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ORIGINAL ARTICLE

Molecular epidemiology of extended-spectrum ß-Lactamase-producing *Klebsiella pneumoniae* isolated from neonatal intensive care unit patients involved in hospital infection cases in Rio de Janeiro, Brazil

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ABSTRACT. During a two years period, in this study was analyzed the demographic and bacteriologic data of 42 hospitalized newborns attempted by extended-spectrum ß-lactamase-producing *Klebsiella pneumoniae* sepsis in a neonatal intensive care unit of a public maternity hospital in Rio de Janeiro, Brazil. The newborns mean age was 10.4 days, with a major prevalence of hospital infection in males (55.0%) than females (45.0%), and a major frequency in vaginal delivery (65.0%) than cesarean delivery (35.0%). 31 patients (77.5%) received a mean of 3 antimicrobials during a 7.9 days before positive blood cultures. The most important underlying risk conditions were prematurely (87.5%), very low birth weight (55.0%) and asphyxia (40.0%). Among the isolated strains were detected high resistance proportion to ß-lactams, aminoglycosides, chloramphenicol and trimethoprim-sulfamethoxazole. 6 distinct clones in a cluster of 42 epidemiologically related strains were detected through PFGE profiles. The isolated strains presented 9 different antimicrobial resistance profiles (ARPs), where the most frequent clones (A, B and D) were distributed in 5, 3 and 5 ARPs, respectively. Based in the PFGE profiles and isolation periods, apparently the clones A plus A1, B and D caused 3 distinct outbreaks during the study period.

Key words: *Klebsiella pneumoniae*, extended-spectrum ß-lactamase, hospital infection, sepsis.

RESUMEN. Durante dos años, en este estudio se analizaron datos demográficos y bacteriológicos de 42 neonatos afectados por sepsis causada por cepas de *K. pneumoniae* con un espectro extendido de ß-lactamasa hospitalizados en una unidad de tratamiento intensivo neonatal de una maternidad pública de Rio de Janeiro, Brasil. La edad media de los neonatos fue 10.4 días, con una prevalencia de infección mayor en el sexo masculino (55.0%) que en el femenino (45.0%), y una mayor frecuencia en neonatos de partos normales (65.0%) que por cesárea (35.0%). 31 (77.5%) pacientes recibieron antimicrobianos antes de presentar hemocultivos positivos, con un promedio de uso de 3.0 antimicrobianos por paciente durante 7.9 días de uso. Las condiciones de riesgo perinatales más importantes fueron: parto prematuro (87.5%), peso muy bajo al nacer (55.0%) y asfixia (40.0%). Entre las cepas aisladas, se detectaron elevados porcentajes de resistencia a ß-lactámicos, aminoglucósidos, cloranfenicol y trimetroprim-sulfametoxazol. 6 distintas clonas fueron detectadas en un grupo de 42 cepas relacionadas epidemiológicamente a través del perfil de la PFGE. Las cepas aisladas presentaron 9 diferentes perfiles de resistencia a los antimicrobianos (PRAs), donde las clonas más frecuentes (A, B y D) se distribuyeron en 5, 3 y 5 PRAs, respectivamente. Con base en los perfiles de PFGE y los periodos de aislamiento, los clones A más A1, B y D causaron aparentemente 3 brotes distintos durante el periodo de estudio.

Palabras clave: *Klebsiella pneumoniae*, ß-lactamasas de espectro extendido, infección hospitalaria, sepsis.

INTRODUCTION

Klebsiella pneumoniae is a pathogen, able to infect debilitated and immunocompromised patients, and newborns, especially those with a risk factors to acquire hospital infection (HI) in a neonatal intensive care units (NICU). *K. pneumoniae* infections are associated with a significantly increase in the hospitalization costs related to patient treatment, morbidity and mortality rates.[4,19,24,32]

In addition to host factors, bacterial virulence determinants, as capsular polysaccharide, the aerobactin-mediated iron uptake system, the mucoid phenotype, and the toxic lipopolysaccharide, contribute to the outcome of *K. pneumoniae* infections.[24]

