

Nosocomial and community infections due to class A extended-spectrum β -lactamase (ESBLA)-producing *Escherichia coli* and *Klebsiella* spp. in southern Brazil

Authors

Claudia Wollheim¹
Ivani Maria F Guerra²
Vania D Conte³
Sheila P Hoffman³
Fernando J Schreiner¹
Ana Paula L Delamare⁴
Afonso L Barth⁵
Sérgio Echeverrigaray⁶
Sérgio Olavo P da Costa⁶

¹PhD, Medical Microbiology Laboratory, Universidade de Caxias do Sul, RS, Brazil
²MS, Medical Microbiology Laboratory, Universidade de Caxias do Sul, RS, Brazil
³Pharmacist, Medical Microbiology Laboratory, Universidade de Caxias do Sul, RS, Brazil
⁴PhD, Instituto de Biotecnologia, Universidade de Caxias do Sul, RS, Brazil
⁵PhD, Hospital de Clínicas de Porto Alegre, RS, Brazil
⁶PhD, Instituto de Biotecnologia, Universidade de Caxias do Sul, RS, Brazil

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Correspondence to:

Cláudia Wollheim
Laboratório de Microbiologia Médica Humana
Universidade de Caxias do Sul
Rua Francisco Getúlio Vargas, 1130, Petrópolis, Caxias do Sul, RS, Brazil 95001-970
Phone: + 55 54 3218-2548
Fax: + 55 54 3218-2041
cwollhei@ucs.br

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ABSTRACT

Objectives: To determine the prevalence of class A extended spectrum β -lactamases (ESBL)-producing *Escherichia coli* and *Klebsiella* spp., and to investigate clonality among ESBL-producing isolates of nosocomial and community infections. **Methods:** The study involved 354 nosocomial infections samples and 992 community infections samples, obtained between 2003 and 2006 at Caxias do Sul, RS. The detection of ESBL was performed by the disk-diffusion test. Presence of *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} β -lactamase genes was evaluated by PCR, and genomic typing was determined by pulsed-field gel electrophoresis analysis. **Results:** Higher frequency of ESBL-producing isolates were detected among nosocomial samples of *E. coli* (6.7%) and *Klebsiella* (43.7%), than those obtained from community infections (0.4% and 2.6%). *bla*_{TEM} and *bla*_{CTX} were the most prevalent ESBL gene families in both *E. coli* and *Klebsiella* isolates. Different pulsotypes were obtained among ESBL-producing *E. coli* and 11 clones for *Klebsiella* spp., which occurred over the years and in different hospital wards. Among ESBL-producing *K. pneumoniae*, 74.3% transferred ESBL genes by conjugation and exhibited concomitant decreased aminoglycosides susceptibility. **Conclusion:** ESBL-producing *E. coli*, and especially *K. pneumoniae* are essentially a nosocomial problem, and their dissemination to the community is relatively limited. The great genetic variability observed among ESBL-producing bacteria indicates polyclonal spread and high transference of ESBL genes between bacteria in the hospital environment. This information is of paramount importance for nosocomial infection control.

Keywords: β -lactamases; polymerase chain reaction; bacterial typing techniques.

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INTRODUCTION

Extended-spectrum β -lactamase-producing clinical isolates among members of the *Enterobacteriaceae* family, especially *Klebsiella pneumoniae* and *Escherichia coli*, represent one of the most important world problems of β -lactam antimicrobial resistance, commonly used in the treatment of many bacterial nosocomial and community infections.¹

Traditionally ESBLs appeared just after the introduction of third-generation cephalosporins, and evolved from parent enzymes due to point mutation in the *bla*_{TEM-1/2} and *bla*_{SHV-1} genes around the β -lactamase active site.² The majority of ESBLs belong to the functional class 2be/molecular class A, particularly TEM and SHV types, and the CTX-M family β -lactamases encoded by genes captured by mobile elements from the chromosomes of the environmental bacteria *Kluyvera* spp.¹

