—

^aValue of only one isolate.

^bIndicates the protein contained a change of glutamine to histidine at position 150 (mutation Q150H). This was not enough to identify the β -lactamase type.

MIC, minimal inhibitory concentration; NP, not performed.

percentages ranging from 7.1% to 7.6%,^{21,22} and elevated in comparison with developed countries such as France (2.4%) and Sweden (2.8%).²³ Nevertheless, ESBL was more associated with nosocomial infections (2.9-fold higher risk than in community-acquired infections), male gender, and *Klebsiella* spp. infections. Previous studies that compared the frequency of ESBL from inpatients and outpatients treated in the same hospital also showed a predominance of ESBL producers in hospital-acquired infections.²⁴ This environment is where ESBL originally emerged and where more third-generation cephalosporins are consumed, which are often used to treat complicated UTI.²⁵ In addition, men were more prone to develop hospital-acquired UTI and

consequently more at risk for acquisition of ESBL producers than women. In our study, the relationship can be supported since 45.8% of UTI in males were hospital acquired, while in females, the percentage was only 12.7%.

With ESBL producers presenting a significantly higher frequency of resistance to 21 of 23 ATMs tested (except to imipenem and ampicillin with sulbactam), appropriate treatment is limited, and the identification of risk groups for ESBL is critical. Aside from fourth-generation cephalosporins, aztreonam and ampicillin, ESBL presented resistance rates in over 50% of cases to fluoroquinolones (LEV and CIP), tetracycline (TET), sulfa-trimethoprim (SXT), aminoglycoside (TOB), and beta-lactams associated with beta-lactamase inhibitor (AMC, TZP, and SAM). The associated resistance to these ATMs has been shown to be related to the presence of a conjugative plasmid that has accumulated genes that confer resistance. These genes can include *rmtA* to *rmtD*-, *armA*-, and *npmA*-encoded 16S rRNA methylases associated with aminoglycoside resistance,²⁶ the *bla*_{ampC} gene encoding the AmpC enzyme, resulting in resistance to the β -lactamase inhibitor- β -lactam combination²⁷; and *qnr*, *qepA*, and *aac(6')*-*Ib-cr* genes associated with resistance to some fluoroquinolones.²⁵ Recently, our group showed that the presence of ESBL producers in UTI isolates increased the risk to fluoroquinolone resistance 11-fold,²⁸ and we found an elevated presence of the *aac(6')*-*Ib-cr* gene associated with chromosomal mutations in *gyrA* and *parC*, which encode the main targets of fluoroquinolones in *E. coli* and *Klebsiella* spp.²⁹ Fortunately, in our study, carbapenems and amikacin displayed activity over 90% in ESBL producers, allowing these ATMs to remain as options for the treatment of complicated UTI. However, it is important to ration their

FIG. 3. Minimal inhibitory concentration (mg/L) to ceftaxime of ESBL-producing *Escherichia coli* and *Klebsiella* spp. Dark gray bar: *E. coli*; light gray bar: *Klebsiella* spp.

use, since the resistance rates are increasing in Enterobacteriaceae members, especially in *K. pneumoniae*. This emergence is mainly associated with the spread of plasmids containing genes encoding carbapenemases and has resulted in outbreaks in several countries, including the United States and China.³⁰

The profile of multidrug resistance associated with ESBL has previously been shown in *E. coli* and *K. pneumoniae* to involve the presence of acquired *bla*_{CTX-M} genes, which are carried on conjugative plasmids.³¹ Actually, in our study, CTX-M was the major ESBL family, identified in 85.6% of *E. coli* and in 100% of *Klebsiella* spp. Among the CTX-M family, the predominant group was CTX-M-1, followed by CTX-M-8/25, and in lower frequency, the groups CTX-M-2 and CTX-M-9. Worldwide, the CTX-M-1 is the most widely disseminated ESBL group in the clinical settings, mainly for pandemic *E. coli* clones bearing the CTX-M-15 type.¹² In relation to the CTX-M-2-group, our results differ from a systematic review of the spread of CTX-M in Brazil that determined the prevalent groups are CTX-M-1 and CTX-M-2.³² While we found a rate of 4.1% of CTX-M-2-group, the rate of CTX-M-2-group reached nearly 60% in some studies from southern and southeastern Brazil.^{33,34} Previous reports from Brazil and Spain determined that CTX-M-9 was more prevalent in Spain,^{35,36} as well as the CTX-M-2-group; we found a low frequency of the CTX-M-9 group (4.1%), but outside of Brazil, its frequency ranges from 11.0% to 24.2%.^{34,37} Finally, in relation to CTX-M-8/25 groups, the primer pair can amplify the *bla* gene of both groups, but 2 of the 13 isolates detected were sequenced and showed similarity with the CTX-M-8 type, which belongs to CTX-M-8-group. The first report of CTX-M-8 was in Brazil,³⁸ and it has subsequently been found at a frequency ~12% in *E. coli* ESBL producers from a different region of Brazil,^{33,39} which is lower than the 25% rate that was found in our study. In addition, in *Klebsiella* spp., the frequency of this group was 30%, demonstrating its successful spread in both *Enterobacteria* species.

