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EXTENDED-SPECTRUM BETA-LACTAMASES AMONG *Enterobacteriaceae* ISOLATED IN A PUBLIC HOSPITAL IN BRAZIL

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SUMMARY

Extended-spectrum β -lactamases (ESBL) in enterobacteria are recognized worldwide as a great hospital problem. In this study, 127 ESBL-producing *Enterobacteriaceae* isolated in one year from inpatients and outpatients at a public teaching hospital at São Paulo, Brazil, were submitted to analysis by PCR with specific primers for bla_{SHV} , bla_{TEM} and bla_{CTX-M} genes. From the 127 isolates, 96 (75.6%) *Klebsiella pneumoniae*, 12 (9.3%) *Escherichia coli*, 8 (6.2%) *Morganella morganii*, 3 (2.3%) *Proteus mirabilis*, 2 (1.6%) *Klebsiella oxytoca*, 2 (1.6%) *Providencia rettgeri*, 2 (1.6%) *Providencia stuartii*, 1 (0.8%) *Enterobacter aerogenes* and 1 (0.8%) *Enterobacter cloacae* were identified as ESBL producers. bla_{SHV} , bla_{TEM} and bla_{CTX-M} were detected in 63%, 17.3% and 33.9% strains, respectively. Pulsed field gel electrophoresis genotyping of *K. pneumoniae* revealed four main molecular patterns and 29 unrelated profiles. PCR results showed a high variety of ESBL groups among strains, in nine different species. The results suggest the spread of resistance genes among genetically different strains of ESBL-producing *K. pneumoniae* in some hospital wards, and also that some strongly related strains were identified in different hospital wards, suggesting clonal spread in the institutional environment.

KEYWORDS: ESBL; *Enterobacteriaceae*; *Klebsiella pneumoniae*; Pulsed Field Gel Electrophoresis; Antimicrobial resistance.

INTRODUCTION

Extended-spectrum beta-lactamases (ESBL) production by nosocomial pathogens are a major challenge for infection control committees in hospitals all over the world, once ESBL-producing strains and their encoding genes can stay permanently in hospitals, causing outbreaks and colonization (BRADFORD 2001, GUPTA *et al.* 2003, DENTON 2007). There are more than 300 ESBL described so far, most of them derived from groups TEM, SHV and CTX-M, with 150, 88 and 69 variants, respectively (see <http://www.lahey.org/Studies>).

In Latin America there is a high rate of ESBLs, although their distribution is still restricted to some genes and to specific locations (VILLEGAS *et al.* 2008). In SHV group, the variants SHV-2, SHV-5 and SHV-12 are the most common (PATERSON *et al.* 2003, VILLEGAS *et al.* 2008), although in Brazil there have already been reported the detection of SHV-4 (MENDES *et al.* 2004) and the characterization of SHV-27 (CORKILL *et al.* 2001). TEM variants have been described very recently in South America (PATERSON *et al.* 2003, VIGNOLI *et al.* 2005, VILLEGAS *et al.* 2008), and the most common are TEM-10, TEM-12 and TEM-26 (VILLEGAS *et al.* 2008). CTX-M ESBLs are endemic and widely distributed in our continent. They were reported in 1989 in Argentina (CTX-M-2) and, since then, several different enzymes from this group were reported in many countries, including Brazil, where CTX-M-8, -9

and -16 were described (VILLEGAS *et al.* 2008). Some recent Brazilian studies show the high prevalence of ESBL-carrying *Enterobacteriaceae* in healthcare centers and even in the community, especially in *Klebsiella pneumoniae* isolates (NOGUEIRA *et al.* 2006, MINARINI *et al.* 2007, MINARINI *et al.* 2008, SILVA DIAS *et al.* 2008).

The aim of this study was to identify groups of ESBL disseminated among enterobacteria isolated during one year in a teaching hospital, as well as to establish the genetic relationship among all ESBL-producing *K. pneumoniae* isolates.

MATERIAL AND METHODS

Setting: This study was carried out in a general public university hospital with 258 beds distributed among ten wards (Emergence Room - ER, Surgical Ward - SW, General Ward - AMW, Pediatric Ward - PW, Adult, Neonatal and Pediatric Intensive Care Units - A.ICU, N.ICU and P.ICU, Intermediate Care Unit - IMC, Neonatal Ward - NW and Maternity Ward - MW), with a mean turnover of 5500 patients-day/month.