K. pneumoniae is a pathogen that frequently presents resistance to cephalosporins through the production of extended-spectrum ß-lactamases (ESBL),[5,24] that are encoded mainly by SHV and/or TEM genes.[37] Generally, these genes are harbored on transferable plasmids, that often carry other resistance factors, including resistance to aminoglycosides.[39]

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These plasmids can be transferred to others species of the Enterobacteriaceae family during outbreaks.[2,5,8]

The epidemiology of *K. pneumoniae* infections are usually studied by the analysis of phenotypic markers, including biotypes, serovars and antimicrobial susceptibility patterns.[5,20,22] However, with the advent of molecular microbiology technologies, nucleic acid-based methods have been used to assist identification of bacteria to subspecies level in epidemiological studies, providing a higher discriminating power than phenotypic data.[20,22]

The chromosomal DNA analysis by pulsed field gel electrophoresis (PFGE) is worldwide considered the most powerful tool for the study of the hospital epidemiology of *K. pneumoniae* due to its high discriminatory capacity, good reproducibility and easy interpretation. These technique facilitates the elucidation of transmission routes, genetic variability, phylogenetic distances between strains and can give insight into the interrelationship of bacterial isolates.[2,5,19,22,35]

This study reports the demographics data of the hospitalized newborns attempted by ESBL-producing *K. pneumoniae* sepsis associated with HI, during a period ranged from July 1997 to July 1999, in a NICU of a public maternity hospital from Rio de Janeiro, Brazil. As well as determine the rates of antimicrobial resistance, the antimicrobials resistance profiles and PFGE patterns of the isolated strains during this period.

MATERIAL AND METHODS

Bacterial strains, patients and hospital. 42 ESBL-producing *K. pneumoniae* strains isolated from blood cultures of newborns involved in HI well documented cases, between July 1997 and July 1999, at Hospital Maternidade Alexander Fleming II (HMAF) were analyzed. HMAF is a maternity hospital in Rio de Janeiro, Brazil, providing assistance and perinatal cares, including a neonatal intermediate care unity with 40 beds and a NICU with 15 beds. HI were defined by CDC standard definitions[9] adapted to neonatal pathology. In general, infections that occurred after 48 h of hospitalization were assumed to be acquired in the hospital, but each instance of infection was considered individually because of the late onset of some perinatal acquired infections.

Blood cultures, strains identification and susceptibility testing. 0.2 ml of venous blood obtained from newborns were drawn into bottles with 10 ml of supplemented TSB (Roche) and incubated at 37ºC. After 24 h, the blood cultures were inoculated into Thioglycolate Broth (Difco Laboratories, Detroit, Mich.), and plated onto Blood Agar and EMB Agar (Difco Laboratories, Detroit, Mich.) during a period ranging from 1 to 7 days. The *K. pneumoniae* strains identification was performed according to Ewing,[7] using classical biochemical tests.

The antimicrobial susceptibility test were carried out using the disk diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) recommendations.[29] Quality control was carried out using standard strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27953) and *Staphylococcus aureus* (ATCC 25923). Concentrations of antimicrobial drugs used were: cephalothin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), tetracycline (30 µg). The phenotypic detection of ESBL production were performed using E-test strips (AB Biodisk, Solna, SW).

PFGE. The chromosomal DNA restriction analysis of the strains were performed by PFGE. Chromosomal DNA extraction of the strains were performed according to procedure previously described by Sader *et al*.[36] Nucleic acids included into agarose plugs were digested with 40 U of *Xba*I restriction enzyme at 37ºC during 20 h. Electrophoresis in agarose gels was performed at 6 V/cm during 24 h in 0.5X TBE buffer at 13ºC with a ramping switch time of 10-70 seg with a CHEF DR III pulsed field electrophoresis system (Bio-Rad, Laboratories, Richmond, Calif.). Fragments were stained with ethidium bromide and photographed. All agarose gels were performed twice to verify the reproducibility of the tests.

Band patterns were analyzed using GelCompar II (Applied Maths, Belgium), any internal markers were used. Relations between fingerprints were determined by construction of a similarity matrix using the Dice's coefficient with 1.5% position tolerance, which generated a dendrogram using the UPGMA clustering algorithm. Clonal structure definitions of the *K. pneumoniae* strains were made according to Tenover *et al*.[38]

Epidemiological analysis of patients. For each HI case an epidemiological record with demographics and microbiologic data was done, unfortunately demographic data from two newborns were not available. Data were collected in Excel 7.0 software (Microsoft) and analyzed in Epi-Info (version 6.04b; CDC, Atlanta, USA).

