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Epidemic diffusion of *Klebsiella pneumoniae* isolates producing extended-spectrum beta-lactamases in neonatal and pediatric wards in Rabta hospital of Tunisia

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***Klebsiella pneumoniae* producing extended-spectrum β-lactamase (ESBL) cause severe life threatening infections resulting in considerable morbidity and mortality especially in neonatology ward. The aim of this study was to evaluate the epidemiology and resistance of 131 strains collected between 2007 and 2008 in neonatology and pediatric wards and to determine the mode of their epidemic spread. The isolates were identified, tested for antimicrobial susceptibility with the disk-diffusion on Mueller-Hinton agar. The type of ESBL was determined by polymerase chain reaction (PCR) followed by sequencing for CTX-M enzymes. The epidemiological relationships between epidemic strains were analysed by pulsed-field gel electrophoresis. In this study, antibiotic susceptibility testing showed resistance to all β-lactams except imipenem with a concomitant resistance to aminoglycosides, tetracycline and co-trimoxazole. Fluoroquinolones still have activity against strains. Characterization of β-lactamases encoding genes revealed that all strains have SHV β-lactamases. TEM-type and CTX-M-1 group were encoded, respectively, in 21 and 57% of ESBLs isolates. Among 84 strains tested by PFGE, 14 pulsotypes were identified. DNA sequencing of amplified CTX-M β-lactamase genes justified diffusion of CTX-M-15 between epidemic strains. In conclusion, this study revealed a high degree of clonal diversity of isolates and complexity of outbreaks that involve more than two epidemic pulsotypes and indicated that both clonal spread of epidemic strains and transfer of β-lactamases might contribute to epidemic dissemination of ESBL in neonatology ward.**

Keywords: *K. pneumonia*, CTX-M β-lactamases, PFGE, sequencing, clonality.

INTRODUCTION

Since the early 1980s, the number of nosocomial infections with extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria has been increasing worldwide (Chong et al., 2011). ESBLs are plasmid-mediated enzymes and were derived from genes for the narrower-spectrum TEM-1, TEM-2 or SHV-1 β–lactamases

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Abbreviations: ESBL, Extended-spectrum β-lactamase; **PCR,** polymerase chain reaction; **PFGE,** pulsed-field gel electrophoresis.

or are from a rapidly evolving class called CTX-M (Bradford et al., 2001; Bonnet, 2004). Moreover, others ESBLs including PER, IBC, GES, SFO, TLA, VEB, BES have also been described in various Gram-negative bacilli (Paterson and Bonomo, 2005). ESBLs have emerged as an important cause of hospital-acquired infections, over the world mostly in *Klebsiella pneumoniae* strains (Paterson and Bonomo, 2005). As opportunistic pathogen, *K. pneumoniae* can cause severe infections in immuno compromised hosts such as newborns which are one of the major susceptible groups (Ruiz et al., 2010; Kristóf et al., 2007). Furthermore, several infections caused by ESBL-producing *K. pneumoniae* have been widely repor-ted particularly in neonatal and intensive care units (Ruiz et al., 2010; Dhillon and Clark, 2012) with a significant proportion of hospital-acquired pneumonia, urinary tract infections and septicemias (Ruiz et al., 2010; Szilagyi et al., 2010; Elhani et al., 2006). These infections, which present a major therapeutic dilemma with restricted choice of antibiotics, were associated with severe morbidity and mortality (Dhillon and Clark, 2012; Szilagyi et al., 2010; Ben Jaballah et al., 2007). In Tunisia, ESBL-producing *K. pneumoniae* has been described as one of the most important pathogens causing serious endemic and epidemic nosocomial infections, especially in neonatal units (Bouallègue-Godet et al., 2005; Ben Hamouda et al., 2003). The prevalence of this multidrug resistant strain in Tunisian hospitals varies from 10 to 32.4% with a high incidence (87.5%) in pediatric intensive care units (Elhani et al., 2010). During 2007, nosocomial infections caused by multidrug resistant *K. pneumoniae* were observed with an increasing frequency particularly in neonatal and lesser in pediatric wards of la Rabta university hospital of Tunisia. In order to monitor dissemination, an epidemiological survey was conducted to study evolution of ESBL-producing *K. pneumoniae*, the first one in our hospital was to determine whether we are in front of emergence of new resistance and new β-lactamases and especially to understand the tendency spread of strains.