Bacterial strains: During one year, 127 ESBL-producing *Enterobacteriaceae* strains were recovered from clinical samples of hospitalized patients. Isolates were responsible for community- or hospital-acquired infections, and for colonization.

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Susceptibility test and ESBL production: Antimicrobial susceptibility tests, screening and confirmatory tests for ESBL production were carried out in Vitek System (BioMerieux, Hazlewood, MO). Double-disk synergy (Oxoid, Cambridge, UK) was also performed as a confirmatory test for all positive isolates (CLSI 2008).

Detection of *bla* genes: Total DNA extraction was carried out for all samples using the heat-shock technique (CHAPMAN *et al.* 2001). Isolates were tested by PCR for the β -lactamase groups SHV, TEM and CTX-M, with previously described oligonucleotides and reaction conditions (CAO *et al.* 2002). PCR products were analyzed by agarose gel electrophoresis.

Pulsed Field Gel Electrophoresis (PFGE): All *K. pneumoniae* isolates were submitted to DNA extraction (CDC 2004) and restriction endonuclease digestion with 10U *Xba*I. Separation of fragments was carried out at 6V/cm at 14 °C for 20h. The linear pulse time was 3,51s to 30,82s. Band polymorphism generated by PFGE was analyzed and the molecular patterns were grouped primarily according to previously established criteria (TENOVER *et al.* 1995), and then according to Dice similarity coefficient and demonstrated graphically in dendrograms. PFGE patterns were also analyzed according to their distribution among hospital wards during the studied period, using the software Pajek as a tool (BATAGELJ and MRVAR 1996).

RESULTS

Bacterial strains: 127 ESBL-producing isolates were recovered

from 86 patients among all hospital wards, except for the Maternity. Ninety-six (75.6%) strains were *K. pneumoniae* (ESBL-*Kp*), 12 (9.3%) *Escherichia coli*, 8 (6.2%) *Morganella morganii*, 3 (2.3%) *Proteus mirabilis*, 2 (1.6%) *Klebsiella oxytoca*, 2 (1.6%) *Providencia rettgeri*, 2 (1.6%) *Providencia stuartii*, 1 (0.8%) *Enterobacter aerogenes* and 1 (0.8%) *Enterobacter cloacae*. According to the hospital infection control committee, 29 (22.8%) isolates were responsible for hospital-acquired infections, 14 (11%) for community-acquired infections, and 84 (66.1%) for cases of colonization, from which 57 (67.9%) were recovered from routine samples and 27 (32.1%) were from surveillance cultures carried out by the committee.

Antimicrobial susceptibility tests and detection of ESBL production and genes: All strains were resistant to ampicillin and cephalotin, and susceptible to imipenem. Except for these results, the predominant antimicrobial susceptibility profiles for each PFGE pattern of *K. pneumoniae* strains are shown in Table 1, and for the other *Enterobacteriaceae* isolates are in Table 2. The combined discs method was able to detect the production of ESBL in all strains.

PCR: Genes *bla*_{SHV} were detected in 80 (63%) isolates, *bla*_{CTX-M} in 43 (33.9%) and *bla*_{TEM} in 22 (17.3%). Among *K. pneumoniae*, *bla*_{SHV} genes were detected in 71 (74%) strains. Twenty one (16.5%) strains produced some type of ESBL that could not be detected in PCR reactions, and thus were not classified as any of the groups studied. It was observed the detection of one to three groups of *bla* genes in each ESBL-producing isolate. All combinations of *bla* genes detected are shown in Tables 1 and 2.

Table 1
Antimicrobial susceptibility profiles for *K. pneumoniae* PFGE patterns, with respective PCR results

PFGE	n	FOX	CTX	CAZ	FEP	ATM	IPM	SAM	TZP	TIM	AMK	GEN	CIP	SXT	PCR (<i>bla</i>)
A ^a	34	S	R ^a	R	S	R	S	R	R ^a	R	R ^a	R	S	S	SHV SHV+TEM SHV+CTX-M SHV+TEM+CTX-M None
B ^a	14	S	R ^a	R	S ^b	R	S	R	R ^a	R	S	R ^a	S	R ^a	SHV None
C	15	S ^b	R	R	S ^b	R	S	R	R ^a	R	S ^b	R	S	R	SHV SHV+TEM None
E	5	S	R	S	R	R	S	R	R	R	S	R	S	S	CTX-M SHV+CTX-M
NR	29	S ^b	R ^a	R ^a	S ^b	R ^a	S	R	R ^a	R	S ^b	R ^a	R ^a	R ^a	SHV SHV+TEM SHV+CTX-M CTX-M TEM+CTX-M SHV+TEM +CTX-M