RESULTS

K. pneumoniae were the most frequent pathogen isolated from NICU patients in the HMAF, accounting 22.9% (69/301) of the isolates during two years period (07/97 to 07/99). From these 69 isolates, 42 strains were ESBL-producers accounting a total of 60.9% of the isolated *K. pneumoniae* strains in this period (data not shown).

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Table 1 shows the general demographic characteristics of the hospitalized newborns in NICU-HMAF, Rio de Janeiro, Brazil, affected by ESBL-producing *K. pneumoniae* sepsis and classified as HI cases. The newborns mean age was 10.4 days. Major prevalence of IH in males (55.0%) than females (45.0%), as well as a major frequency in vaginal delivery (65.0%) than cesarean delivery (35.0%) was verified. 31 (77.5%) newborns received antimicrobial drugs before positive blood cultures presentation with mean use of 3.0 antimicrobials per patient during 7.9 days. The most important underlying risk conditions were prematurity (87.5%), very low birth weight (55.0%), asphyxia (40.0%) and hyaline membrane disease (25.0%).

A total of 13 different antimicrobials (Table 2) were used for treatment of 31 (77.5%) patients before positive blood cultures presentation, with the highest frequency of use for ampicillin (29/31 patients; 93.5%), amikacin (27/31; 87.1%), cefotaxime (11/31; 35.5%) and oxacillin (10/31; 32.2%).

The analysis of the antimicrobial resistance percentages (Table 3) showed a high antimicrobial resistance percentage (70 to 100%) to cephalotin, ceftriaxone, cefuroxime, gentamicin, amikacin, chloramphenicol and trimethoprim-sulfamethoxazole in the two analyzed periods (07/97 to 07/98 and 08/98 to 07/99). High antimicrobial resistance percentage to tetracycline was detected only in the second period (72.7%). A low antimicrobial resistance percentage to cefoxitin (3.2% in first year and 18.2% in second) and no strain resistant to imipenem and ciprofloxacin were demonstrated in both periods.

Table 1. Demographic characteristics of the hospitalized newborns in two years period (07/97 to 07/99).

Demographics data	*Frequency*
Mean age[a]	10.4 days
Male Sex[a]	22/40 (55.0%)
Female Sex[a]	18/40 (45.0%)
Vaginal delivery[a]	26/40 (65.0%)
Cesarean delivery[a]	14/40 (35.0%)
Twins[a]	1/40 (2.5%)
Patients in antimicrobial drugs use before positive blood cultures presentation[a]	31/40 (77.5%)
Mean of antimicrobial drugs used by patients before positive blood culture presentationb	94/31 (3.0)
Mean of antimicrobial days use before positive blood culture presentation[b]	7.9 days
Underlying risk conditions	
Prematurity[a]	35/40 (87.5%)
Very low birth weight (VLBW; < 1500 g)[a]	22/40 (55.0%)
Asphyxia[a]	16/40 (40.0%)
Hyaline Membrane Disease[a]	10/40 (25.0%)
Prolonged amniotic membrane rupture time (PAMR; > 24 hs)[a]	8/40 (20.0%)

[a]Data from 40 patients. [b]Data from 31 patients who received antimicrobial drugs before positive blood culture presentation.

Table 2. Antimicrobial drugs administrated to newborns before positive blood culture presentation in a two years period (07/97 to 07/99).[a]

Antimicrobials	*Frequency*	*Percentage (%)*
Ampicillin	29	93.5
Amikacin	27	87.1
Cefotaxime	11	35.5
Oxacillin	10	32.2
Ceftazidime	3	9.7
Gentamicin	3	9.7
Imipenem	3	9.7
Metronidazole	2	6.4
Vancomycin	2	6.4
Amphotericin	1	3.2
Cefoxitin	1	3.2
Ceftriaxone	1	3.2
Penicilina	1	3.2
Total	**94**	—

[a] Data from 31 hospitalized newborns who received antimicrobial drugs before positive blood culture presentation.