The detection of the *bla*_{CTX-M}-group was successfully performed by multiplex PCR.¹⁷ This tool could be useful for rapid epidemiological studies screening for *bla*_{CTX-M} groups. However, the multiplex PCR to detect *bla*_{SHV}, *bla*_{TEM}, and *bla*_{OXA} had limited efficacy, since the presence of amplified PCR product did not differentiate between ESBL or simply penicillinases. For *bla*_{SHV} and *bla*_{TEM}, it was necessary to sequence the amplified product to differentiate ESBL from penicillinases (e.g., TEM-1 or 2 and SHV-1). The *bla*_{SHV} presented at frequency of 8.3% of the ESBL in the studied population, and this lower frequency of SHV is in agreement with a previous study.⁴⁰ This result demonstrates an inversion in the prevalence of CTX-M enzymes on TEM and SHV. Although two samples did not show any gene that was screened for and one showed just penicillinase TEM-1, these bacteria should contain another ESBL family that was not studied in this work. Some possible ESBL would be the PER, GES, and BES enzymes, which have already been detected in Brazil.^{33,38,41}

Due to variation in the combination of genes encoding β -lactamase enzymes, no statistical analysis was able to be performed to associate MICs with ESBL enzymes. Nevertheless, it was possible to observe that *Klebsiella* spp. presented higher levels of resistance to CTX than *E. coli*. *Klebsiella*

spp. also contained more combinations of genes, such as the presence of penicillinases TEM-1, SHV-1, SHV-derived-ESBL, and CTX-M groups. The variation in MIC may indirectly represent the levels of expression of the genes involved,⁴² but they may also represent the involvement of other resistance mechanisms to CTX, such as efflux pumps, lower permeability due to the reduction of porins, or the production of AmpC.^{43–45}

This study showed a relevant frequency of ESBL principally in *Klebsiella* spp. isolated from male gender with UTI of hospital origin. CTX-M was the major family of ESBL identified, with a higher frequency of the CTX-M-1-group, but the CTX-M-8/25 groups were also present. *Klebsiella* spp. also presented more combinations of β -lactamase enzymes and higher levels of resistance to CTX. The strong association with different ATM classes, but low resistance to carbapenems, shows that despite having therapeutic options for ESBL producers, it is necessary to ration ATM use to control the spread of ESBL producers in the studied populations.

Acknowledgments

We would like to thank Dr. Jorge Luiz Mello Sampaio for kindly providing strains as positive controls and our colleague Lourdes Helena Rodrigues Martins for the support with the bench work. *E. coli* INCQS 00033—(ATCC 25922) was courtesy of Coleção de Microrganismos de Referência em Vigilância Sanitária-CMRVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ.

Disclosure Statement

No competing financial interests exist.

References

1. Holmes, A.H., L.S.P. Moore, A. Sundsfjord, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P.J. Guerin, and L.J. Piddock. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet* 387:176–187.
2. Foxman, B. 2014. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect. Dis. Clin. North Am.* 28:1–13.
3. World Health Organization (WHO). 2014. Antimicrobial resistance: global report on surveillance. Available at www.who.int/drugresistance/documents/surveillance-report/en/ (accessed September 4, 2017).
4. Paterson, D.L., and R.A. Bonomo. 2005. Extended-spectrum beta-lactamases: a clinical update. *Clin. Microbiol. Rev.* 18: 657–686.
5. Bradford, P.A. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* 14:933–951.
6. Shaikh, S., J. Fatima, S. Shakil, S.M.D. Rizvi, and M.A. Kamal. 2015. Antibiotic resistance and extended spectrum beta-lactamases: types, epidemiology and treatment. *Saudi J. Biol. Sci.* 22:90–101.
7. Evans, B.A., and G.B. Sebastian. 2014. OXA β -lactamases. *Clin. Microbiol. Rev.* 27: 241–263.
8. Torres, E., I. López-Cerero, J.M. Rodríguez-Martínez, and A. Pascual. 2016. Reduced susceptibility to cefepime in clinical isolates of Enterobacteriaceae producing OXA-1 beta-lactamase. *Microb. Drug Resist.* 22:141–146.