At present, the definition of ESBL as 2be/molecular class A, clavulanic acid inhibited enzymes that cause resistance to penicillins, to all cephalosporin generations, and to the monobactam aztreonam, but not to cephamycins or carbapenems, is narrow and excludes other clinically important acquired β -lactamase with wider spectrum.¹ In the effort to prevent the spread of acquired β -lactamase, a new classification had been proposed including β -lactamase with activity against extended-spectrum cephalosporins and/or carbapenems, making the classification more accessible to clinicians, infection control professionals, hospital managers, and politicians. Three groups of ESBL were projected: (1) ESBL_A (classical class A ESBLs); (2) ESBL_M (plasmid-mediated AmpC and OXA-ESBLs classed as miscellaneous ESBLs); and (3) ESBL_{CARBA} (carbapenemases).^{1,3}

The correct identification of ESBL-producing bacteria has important clinical-epidemiological and laboratory implications. First, patients may experience a delay in appropriate treatment if ESBL-producing bacteria are not correctly detected by routine antimicrobial susceptibility tests.⁴ Second, while carbapenemics are the most effective therapy for ESBL bacterial infections, their routine use can select resistant strains, as the emergence of imipenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *K. pneumoniae*.^{5,6} Third, ESBL genes are located on large plasmids that can harbor genes for resistance to other non- β -lactams antibiotics, and therefore, ESBL-producing bacteria often exhibit multidrug-resistant phenotypes, reducing the drug arsenal even further.¹ Fourth, genes encoding ESBLs are typically located in conjugative plasmids or integron-like structures and can be effectively transferred to other strains and species.^{7,8} Finally, ESBL-producing organisms, especially *K. pneumoniae*, but also *E. coli*, have been responsible for serious nosocomial infection outbreaks that lead to prolonged hospital stay, increased morbidity and mortality, and consequently increase healthcare associated costs.⁹⁻¹¹

Since the first description of ESBL in 1983, in Germany, ESBL-producing clinical isolates have spread rapidly throughout Europe and the United States and are now disseminated worldwide.^{12,13} However, the world prevalence of ESBL producers varies greatly, even among health institutions in the same country.¹⁴ Although it is a major threat for patients in the hospital, currently ESBL are becoming an emerging problem for patients in long-term care facilities, and also in the community in some areas of the world.¹⁵⁻¹⁷

The aims of this study were to determine the prevalence of ESBL among *E. coli* and *Klebsiella* spp. in Caxias do Sul, in the southern region of Brazil, to identify the prevalence of β -lactamase genes (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}), and to investigate clonality among clinical isolates of ESBL-producing *E. coli* and *Klebsiella* spp. of nosocomial and community infections during a period of three years.

MATERIAL AND METHODS

Patient population and definitions

The study was carried between April 2003 and May 2006 at a private clinical analyses laboratory, and at the General Hospital of Caxias do Sul (HG), located in Rio Grande do Sul State Brazil. The HG is a 257-bed public university teaching hospital and acts as a referral hospital for a wider area of Northeast RS, covering a population of about one million inhabitants. Personal data (age, sex), type of specimens, and the hospital ward at the HG where the patient had been assigned were obtained. Nosocomial infection was defined as infection which occurred more than 48 hours after hospital admission; infection which occurred less than 48 hours af-

ter hospital admission, if that patient had been hospitalized within the prior 30 days; or infection which occurred in a patient transferred from another hospital or from a nursing home.¹⁸ Patients with community infection were those presenting it at a community clinical analyses laboratory and those who had a positive culture at the time of or within 48 hours of hospitalization; in both cases, patients had no previous contacts with hospitals or long-term care facility in the previous two weeks.¹⁹

Bacterial isolates

Clinical specimens obtained from patients with nosocomial infection yielded 354 isolates (209 *E. coli*, 142 *K. pneumoniae* and 3 *K. oxytoca*), and a total of 992 isolates (953 *E. coli*, 38 *K. pneumoniae* and 1 *K. oxytoca*) were obtained from patients with community infection. Isolates were identified using the conventional biochemical tests and stored at -70°C in skim milk. Only one isolate from each patient was included in the study.