Table 3. Antimicrobial resistance percentage detected in 42 isolated *K. pneumoniae* strains during two years period (07/97 to 07/99).

	Period	
Antimicrobials	*07/97 to 07/98[a] Percentage (%)*	*08/98 to 07/99[b] Percentage (%)*
Cephalothin	100	100
Cefoxitin	3.2	18.2
Ceftriaxone	100	100
Cefuroxime	100	100
Imipenem	0	0
Gentamicin	80.6	100
Amikacin	100	100
Ciprofloxacin	0	0
Chloramphenicol	96.8	100
Trimethoprim/sulfamethoxazole	90.3	100
Tetracycline	29.0	72.7

[a] 31 strains were isolated during this period.
[b] 11 strains were isolated during this period.

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Figure 1 shows 6 distinct clones (A, A1, B, C, D and E) detected in a cluster of 42 epidemiologically related strains through of PFGE. These clones were isolated during a two years period, with the highest frequency of isolation for clone A (15 strains; 35.7%), followed by clones D and B with frequencies of 30.9% and 23.8% respectively. Isolation of the clones A1, C and E accounted for 9.5% of the isolated strains.

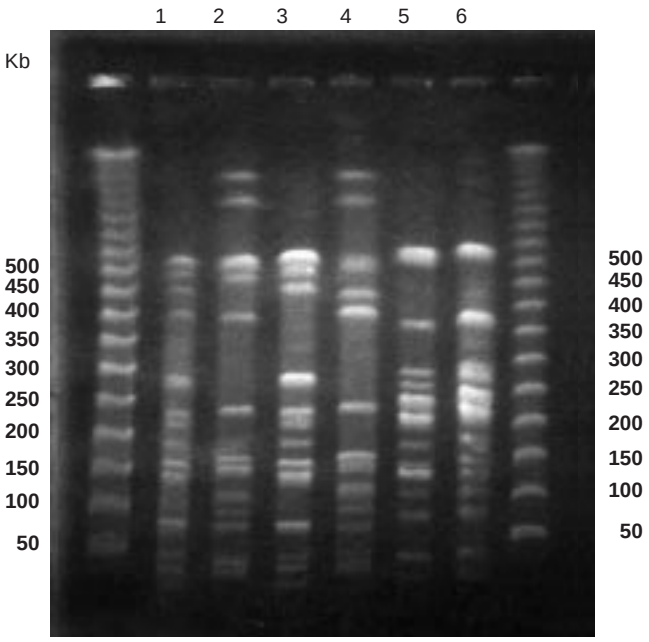

Figure 1. *PFGE of* K. pneumoniae *strains isolated from blood culture of NICU newborns at HMAF during two years period.*
λ, Lambda ladder marker (50-1000 Kb); Lanes 1-6, PFGE patterns detected in 42 ESBL-producing K. pneumoniae strains isolated from blood culture of NICU newborns at HMAF during period from 07/97 to 07/99. Lane 1, A (15 strains); lane 2, D (13 strains), lane 3, A1 (2 strains); lane 4, E (1 strain), lane 5, B (10 strains), and lane 6, C (1 strain).

The dendrogram generated by GelCompar II (Figure 2), revealed that the clones A and A1 are genetically related (similarity percentage >95%), and genetically unrelated to the others clones (similarity percentage <80%), under stringency conditions above cited. In general, the detected clones presented low cophenetic correlation (44.7%). Four clones (A, A1, D and E) were detected in the first year of analysis, whereas two clones (B and C) were isolated in the second year.

The distribution of the isolated strains according to their antimicrobial resistance profiles (ARP) and PFGE profiles (Table 4), demonstrated 9 ARPs with resistance ranged from 6 to 9 different antimicrobials. Isolated strains associated to profile 4 (42.8%) had resistance to 7 anti-microbials, followed by the profiles 7 (26.1%) and 1 (9.5%) had resistance to 8 and 6 antimicrobials respectively. In relation to distribution of the more frequent clones (A, D and B) in the ARPs, we verified that the clone A was distributed in 5 different ARPs (2, 4, 5, 6 and 7); the clone D was detected in 5 ARPs (1, 3, 4, 7 and 8); and the clone B was observed in 3 ARPs (4, 7 and 9).