^a More than 50% of strains expressing resistance to the referred antibiotic; ^b More than 50% of strains expressing susceptibility to the referred antibiotic; n: number of strains; FOX: Cefoxitin; CTX: Cefotaxime; CAZ: Ceftazidime; FEP: Cefepime; ATM: Aztreonam; SAM: Ampicillin/Sulbactam; TZP: Piperacillin/Tazobactam; TIM: Ticarcillin/clavulanic acid; AMK: Amikacin; GEN: Gentamicin; CIP: Ciprofloxacin; SXT: Trimethoprim/sulfamethoxazole.

Table 2
Antimicrobial susceptibility profiles for other *Enterobacteriaceae*, with respective PCR results

Species (n)	FOX	CTX	CAZ	FEP	ATM	SAM	TZP	TIM	AMK	GEN	CIP	SXT	PCR (<i>bla</i>)
<i>Escherichia coli</i> (12)	S	S	R	S	R	R	R	R	S	S	S	S	SHV
	S	R	R	S	R	R	S	R	S	R	R	S	
	S	S	R	S	R	R	S	R	S	R	S	S	SHV+TEM
	S	R	S	R	R	R	S	S	S	S	S	R	SHV+CTX-M
	S	R	S	R	R	R	R	R	S	S	R	R	TEM+CTX-M
	S	R	R	R	R	R	R	R	S	R	R	R	
	S	R	S	R	R	R	R	R	S	R	S	R	CTX-M
	S	R	S	R	R	R	S	S	S	R	S	S	
	S	R	S	R	R	R	S	S	S	R	S	S	
	S	R	S	S	R	R	S	S	S	S	S	R	None
	S	S	R	S	R	R	S	R	S	R	R	S	
	<i>Morganella morganii</i> (7)	R	S	S	S	S	R	S	R	S	R	R	S
R		R	S	S	S	R	S	R	S	S	R	S	SHV+CTX-M
R		R	S	S	S	S	S	R	S	R	R	R	CTX-M
R		S	S	S	S	R	S	R	S	R	R	S	
R		R	S	S	S	R	S	S	R	R	R	R	None
R		S	S	S	S	R	S	R	S	R	R	S	
S		S	S	S	S	R	S	R	S	S	R	R	
<i>Proteus mirabilis</i> (4)	S	S	R	S	S	R	S	S	S	R	R	R	SHV
	S	S	S	S	S	R	S	S	S	R	R	R	CTX-M
	S	S	S	R	S	R	S	R	S	R	R	R	TEM+CTX-M
	S	S	S	S	S	S	S	S	S	R	R	R	SHV+TEM+CTX-M
<i>Providencia stuartii</i> (2)	S	R	S	R	S	R	S	R	S	R	R	S	CTX-M
	S	R	S	S	S	R	S	R	S	R	R	R	
<i>Klebsiella oxytoca</i> (2)	S	S	S	S	S	R	S	R	R	R	S	R	TEM
	S	R	S	R	R	R	R	R	R	R	R	R	CTX-M
<i>Providencia rettgeri</i> (1)	S	S	S	S	S	R	S	S	R	R	R	R	None
<i>Enterobacter cloacae</i> (1)	R	S	S	S	S	S	I	S	S	S	S	S	SHV+CTX-M
<i>Enterobacter aerogenes</i> (1)	R	R	S	R	I	R	R	R	R	I	R	R	CTX-M

n: number of strains; AMP: Ampicillin; CEF: Cefalotin; FOX: Cefoxitin; CTX: Cefotaxime; CAZ: Ceftazidime; FEP: Cefepime; ATM: Aztreonam; IPM: Imipenem; SAM: Ampicillin/Sulbactam; TZP: Piperacillin/Tazobactam; TIM: Ticarcillin/clavulanic acid; AMK: Amikacin; GEN: Gentamicin; CIP: Ciprofloxacin; SXT: Trimethoprim/sulfamethoxazole.