Screening and confirmation tests of ESBLs

ESBL-producing isolates identification was made by the disk-diffusion screening test with aztreonam, cefotaxime, ceftazidime, ceftriaxone, and cefpodoxime, followed by a confirmatory test with ceftazidime and cefotaxime, combined with clavulanic acid as recommended by the Clinical and Laboratory Standards Institute (CLSI).²⁰ An additional confirmatory test was performed with cefepime and cefpodoxime discs containing clavulanic acid. All the tests were conducted with Oxoid antibiotic discs. The standard strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls of ESBLs production.

PCR amplification for detection of β -lactamase genes

The bacterial isolates with confirmed ESBL phenotype were submitted to PCR assay to identify *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} β -lactamase genes, using the primers and method described by Paterson *et al.*¹⁴ The *K. pneumoniae* ATCC 700603 strain was used as a positive control for the *bla*_{SHV} gene.

Molecular typing

Genomic typing of ESBL producing isolates was performed by the pulsed-field gel electrophoresis (PFGE) technique based on PulseNet PFGE protocol.²¹ Briefly, genomic DNA was prepared in agarose plugs, cut with restriction endonuclease SpeI (Invitrogen), and restriction fragments separated using a CHEF-DRIII system (Bio-Rad). Photographs of ethidium bromide-stained gels were examined visually and the macrorestriction patterns were interpreted according to the criteria proposed by Tenover *et al.*²² A 48.5 Kb bacteriophage λ ladder was used as a DNA size marker.

Gene transfer assays

Conjugation assays were carried out by a broth mating procedure in Luria-Bertani (LB) broth with 35 ESBL-producing *K. pneumoniae* isolates susceptible to streptomycin and ceftriaxone. DH5 α *E. coli* (streptomycin resistant, lactose fermentation negative, and plasmid free) was used as the recipient.²³ Overnight cultures of donor and recipient strains grown in LB broth were added to 8 mL of fresh LB broth at a donor-recipient ratio of 1:1 (300 μ L of cultures each), and incubated for 4 hours at 37°C. The mixed culture was plated onto MacConkey agar containing 30 μ g/mL of ceftriaxone and 300 μ g/mL of streptomycin. Six colonies growing on the selections plates and again on subculture on selective MacConkey agar were subjected to confirmatory tests of ESBLs and to antimicrobial susceptibility by disk diffusion assays according to CLSI recommendation with amikacin, gentamicin, tobramycin, ciprofloxacin, gatifloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole and chloramphenicol. Resistance was considered nontransferable when the isolates failed to transfer in more than two experiments.

RESULTS

Prevalence of ESBL isolates

A total of 1,346 non-replicated isolates (354 from nosocomial and 992 from community infection patients) were analyzed. Overall, 21.8% (77/354) and 0.5% (5/992) of the isolates were confirmed as ESBL producers by the clavulanic acid inhibition disk diffusion assay. Fourteen (6.7%) of 209 patients with *E. coli* infection had ESBL-producing strain, compared with 4 (0.4%) of 953 patients with community-acquired *E. coli* infection. ESBL-producing *K. pneumoniae* were isolated from 62 (43.7%) of 142 patients with nosocomial infection, and from one (2.6%) of 38 isolates with community-acquired infection. Among the four *K. oxytoca* isolates, one obtained from a nosocomial patient exhibited ESBL phenotype (Table 1).

Table 1. Prevalence of ESBL-positive *Escherichia coli* and *Klebsiella spp.* by origin of the infection

Origin in infection species	Total no. of isolates (n = 1,346)	ESBL positive isolates	
		No. of isolates	% in species
Nosocomial			
<i>E. coli</i>	209	14	6.7
<i>K. pneumoniae</i>	142	62	43.7
<i>K. oxytoca</i>	3	1	33.3
Total	354	77	21.8
Community			
<i>E. coli</i>	953	4	0.4
<i>K. pneumoniae</i>	38	1	2.6
<i>K. oxytoca</i>	1	0	0
Total	992	5	0.5

Patient population and source of specimens

Of the 1,346 patients studied, the mean age and gender of patients with nosocomial infection were different among those with community infections (51.4 \pm 28.0 years and 54.8% female versus 39.1 \pm 22.4 years and 89.3% female). In both patient populations, the most frequent source of infection was urinary. The high frequency of urinary infections (97.7%) among community patients explains the difference on gender proportion. The mean age of the patients with nosocomial infection due to ESBL-producing *E. coli* and *Klebsiella spp.* differed significantly (40.7 \pm 31.7 years, range: 8.3 months to 88 years versus 4.8 \pm 2.9 years, range: 4 days to 92 years), respectively. Most patients (65.1%) with nosocomial infection due to ESBL-producing *Klebsiella spp.* were male, while no gender difference was observed among community infection patients.