DISCUSSION

Nosocomial sepsis are among the most common nosocomial infections,[35] and are a major problem for newborns admitted to intensive care units. These infections, associated to immaturity and invasive procedures, cause a greater need for patient cares and handling.[12,39] In this study, realized between July 1997 to July 1999, the hospitalized newborns affected by ESBL-producing *K. pneumoniae* sepsis

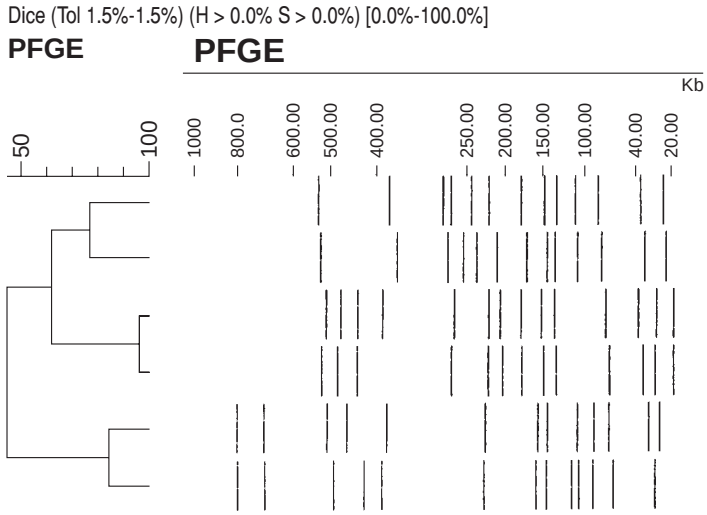

Figure 2.

A comparative dendrogram and schematic representation of 6 PFGE patterns (A, A1, B - E) detected in 42 ESBL-producing K. pneumoniae *strains isolated from blood culture of NICU newborns at HMAF during two years period (07/97 to 07/99).*
In brackets: number of strains and period of the isolation.

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Table 4. Distribution of the 42 isolated *K.pneumoniae* strains according to the antimicrobial resistance profiles and PFGE profiles detected in two years period (07/97 to 07/99).

ARP number	ARP	Isolated strains		Isolated clones (N)
		N	(%)	
1	CFL/CRO/CRX/AMI/CLO/SXT	4	(9.5)	D (3), E (1)
2	CFL/CRO/CRX/GEN/AMI/SXT	1	(2.4)	A (1)
3	CFL/CRO/CRX/GEN/AMI/CLO	1	(2.4)	D (1)
4	CFL/CRO/CRX/GEN/AMI/CLO/SXT	18	(42.8)	A (8), B (2), C (1), D (7)
5	CFL/CRO/CRX/GEN/AMI/CLO/TET	2	(4.8)	A (2)
6	CFL/CRO/CRX/AMI/CLO/SXT/TET	2	(4.8)	A (2)
7	CFL/CRO/CRX/GEN/AMI/CLO/SXT/TET	11	(26.1)	A (2), A1 (2), B (6), D (1)
8	CFL/CFO/CRO/CRX/GEN/AMI/CLO/SXT	1	(2.4)	D (1)
9	CFL/CFO/CRO/CRX/GEN/AMI/CLO/SXT/TET	2	(4.8)	B (2)

ARP: Antimicrobial resistance profiles. A, A1, B – E: detected clones among 42 isolated strains by pulsed field gel electrophoresis method.
CFL: Cephalothin; CFO: Cefoxitin; CRO: Ceftriaxone; CRX: Cefuroxime; IMI: Imipenem; GEN: Gentamicin;
AMI: Amikacin; CIP: Ciprofloxacin; CLO: Chloramphenicol; SXT: Trimethoprim/sulfamethoxazole; TET: Tetracycline.

at HMAF presented some risk conditions for HI acquisition. The most predominant were submission to various invasive procedures (tracheal intubations, mechanical ventilation, respiratory ways aspiration, intravascular catheters, parenteral nutrition), and presence of underlying risk conditions (prematurity, asphyxia, hyaline membrane disease, very low birth weight, small gestational age, and prolonged amniotic membrane rupture of the mothers, birth defects, etc.). Routinely, the analyzed newborns presented association of diverse risk factors during their stay in the NICU from HMAF. These observations agree with several authors, who described risk conditions to HI acquisition in NICUs.[4,12,19,32,35,39]