PFGE: Four main PFGE patterns (A, B, C and E) and four subtypes (A1, A3, A4 and B1) were obtained from 67 (69.8%) ESBL-Kp isolates (Fig. 1). The subtypes were classified according to previously established interpreting criteria (TENOVER *et al.* 1995). The other 29 (30.2%) strains presented unrelated profiles. Pattern A and subtypes A₁, A₃ and A₄ were grouped and called only A*, and pattern B and subtype B1 were called only B*. Pattern A* was observed in 33 (34.4%) strains, pattern B* in 14 (14.6%) strains, pattern C in 15 (15.6%) strains, and pattern

E in five (5.2%) strains. The distribution of the different PFGE patterns among the hospital wards during the studied period is shown in Fig. 2. From isolates showing genetically related profiles, 53 (79.1%) were responsible for colonizations. For this group detection of *bla*_{SHV} genes was observed in 56 (83.6%) of 67 strains, *bla*_{TEM} in five (7.5%) and *bla*_{CTX-M} in seven (10.4%) isolates. In strains showing unrelated patterns (29), it was possible to observe detection of *bla*_{SHV} genes in 22 (75.9%), *bla*_{TEM} in 11 (37.9%) and *bla*_{CTX-M} in 16 (55.2%) strains.

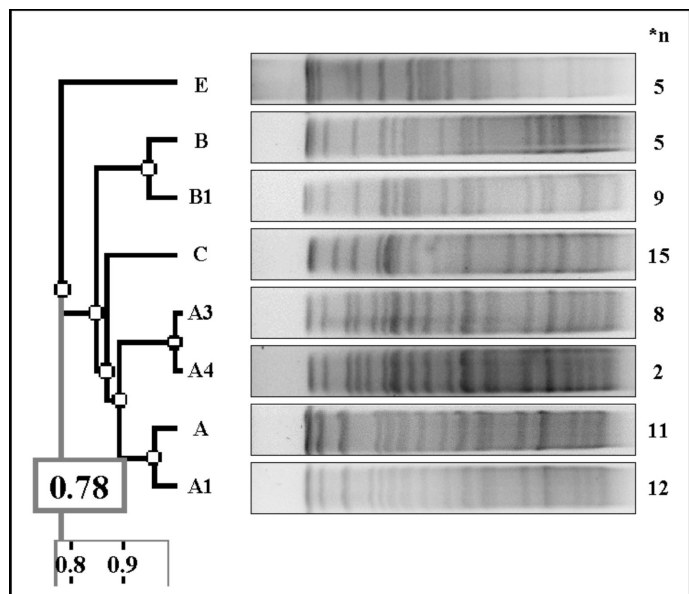


Fig. 1 - Estimates of genetic relationship, according to Dice similarity coefficient, of *Xba*I patterns obtained for ESBL-producing *K. pneumoniae* strains isolated from clinical and surveillance samples of patients in the university hospital. On the left are quoted the PFGE profiles identified and on the right the numbers of isolates (*n) exhibiting each PFGE pattern.

DISCUSSION

Production of ESBL has been frequently described worldwide, in several *Enterobacteriaceae* species, isolated from diverse clinical samples (QUINTEROS *et al.* 2003, CELENZA *et al.* 2006). In the present study, during one year it was detected one to three ESBL encoding genes (*bla*) in 127 isolates of nine *Enterobacteriaceae* species. It was not possible to establish the relationship among the several antimicrobial susceptibility patterns observed with the *bla* genes detected, once we carried out only a group identification. In regard to PFGE patterns, each of them presented a predominant susceptibility profile, although they had different *bla* genes combinations.

All the three groups of genes searched by PCR were detected, alone or in several combinations. Encoding genes for the SHV family were detected in 63% of the isolates, in six different species, from which 88.7% were *K. pneumoniae*. Most enzymes from this group are ESBL, except for SHV-1 and SHV-11, and according to the literature most *K. pneumoniae* strains possess the chromosomal gene *bla*_{SHV-1} (BABINI & LIVERMORE 2000, HOWARD *et al.* 2002, LIVERMORE 2008). Hence, the identification of *bla*_{SHV} genes in the major part of ESBL-*Kp* strains in this study was already expected, although we cannot affirm those are ESBL-encoding genes, mostly in strains presenting more than one *bla* gene. On the other hand, *bla*_{SHV} genes were also detected in other four species (*E. coli*, *M. morgani*, *P. mirabilis* and *E. cloacae*), what