The ESBL-producing bacteria obtained from patients with nosocomial infections were isolated from the urinary tract (5.8% *E. coli* and 49% *Klebsiella spp.*), respiratory tract (14.3% *E. coli* and 41.9% *Klebsiella spp.*), blood/catheter (12.5% *E. coli* and 48.4% *Klebsiella spp.*), peritoneal liquid (25% *Klebsiella spp.*), and skin/soft tissue (25% *Klebsiella spp.*). Most ESBL-producers were detected on patients hospitalized in Intensive-Care Units, including the Neonatal, Pediatric and Adult ICUs (15.9% *E. coli* and 51.7% *Klebsiella spp.*), in Clinical-Surgical Units (4.3% *E. coli* and 45.5% *Klebsiella spp.*), and Urgency and Emergency wards (31.6% *Klebsiella spp.*).

Prevalence of β -lactamase genes and genotyping

As shown in Figure 1, 61.1% of ESBL-producing *E. coli* were isolated during 2005 to 2006, whereas 67.2% of ESBL *Klebsiella spp.* isolates were collected between 2003 to 2004.

The PCR assay allowed detection of *bla*_{TEM} gene family in 66.7% of the isolates of ESBL-producing *E. coli*, followed by *bla*_{CTX-M} gene (50%) and the *bla*_{SHV} gene (11.1%) (Figure 1A). Several *E. coli* isolates exhibited β -lactamase gene combinations (*bla*_{CTX-M} and *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{TEM}), and no amplification products were observed in one ESBL isolate. Among *Klebsiella spp.* with ESBL-phenotype, *bla*_{TEM} gene was the most prevalent (95.3%), followed by the *bla*_{CTX-M} (82.8%), and the *bla*_{SHV} gene (42.2%) (Figure 1B). Combination of two and three ESBL genes was detected in 59.4% and 31.3% of ESBL-producing *Klebsiella* isolates.

The clonal relatedness was studied by PFGE. Eighteen different pulsotypes were obtained among 18 ESBL-producing *E. coli* isolates, and 23 pulsotypes among 63 ESBL-producing *K. pneumoniae* (Figure 1). One isolate was consistently untypeable by the applied method.

Genotyping revealed that 82.3% of *K. pneumoniae* isolated were non-unique and could be separated into 11 clones. The most prevalent (Clone 5) with 13 isolates,

emerged in 2003 with four isolates in two hospital wards (Clinical-Surgical floor 5 and Adult ICU floor 3), and was detected again in 2004, with 9 isolates recovered from the same hospital wards. The occurrence of different clones over the years and hospital wards are shown in Table 2.

Among the 35 ESBL-producing *K. pneumoniae* isolates selected for conjugation experiments, 74.3% (26 isolates) transferred ESBL genes by conjugation. All donor strains harbored the *bla*_{CTX-M} gene. The transconjugants obtained exhibited decreased susceptibilities to the aminoglycosides, amikacin, gentamicin and tobramycin.

Figure 1: Distribution of different β -lactamase genes among ESBL-positive 18 *E. coli* (A) and 64 *Klebsiella* spp. (B) isolates from 2003 to 2006. Values between brackets represent the number of pulsotypes.