In this study, a large number of patients (77.5%) received a mean of 3 antimicrobial drugs during 7.9 days before positive blood culture presentation, due to the clinical picture suggestive of sepsis. In these cases, the first scheme of therapeutic treatment adopted in our institution was ampicillin plus amikacin, followed by oxacillin plus cefotaxime (second scheme). Empirical treatment according to schemes previously adopted in hospital routine induced selective pressures to select of multi-drug resistant strains. Several authors described that prolonged use of antimicrobials before and during NICU stay can lead to selection of ESBL-producing *K. pneumoniae* strains, resulting in treatment failure, due to indiscriminate use of extended spectrum ß-lactams and aminoglycosides principally.[4,6,17,20,25,28,32,33,35]

The first empirical antimicrobial treatment scheme (ampicillin plus amikacin) reserves the use of more potent antimicrobials after culture confirmation and antimicrobial susceptibility tests, to control the emergence of resistant mutants to third generation cephalosporins, ciprofloxacin and imipenem. Cordero, Sananes and Ayers[4] recommend the monitoring and restrict use of antimicrobials, because imipenem and cephalosporins induce ß-lactamase production very rapidly and efficiently. Other authors6,13,37 described in ICUs that the low levels of antimicrobial resistance among *K. pneumoniae* to cephalosporins, aminoglycosides, imipenem and fluoroquinolones were due a still relatively restricted use of these drugs in the unit.

K. pneumoniae is the most frequent pathogen isolated from nosocomial sepsis in the NICU-HMAF, accounting a total of 22.9% (69/301) of the isolates in a two years period, and 60.9% of these strains were ESBL-producers. This high rate of ESBL-producing *K. pneumoniae* in our NICU is more elevated than existing in other hospitals located on North America, Europe and Latin America.[25,28,34,35]

The analysis of the antimicrobial resistance demonstrated that cephalothin, ceftriaxone, cefuroxime, gentamicin, amikacin, chloramphenicol and trimethoprim-sulfamethoxazole are not effective agents for treatment of sepsis caused by ESBL-producing *K. pneumoniae* in HMAF, due the high resistance rates detected during two years period. Also, were detected percentage of resistance to tetracycline in the second year of the study, even though this drug is not used in HMAF routine. Some authors[8,15,23,27] related the high resistance rates to these drugs with the presence of transferable plasmids commonly harbored in *K. pneumoniae* strains. These plasmids generally carry SHV and/or TEM genes encoding ESBLs production and other resistance factors, including resistance to aminoglycosides, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole.

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We detected low resistance percentage to cefoxitin in two years of analysis, and these results demonstrate the emergence of resistant strains to this drug. The resistance mechanisms to cefoxitin and others cephalosporins, and the increase of the ciprofloxacin MIC in *K. pneumoniae* correspond to production of class C ß-lactamase (AmpC-type ß-lactamases) and/or reduced or null production of the OmpK35 and OmpK36 porins, that decrese the penetration of the drugs through of outer membrane.1,10,14-16,18,27,[30] Martínez-Martínez *et al*.[27], suggested that cefoxitin may not be suitable for treatment of the ESBL-producing *K.pneumoniae*, because mutants selected *in vivo* that lack porins are resistant to cefoxitin. Cefoxitin-resistant porin-deficient mutants are easily selected *in vitro* and show decreased susceptibilities to other ß-lactams, including expanded-spectrum cephalosporins.

According with our results, ciprofloxacin and imipenem were considered the most effective agents for treatment of ESBL-producing *K. pneumoniae* sepsis at HMAF, because no resistant strain to these drugs was detected during the analysis period. These antibiotics are used in HMAF routine only as the last option after antimicrobial susceptibility test and culture results, due to imipenem induce the ESBL production very rapidly and efficiently,[4] and ciprofloxacin is not yet licensed for use in children due to its demonstrated toxicity to cartilage and central nervous system in animal models. [21] Several authors,[13,17,27,34,35] consider these drugs as potent agents in treatment of sepsis caused by these strains, principally imipenem, because this drug is effective even against porin-less mutants, due to the small size of the molecule and its zwitterionic structure, which facilitates its diffusion through the outer membrane.