9. Brolund, A. 2014. Overview of ESBL-producing Enterobacteriaceae from a Nordic perspective. *Infect. Ecol. Epidemiol.* 4:1–9.
10. Reuland, E.A., N.A. Naiemi, A.M. Kaiser, M. Heck, J.A.J.W. Kluytmans, P.H.M. Savelkoul, P.J.M. Elders, and C.M.J.E. Vandenbroucke-Grauls. 2016. Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam. *J. Antimicrob. Chemother.* 71:1076–1082.
11. Shodavaram, U.V.R., M.V. Ramanamma, A.L. Mukherjee, and A.S. Jacob. 2016. Prevalence of ESBL-mediated resistance among hospital and community isolates of *Klebsiella pneumoniae* in Warangal. *Int. J. Med. Res. Rev.* 4: 1005–1009.
12. Rogers, B.A., H.E. Sidjabat, and D.L. Paterson. 2011. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *J. Antimicrob. Chemother.* 66:1–14.
13. Dudley, M.N., P.G. Ambrose, S.M. Bhavnani, W.A. Craig, M.J. Ferraro, R.N. Jones, for the Antimicrobial Susceptibility Testing Subcommittee of the Clinical and Laboratory Standards Institute. 2013. Background and rationale for revised clinical and laboratory standards institute interpretive criteria (breakpoints) for Enterobacteriaceae and *Pseudomonas aeruginosa*: I. cephalosporins and aztreonam. *Clin. Infect. Dis.* 56:1301–1309.
14. Ministry of Health, Brazil. 1998. Ordinance 2616/98, of May 12, 1998. Regulates the actions to control hospital infection in the country [In Portuguese]. Available at http://bvsms.saude.gov.br/bvs/saudelegis/gm/1998/prt2616_12_05_1998.html (accessed October 17, 2017).
15. Clinical and Laboratory Standards Institute Edition. Approved standard—ninth. 2012. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
16. Xu, L., V. Ensor, S. Gossain, K. Nye, and P. Hawkey. 2005. Rapid and simple detection of blaCTX-M genes by multiplex PCR assay. *J. Med. Microbiol.* 54:1183–1187.
17. Dallenne, C., A. Da Costa, D. Decré, C. Favier, and G. Arlet. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β -lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* 65:490–495.
18. Bansal, S., and V. Tandon. 2011. Contribution of mutations in DNA gyrase and topoisomerase IV genes to ciprofloxacin resistance in *Escherichia coli* clinical isolates. *Int. J. Antimicrob. Agents* 37:253–255.
19. Wu, L.T., S.Y. Chang, M.R. Yen, T.C. Yang, and Y.H. Tseng. 2007. Characterization of extended-host-range pseudo-T-even bacteriophage Kpp95 isolated on *Klebsiella pneumoniae*. *Appl. Environ. Microbiol.* 73:2532–2540.
20. Bush, K., G.A. Jacoby, and A.A. Medeiros. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob. Agents Chemother.* 39:1211–1233.
21. Abreu, A.G., S.G. Marques, V. Monteiro-Neto, and A.G. Gonçalves. 2013. Extended-spectrum beta-lactamase-producing Enterobacteriaceae in community-acquired urinary tract infections in São Luís, Brazil. *Braz. J. Microbiol.* 44:469–471.
22. Gonçalves, L.F., P. de Oliveira Martins-Júnior, A.B.F. de Mello, R.C.R.M. da Silva, V. de Paulo Martins, A. Pitondo-Silva, and T.A. de Campos. 2016. Multidrug resistance dissemination by extended-spectrum beta-lactamase-producing *Escherichia coli* causing community-acquired urinary tract infection in the Central-Western Region, Brazil. *J. Glob. Antimicrob. Resist.* 6:1–4.
23. Kahlmeter, G., J. Åhman, and E. Matuschek. 2015. Antimicrobial resistance of *Escherichia coli* causing uncomplicated urinary tract infections: a European update for 2014 and comparison with 2000 and 2008. *Infect. Dis. Ther.* 4:417–423.
24. Manyahi, J., S.J. Moyo, M.G. Tellevik, F. Ndugulile, W. Urassa, B. Blomberg, and N. Langeland. 2017. Detection of CTX-M-15 beta-lactamases in Enterobacteriaceae causing hospital- and community-acquired urinary tract infections as early as 2004, in Dar es Salaam, Tanzania. *BMC Infect. Dis.* 17:282.
25. Melekos, M.D., and K.G. Naber. 2000. Complicated urinary tract infections. *Int. J. Antimicrob. Agents* 15:247–256.
26. Doi, Y., and Y. Arakawa. 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clin. Infect. Dis.* 45:88–94.
27. Jacoby, G.A. 2009. AmpC β -lactamases. *Clin. Microbiol. Rev.* 22:161–182.
28. Volcão, L.M., J.L. Pereira, I.B. Ramis, D.F. Ramos, G.S. Damati, C.V. Gonçalves, P.E.A. Silva, and A.V. Groll. 2016. Factors associated with resistance to ciprofloxacin and levofloxacin in Gram-negative isolates from urinary tract infections. *Rev. Epidemiol. Control. Infecção.* 6:18–23.
29. Volcão, L.M., J.L. Pereira, M.F. Gewehr, V.L. Real, D.F. Ramos, C.V. Gonçalves, L.G. Possuelo, L.A.R. Minarini, P.E.A. da Silva, and A. von Groll. 2018. High frequency of the aac (6′)-Ib-cr gene associated with double mutations in gyrA and parC in *Escherichia coli* isolates from patients with urinary tract infections. *J. Glob. Antimicrob. Resist.* 13:180–183.
30. Nordmann, P., G. Cuzon, and T. Naas. 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet. Infect. Dis.* 9:228–236.
31. Carattoli, A. 2009. Resistance plasmid families in Enterobacteriaceae. *Antimicrob. Agents Chemother.* 53:2227–2238.
32. Rocha, F.R., V.T.P. Pinto, and F.C.B. Barbosa. 2016. The spread of CTX-M-type extended-spectrum β -lactamases in Brazil: a systematic review. *Microb. Drug Resist.* 22:301–311.
33. Nogueira, K.S., D. Conte, F.V. Maia, and L.M. Dalla-Costa. 2015. Distribution of extended-spectrum β -lactamase types in a Brazilian tertiary hospital. *Rev. Soc. Bras. Med. Trop.* 48:162–169.
34. Chagas, T.P.G., R.M. Alves, D.C. Vallim, L.M. Seki, L.C. Campos, and M.D. Asensi. 2011. Diversity of genotypes in CTX-M-producing *Klebsiella pneumoniae* isolated in different hospitals in Brazil. *Braz. J. Infect. Dis.* 15:420–425.
35. Sabaté, M.R., F. Tarrago, E. Navarro, E. Miró, C. Vergés, J. Barbé, and G. Prats. 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-9) from *Escherichia coli* in Spain. *Antimicrob. Agents Chemother.* 44:1970–1973.
36. Bonnet, R., C. Dutour, J.L.M. Sampaio, C. Chanal, D. Sirot, R. Labia, C. De Champs, and J. Sirot. 2001. Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240 $\rightarrow$ Gly. *Antimicrob. Agents Chemother.* 45:2269–2275.
37. Dexheimer, G.M., J. Prediger, L. Weidlich, and A. Pozzobon. 2015. Prevalence of resistance and molecular characterization of extended spectrum beta-lactamase (ESBL)-producing bacteria isolated in a hospital in Southern Brazil. *Afr. J. Microbiol. Res.* 9:294–300.