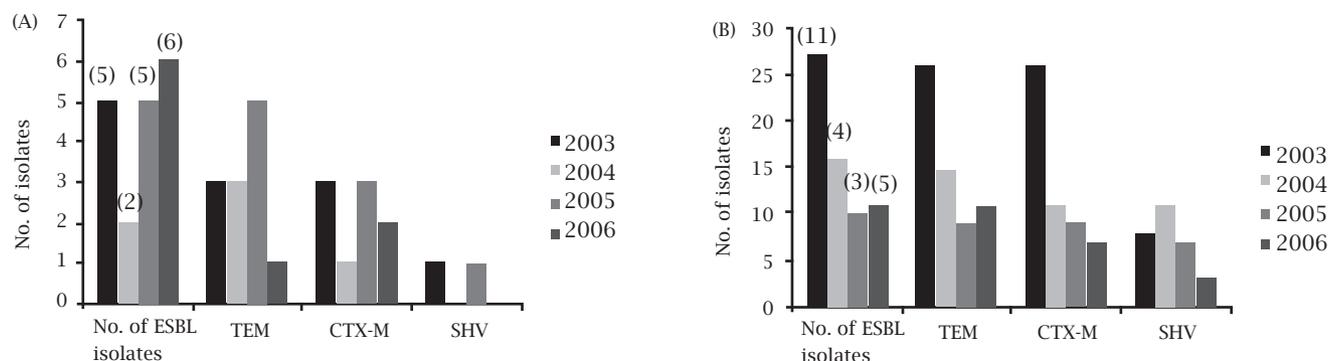


Table 2. Distribution of the 11 clones of ESBL-producing *Klebsiella pneumoniae* by genes of β -lactamases, time of detection the isolates and hospital ward

PFGE type	N° of isolates	β -lactamase genes profile ^a			Time (yr) of isolation	Hospital ward of isolation (No. of isolates)	Floor
		CTX-M	TEM	SHV			
Clone 1	4	+	+	-	2003	Clinical-surgical (1)	6°
		+	+	+	2003/2005	Clinical-surgical (1) ^b	6°
		-	+	+	2006	Adult ICU (1)	3°
Clone 2	6	+	+	-	2003	Neonatal ICU (2), clinical-surgical (2)	3°, 6°
		+	+	-	2003	Urgency-emergency (1), clinical-surgical (1)	1°, 5°
Clone 3	5	+	+	-	2003	Neonatal ICU (2), adult ICU (1)	3°
		+	+	-	2003	Pediatric ICU (1), clinical-surgical (1)	4°, 6°
Clone 4	5	+	-	-	2003	Pediatric ICU (1)	4°
		+	+	-	2003/2004	Clinical-surgical (1), adult ICU (1)/ pediatric ICU (2)	6°, 3°, 4°
Clone 5	13	-	+	-	2003	Clinical-surgical (1)	5°
		+	+	+	2003	Adult ICU (3)	3°
		+	+	+	2004	Adult ICU (3)/ clinical-surgical (2)	3°/5°, 6°
		-	+	+	2004	Adult ICU (2)/ clinical-surgical (2)	3°/5°, 6°
Clone 6	2	+	+	+	2003/2004	Adult ICU (2)	3°
Clone 7	5	+	+	+	2005	Pediatric ICU (1)	4°
		+	-	-	2005	Adult ICU (2)	3°
		+	+	+	2005	Neonatal ICU (1), adult ICU (1)	3°
Clone 8	2	+	+	-	2005	Adult ICU (1), pediatric ICU (1)	3°, 4°
		+	+	+	2005	Urgency-emergency (1)	1°
Clone 9	2	-	+	+	2005	Clinical-surgical (1)	5°
		+	+	-	2006	Clinical-surgical (1)	6°
Clone 10	3	+	+	+	2006	Clinical-surgical (2)	6°
		+	+	-	2006	Clinical-surgical (1)	6°
Clone 11	4	+	+	-	2006	Clinical-surgical (2)	5°, 6°
		-	+	-	2006	Clinical-surgical (2)	6°

a-, absence; +, presence; b, community infection. Adult ICU, Adult intensive-care unit; Neonatal ICU, Neonatal intensive-care unit; Pediatric ICU, Pediatric intensive-care.