K. pneumoniae strains could be grouped in 6 branches according to their EFLP profiles, most strains belonging to groups A, D, and B. 9 different ARPs, with resistance to antimicrobials, were included in two different families, characterizing the strains as multi-drug resistant. This fact suggest the presence of different antimicrobial resistance mechanisms, that have been commonly described in *K. pneumoniae* (enzymes production, efflux pump mechanism, and loss of porins or mutations in porin-encoding genes).[1,10,15,16,25,27] This reinforce the idea of resistance development principally by horizontal gene transfer, gene rearrangements and enzyme overproduction.[1,2,5,8,10,14-16,20,25,27,32,40] Detection of strains belonging to same clone with different ARPs is very common, because ARPs analysis are not considered as a satisfactory method for the typing of *K. pneumoniae*, due the insufficient discriminatory power, poor reproducibility, horizontal transfer of genes, presence of diverse resistance mechanisms and inadequate ability to distinguish different strains.[1,5,10,14,16,20-22,27]

The clones A and D (the most frequent clones), plus clone A1 (clone genetically and epidemiologically related with the clone A) and clone E, had a isolation period limited to the first year of the analysis, probably because all reservoirs were eliminated from the hospital environment. Adoption of reinforcement measures to HI prevention, such as contact isolation, suitable handwashing, gloving and gown use for management of the newborns, treatment of the infected newborns, elimination of possible contamination reservoirs and transmission routes were important decisions. These strategies initiated in May 1998 were adopted in NICU-HMAF to control a methicillin-resistant *Staphylococcus aureus* outbreak, previously described.[26] Two months after reinforcement of the prevention and control measures, new sepsis cases caused by ESBL-producing *K. pneumoniae* were not detected. However, the clone B started to be isolated in the NICU four months after (September 1998). For this clone, the implementation of these measures of prevention and control was not effective to prevent infections.

Hands carriage is largely related in the literature as the principal transmission route of ESBL-producing *K. pneumoniae* strains in NICUs, because the digestive tract of newborns is recognized as the principal reservoir of the bacteria.[5,8,33,39] This reinforces the idea that the measures of HI control and prevention used to control the MRSA outbreak were able to eliminate the *K. pneumoniae* clones A, A1, D and E from NICU.

The PFGE profiles and respective isolation periods demonstrated that the most frequent clones isolated in NICU-HMAF (clone A plus A1, D and B) were responsible for three distinct outbreaks occurred in July 1997 to July 1998 (clone A plus A1); July 1997 to April 1998 (clone D); and September 1998 and July 1999 (clone B). Apparently clones A plus A1 and D caused parallel outbreaks during the first year of analysis. The identification of genetically unrelated clones in a cluster of 42 epidemiologically related strains, is due to the detection of these 3 distinct outbreaks. However, many authors[2,3,5,10,11,31,33,39] related that outbreaks occurred in periods ranging from three months to two years, were caused by a single strain or by genetically related strains. Outbreaks caused by different clones were associated with strains isolated from different hospitals, departments or wards; and that genetic polymorphism was associated with epidemiologically unrelated strains or epidemiologically related strains isolated from different clinical specimens.

These results reinforce the necessity of to use more accurate sub-typing methods as PFGE, because the identification of these outbreaks only were possible after application of this technique. Others authors[2,5,22,25,35] recommend the use of this technique because it facilitates the study of transmission routes, genetic variability, phylogenetic dis-

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tances between strains and can give insight into the interrelationship of bacterial isolates.

The observations related in this study, emphasize the need for appropriate epidemiological monitoring of ESBL-producing *K. pneumoniae* and other strains implicated in nosocomial infections, by using both traditional and molecular methods. As well, monitoring of resistance to broad-spectrum antimicrobials in hospital environment is necessary to control antimicrobial use, reinforce educational measures for prevention of nosocomial transmission, and elimination of possible contamination reservoirs.

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