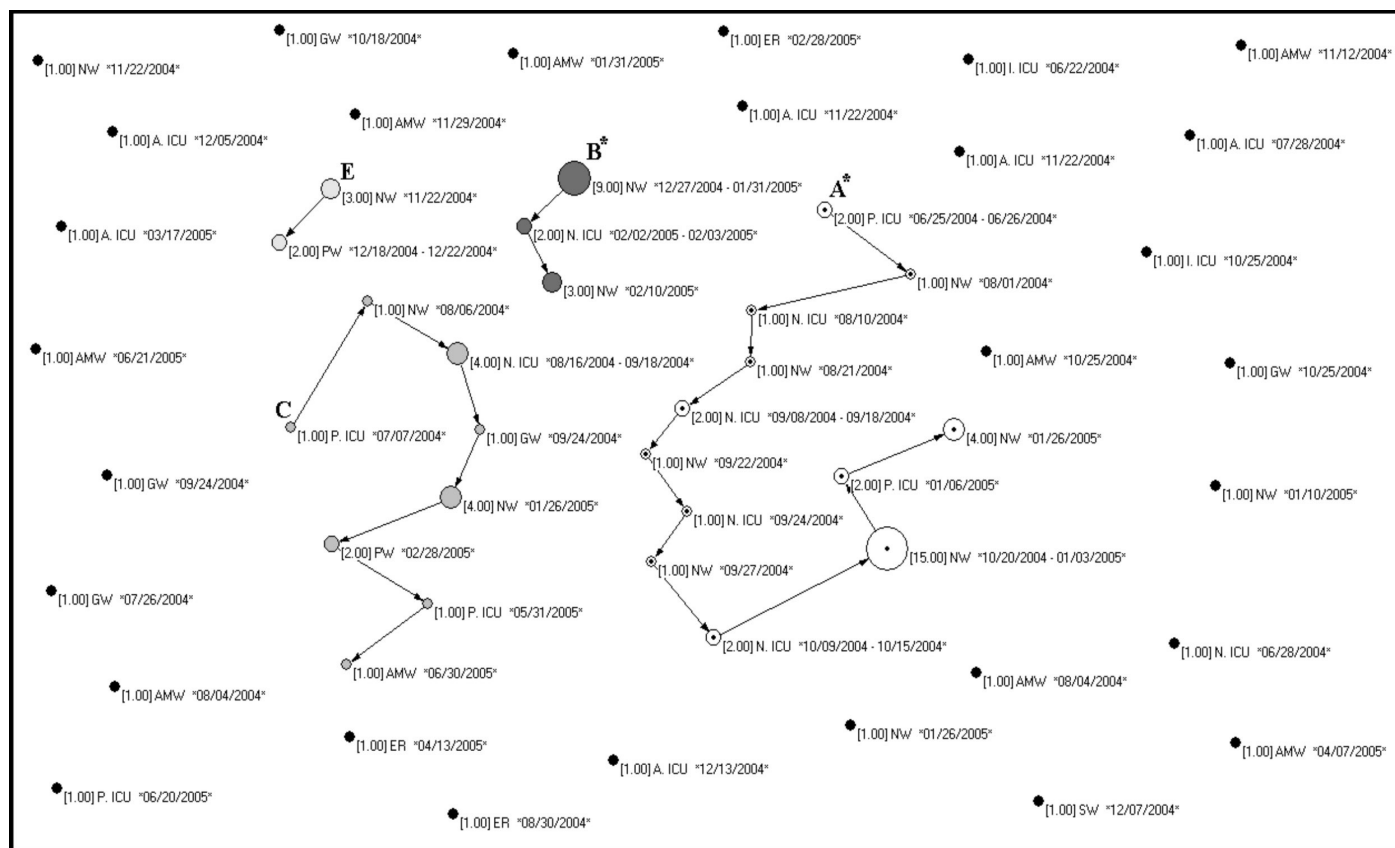


Fig. 2 - Overall clone-sharing relationships among ESBL-*Kp* PFGE patterns, according to the hospital locations and the periods of isolation. A*, B*, C and E correspond to the PFGE related patterns. Small black spots are the 29 non-related patterns generated by PFGE. Arrows indicate the trajectory of each pattern during the studied period.