DISCUSSION

Although, in our study, *E. coli* was more frequently isolated than *K. pneumoniae*, ESBL production was more prevalent in *K. pneumoniae*, with a high prevalence (43.7%) in the hospital environment, and especially in pediatric patients from the neonatal ICU. The high frequency of ESBL-producing bacteria in ICUs is expected due the great selective pressure resulting from the large use of antibiotics on ICU patients. High prevalence of ESBL among isolated *K. pneumoniae* had been detected in numerous studies as reviewed by Paterson and Bonomo.¹ However, the prevalence of ESBL-positive *K. pneumoniae* and *E. coli* varies greatly among different geographical areas, with one of the highest reported rate (*K. pneumoniae* 30% to 60%, *E. coli* 4.5% to 12%) in Latin American countries, including Brazil.^{24,25}

As previously reported in Juiz de Fora, Brazil,²⁶ low prevalence of ESBL phenotype was detected in community isolates, independently of the bacteria species, 2.6% for *K. pneumoniae* and 0.4% for *E. coli*. Moreover, two of the five community-acquired cases reported healthcare contact (undergoing chemotherapy on day hospital) and may be healthcare associated infections.

Although the clinical impact of ESBLs is related to a combination of factors that rely more heavily on functional rather than structural characteristics, in terms of epidemiology or long-term effects of antibiotic use, identification of specific β -lactamases and/or their genes can play an important role. Among ESBL gene families, *bla*_{TEM} was the most prevalent in both *E. coli* and *K. pneumoniae* ESBL-producing isolates obtained from nosocomial and community infections. A very high prevalence of *bla*_{CTX-M} (82.8%) was also detected among *K. pneumoniae* isolates, and *bla*_{SHV} was present in 42.2% and 11.1% of *K. pneumoniae* and *E. coli* ESBL-producing isolates, respectively. *bla*_{TEM} family genes are frequent among enterobacteria,¹ but not all TEM enzymes can be considered ESBL, and sequencing is therefore necessary for allele identification.¹³ Extra-intestinal infections caused by *E. coli* harbouring plasmid-encoded *bla*_{CTX-M} have been increasingly reported worldwide, from both developed and developing countries in the last decades.^{1,15,27-30} Moreover, while most of the SHV β -lactamases are ESBLs, it is known that the chromosomal *bla*_{SHV-1} is highly prevalent in *K. pneumoniae*.¹⁴

Genotyping using PFGE analysis of ESBL-producing *K. pneumoniae* revealed 23 different pulsotypes, consisting of 11 clones and 12 single patterns. Of the 11 clones, clone 5 was predominant and a persistent endemic clone in different wards and throughout the years. While multiclonal dissemination of ESBL-producing *K. pneumoniae* was found, no clonal dissemination of ESBL-producing *E. coli* was observed. Clonal dissemination suggests cross-transmission, which implies adopting precautionary measures of contact, including wearing aprons and gloves for contact with the colonized or infected patient, together with an efficient hy-

gienization and antiseptics. Alternatively, the great genomic variability suggests strong selective pressure on bacterial populations, indicating the necessity of proper management of antibiotic therapy, particularly within healthcare units.

CONCLUSION

To sum up, the data obtained in this study showed that ESBL-producing isolates of *E. coli* and especially of *Klebsiella* spp. are essentially a nosocomial problem, and their dissemination to the community are not very significant. However, public health professionals must remain alert to the advancing issues associated with ESBLs to assist potential healthcare-associated and community outbreaks.

Genes *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{SHV} were detected in high frequency among ESBL-positive *K. pneumoniae* and *E. coli* isolates, occurring alone or in combination. The high prevalence of ESBL genes that are frequently found in conjugative plasmids and/or integrons may be considered a risk factor as they can be efficiently transferred within and between enterobacterial species.

Moreover, it was observed a great genomic variability in the ESBL-producing isolates of *E. coli* and *Klebsiella* spp., indicating a strong selective pressure of antimicrobials. However, it was also found that the polyclonal spread of strains might contribute to the proliferation of ESBL *K. pneumoniae* in the hospital and that endemic ESBL clone persisted throughout the years in the hospital. Finally, we have shown that molecular typing ESBL-producing *K. pneumoniae* was clinically relevant, given that patient-to-patient transmission of organisms harboring ESBLs, which were fundamental for infection control interventions, clearly occurs.

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