38. Bonnet, R., J.L.M. Sampaio, R. Labia, C. De Champs, D. Sirot, C. Chanal, and J. Sirot. 2000. A novel CTX-M β -lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in Brazil. *Antimicrob. Agents Chemother.* 44:1936–1942.
39. Peirano, G., M.D. Asensi, A. Pitondo-Silva, and J.D.D. Pitout. 2011. Molecular characteristics of extended-spectrum β -lactamase-producing *Escherichia coli* from Rio de Janeiro, Brazil. *Clin. Microbiol. Infect.* 17:1039–1043.
40. Cantón, R., J.M. González-Alba, and J.C. Galán. 2012. CTX-M enzymes: origin and diffusion. *Front. Microbiol.* 3:110.
41. Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann. 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β -lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* 44:622–632.
42. Kjeldsen, T.S., M. Overgaard, S.S. Nielsen, V. Bortolaia, L. Jelsbak, M. Sommer, L. Guardabassi, and J.E. Olsen. 2015. CTX-M-1 β -lactamase expression in *Escherichia coli* is dependent on cefotaxime concentration, growth phase and gene location. *J. Antimicrob. Chemother.* 70:62–70.
43. Nikaido, H., M. Basina, V.Y. Nguyen, and E.Y. Rosenberg. 1998. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β -lactam antibiotics containing lipophilic side chains. *J. Bacteriol.* 180:4686–4692.
44. Doménech-Sánchez, A., S. Hernández-Allés, L. Martínez-Martínez, V.J. Benedí, and S. Albertí. 1999. Identification and characterization of a new porin gene of *Klebsiella pneumoniae*: its role in β -lactam antibiotic resistance. *J. Bacteriol.* 181:2726–2732.
45. Winokur, P.L., A. Brueggemann, D.L. DeSalvo, L. Hoffmann, M.D. Apley, E.K. Uhlenhopp, M.A. Pfaller, and G.V. Doern. 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC β -lactamase. *Antimicrob. Agents Chemother.* 44:2777–2783.

Address correspondence to:
Juliano Lacava Pereira, PhD

Faculdade de Medicina
Medical Microbiology Research Center (NUPEMM)
Universidade Federal do Rio Grande—FURG
Campus Saúde
Rua General Osório, s/n, Centro
Rio Grande, RS, CEP: 96200-400
Brazil

E-mail: julianolacava@gmail.com