shows their spread potential among different strains. Group TEM was identified in 17.3% of isolates in five different species (*K. pneumoniae*, *K. oxytoca*, *E. coli*, *M. morgani* and *P. mirabilis*), generally in association with SHV and/or CTX-M genes. Therefore, it was not possible to presume if they were responsible for the ESBL phenotype or not, once they could be non-ESBL encoding genes associated to another gene expressing the extended-spectrum phenotype (LIVERMORE 2008). On the other hand, co-production of different ESBLs by a unique strain is a common finding in several studies (LI *et al.* 2003, PATERSON *et al.* 2003, VIGNOLI *et al.* 2005, MINARINI *et al.* 2007), so the carriage of two or three ESBL encoding genes by the TEM-producing strains of our study could also have happened. CTX-M ESBL was detected in 33.9% of isolates, in all species, except for *P. rettgeri*. This emerging group of enzymes is prevalent and endemic in South America (CELENZA *et al.* 2006, VILLEGAS *et al.* 2008) and, similarly to our work, some other studies in Argentina and Bolivia reported the finding of *bla*_{CTX-M} genes in a great variety of *Enterobacteriaceae* species (QUINTEROS *et al.* 2003, CELENZA *et al.* 2006). ESBL-producing isolates in which none of the genes searched was detected could be carrying genes from other groups of extended-spectrum enzymes.

Klebsiella pneumoniae was the species with the highest number of isolates (75.6%) in this study. It is the most frequent ESBL-producing species, especially in Latin America, and its prevalence in Brazil is around 50%, while in the United States it is 5% and in Europe, 15 to 20% (SADER *et al.* 2001, GALES *et al.* 2002, NOGUEIRA *et al.* 2006, VILLEGAS *et al.* 2008). As a nosocomial pathogen, it can cause severe morbidity and mortality in hospitalized patients, and once it is part of intestinal flora, rectal colonization can be strongly associated to infection by ESBL-*Kp* (GUPTA *et al.* 2003). In addition to its intrinsic resistance to ampicillin due to the presence of chromosomal β -lactamase SHV-1 (BABINI & LIVERMORE 2000), it can also express resistance due to other β -lactamase classes like metalloenzymes and AmpC, and/or due to porine losses (MENDES *et al.* 2004, LINCOPAN *et al.* 2005, DENTON 2007). In Brazil, there are some reports of multi-drug resistant hospital-acquired *K. pneumoniae* (MENDES *et al.* 2004, LINCOPAN *et al.* 2005, PEIRANO *et al.* 2009). In the present study, four main PFGE patterns (A*, B*, C, and E) were obtained from 67 (69.8%) ESBL-*Kp* isolates. Figure 2 shows the distribution of all patterns among the hospital wards during the studied period, emphasizing that there was clearly four groups of genetically related strains and several isolated strains with genetic unrelated profiles. Patterns A* and B* were found mostly in the neonatal ward, neonatal ICU (B only) and pediatric ICU (A only), where a few unrelated patterns were identified (Fig. 2). These wards have patients with fragile host defenses, receiving excessive manipulation and a high antimicrobial intake, which could have been the predisposing factors for infections and spread of resistance genes (BRADFORD 2001, GUPTA *et al.* 2003). An outbreak took place in the Neonatal Ward during our study, and it is described elsewhere (CASSETARI *et al.* 2009). In Figure 2 we can see that pattern A* was isolated from June 2004 to January 2005, and pattern B* from December 2004 to February 2005. The "substitution" of pattern A* by B* in the neonatal ward was coincident with the infection control measures that were reinforced by the institution during the outbreak investigation. Although pattern C was identified in 15 strains, seven of them were isolated from a unique patient who was hospitalized in Neonatal ICU, Pediatric ICU and Pediatric Ward during all the study period (data not shown) (Fig. 2). Pattern E was identified in three strains from patients of Neonatal Ward and in two strains from patients

of Pediatric ICU, between November and December of 2004. After this period, no more strains from this pattern were isolated until the end of the study period (July of 2005) (Fig. 2). *K. pneumoniae* isolated from samples of patients from Adult ICU, IMC, General and Surgical wards, showed only unrelated patterns (Fig. 2). According to another study, when isolates are genetically distinct, the group of resistant microorganisms can be originated due to excessive antimicrobial selective pressure, which favors resistant phenotypes within an unrelated group of strains, turning to be a problem of endemic resistance of different groups of organisms (TOSIN *et al.* 2003). Also, approximately 30% of those unrelated strains were isolated from patients who had been transferred from other hospitals or had previous hospital stays in our institution. In other reports, after genotyping *K. pneumoniae* isolates, it was detected several distinct patterns, suggesting horizontal transfer of resistance genes as a way of spread. Those strains contained *bla*_{CTX-M} genes, which is exclusively located in mobile elements (LI *et al.* 2003, MINARINI *et al.* 2007). Similarly, in our study *bla*_{CTX-M} genes were detected in a high number of isolates with unrelated molecular patterns (55.2%), and in a few strains of patterns A*, B*, C and E, what can suggest that in the environment of the studied institution there was also the spread of these resistance genes by conjugation. Some other observations in our results can reaffirm this kind of spread. TEM ESBLs were not detected in isolates from pattern A, but they were present in the subtype A1, which had one extra band in relation to A. The same occurred for subtypes A3 and A4, in which groups TEM and CTX-M were present only in the last one, what could have happened due to the presence of different plasmids among the isolates (LI *et al.* 2003). Despite the wide spread of ESBL encoding genes through plasmids, the high genetic variability found among Brazilian strains suggests that a continuous selection of resistant mutants occurs mostly due to the use of third-generation cephalosporins (SADER *et al.* 2001, GALES *et al.* 2002). Indiscriminate use of antimicrobials is strongly associated to multidrug resistance, colonization, hospital outbreaks and severe infections due to ESBL-producing microorganisms. This is considered a public health problem, and not only institutional, once solutions and control depend on local policies for restricted antimicrobial use (BRADFORD 2001, PATERSON *et al.* 2000).

According to the infection control committee of the studied hospital, ESBL-producing enterobacteria represent a great challenge for the control of infection and colonization in the institution, especially in Intensive Care Units. Our results suggest that the problem of dissemination of resistant clones and genes in hospital environment can occur due to contact with healthcare workers or to the patient's transferring between hospital units, once isolates presenting identical molecular patterns were observed in different wards, and some isolates showed patterns identical to the strain isolated from the hands of a nursing auxiliary in the institution (CASSETARI *et al.* 2009). Our data corroborate the importance of antibiotic use restriction and enhancement of prevention measures, mostly efficient hand-washing, contact isolation of colonized patients, appropriate cleansing and disinfection of materials and equipment, and monitoring of patients transference between hospital wards. Surveillance cultures are also of great value when appropriately applied (GUPTA *et al.* 2003).

In conclusion, several groups of *bla* genes were detected in clinical samples during one year in the studied institution, and further studies will be necessary to identify them precisely. Moreover, ESBL-*Kp* strains with unrelated molecular patterns carried *bla* genes located exclusively

in mobile elements, suggesting horizontal gene transfer between isolates. On the other hand, similar and/or identical ESBL-*Kp* according to PFGE were disseminated among several hospital wards, suggesting spread in hospital environment.

RESUMO

Beta-lactamases de espectro estendido em *Enterobacteriaceae* isoladas de Hospital Público no Brasil

Beta-lactamases de espectro estendido (ESBL) em enterobactérias são reconhecidas mundialmente como um grande problema hospitalar. Neste estudo, 127 *Enterobacteriaceae* produtoras de ESBL isoladas por um ano, de pacientes internados e ambulatoriais de um hospital público de ensino em São Paulo, Brasil, foram submetidas à análise pela PCR com iniciadores específicos para os genes *bla*_{SHV}, *bla*_{TEM} e *bla*_{CTX-M}. Dos 127 isolados, 96 (75,6%) *K. pneumoniae*, 12 (9,3%) *E. coli*, 8 (6,2%) *M. morgani*, 3 (2,3%) *Proteus mirabilis*, 2 (1,6%) *Klebsiella oxytoca*, 2 (1,6%) *Providencia rettgeri*, 2 (1,6%) *Providencia stuartii*, 1 (0,8%) *Enterobacter aerogenes* e 1 (0,8%) *Enterobacter cloacae* foram identificados como produtores de ESBL. *Bla*_{SHV}, *bla*_{TEM} e *bla*_{CTX-M} foram detectados em 63%, 17,3% e 33,9% das cepas, respectivamente. A genotipagem de *K. pneumoniae* por eletroforese em campo pulsado revelou quatro padrões moleculares principais e 29 perfis não relacionados. Os resultados da PCR demonstraram alta variedade de grupos de ESBL entre as cepas, em nove espécies diferentes. Os resultados sugerem a disseminação de genes de resistência entre cepas geneticamente diferentes de *K. pneumoniae* produtoras de ESBL em algumas unidades do hospital, e também que algumas cepas fortemente relacionadas foram identificadas em unidades hospitalares diferentes, sugerindo disseminação clonal no ambiente da instituição.

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