MATERIALS AND METHODS

Bacterial strains

This study was performed with a consecutive collection of a total of 131 non repetitive ESBL-producing *K. pneumonia*e isolates recovered from invasive specimens. The clinical sites of isolation were as follows: 80 (61%) were recovered from blood specimens, 21 (16%) from urine specimens, 19 (14%) from suppuration, 9 (7%) from catheters tips and 2 (2%) from cerebrospinal liquid. Strains were isolated from 105 and 26 patients hospitalized respectively in neonatal and pediatric wards of "la Rabta" hospital between the 1st January 2007 and December 2008. The bacteria were identified using an automated API 32E system (API-biomérieux ®-France). In our study, *K. pneumoniae* (N = 553) has been the most frequently isolated ESBL-producing pathogen accounting for 70% of all isolated ESBL-producing *Enterobacteriaceae* (N = 786). The incidence of ESBL-producing *K. pneumoniae* among *K. pneumoniae* (N = 1427) during the study period was 39%. In neonatology and pediatric wards, the infection rate by ESBL-producing *K. pneumoniae* was 32 and 8%, respectively.

Antibiotic susceptibility and screening for production of ESBLs

Antibiotic susceptibility was tested by disk diffusion method on Mueller-Hinton (MH) agar plates and results were interpreted according to the standards of the French Antibiogram Committee (CA-SFM) (Comité de l'Antibiogramme de la société Française de Microbiologie, 2009) for the following antibiotics: amoxicillin, amoxicillin-clavulanic acid, ticarcillin, piperacillin, cephalotin, cefsulodine, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, imipenem, streptomycin, kanamycin, gentamicin, tobramycin, amikacin, nalidixic acid, pefloxacin, ofloxacin, ciprofloxacin, levofloxacin, colistin, cotrimoxazole, chloramphenicol and tetracycline. Production of ESBL was detected by the double-disk synergy test performed on MH agar plates between amoxicillin-clavulanic acid surrounded at a radius of 30 mm by cefotaxime and ceftazidime. ESBL production was deduced when the zone of cefotaxime or ceftazidime was expanded by clavulanic acid (Livermore et al., 2001; Jacoby and Han, 1996).

Characterization of β-lactamase genes

Isolates with ESBL phenotypes were subjected to DNA extraction as described by Chen and Kuo (1993). The presence of ESBL genes was determined by polymerase chain reaction (PCR) using universal primers targeting TEM, SHV and CTX-M. Isolates detected to be CTX-M gene positive by PCR were further identified by using specific primers for the CTX-M-1 group. PCR amplification reactions were performed in a volume of 50 µl containing 5 µl of 10X PCR buffer (Invitrogen); 1.25 mM $MgCl_2$; 0.5 mM of each dNTP; 15 pmol of each primer; 1 unit of Go® *Taq* Polymerase (Promega) and 2 µl of DNA template. The oligonucleotide primer sets specific for β-lactamase genes used in the PCR assays are listed in Table 1.

The cycling parameters were as follows: an initial denaturation at 94°C for 10 min ; followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 48°C for CTX-M primers, at 60°C for CTX-M-1 and SHV primers and at 42°C for TEM primers and 1 min at 72°C for polymerization. Final products were extended by incubation for 5 min at 72°C. Four CTX-M PCR products selected from representative isolates belonging to epidemic pulsotypes (P2, P3, P9 and P13) were purified by using Wizard genomic DNA purification Kit (Promega) according to the manufacturer's procedure and sequenced with an ABI prism 310 DNA sequencer using the ABI PRISM® Big Dye™ Terminator. The nucleotide sequences were analyzed with the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nhi.gov).

Molecular typing

Pulsed-field gel electrophoresis (PFGE) genotyping was used to analyze the molecular epidemiology of 84 epidemics strains. Banding patterns generated by PFGE of *Xba*I-digested genomic DNA following the method of Gautom (1997) were analyzed with the fingerprinting™-II Software Version 3.00 (Bio-Rad, Germany) and a dendrogram was computed after band intensity correlation using 2% optimization and unweighted pair group matching average (UPGMA). Strains showing 90% or more of similarity were classified as clonally related and assigned to the same lineage.

RESULTS

Isolation of ESBL-producing *K. pneumoniae* during the study period

The distribution of the strains during the study period

Table 1. Sequences of primers used for polymerase chain reaction for detection β-lactamase genes.

PCR target	Primer name	Sequence	Amplicon Size (bp)	References
TEM	Tem A1	5'- ATA AAA TTC TTG AAG AC-3'	1,075	Leflon-Guibout et al. (2004)
	Tem B1	5'- TTA CCA ATG CTT AAT CA-3'		
SHV	SHV-F	5'- CAC TCA AGG ATG TAT TGT G-3'	822	Leflon-Guibout et al. (2004)
	SHV-R	5'- TTA GCG TTG CCA GTG CTC G-3'		
CTX-M	CTX-C1	5'- ATG TGC AGC ACC AGT AAA GT-3'	545	Leflon-Guibout et al. (2004)
	CTX-C2	5'- ACC GCG ATA TCG TTG GTG G-3'		
CTX-M-1 group	CTX-1F	5'- ATG GTT AAA AAA TCA CTG CGT C-3'	864	Ben Achour et al. (2009)
	CTX-1R	5'- TTG GTG ACG ATT TTA GCC GC-3'		

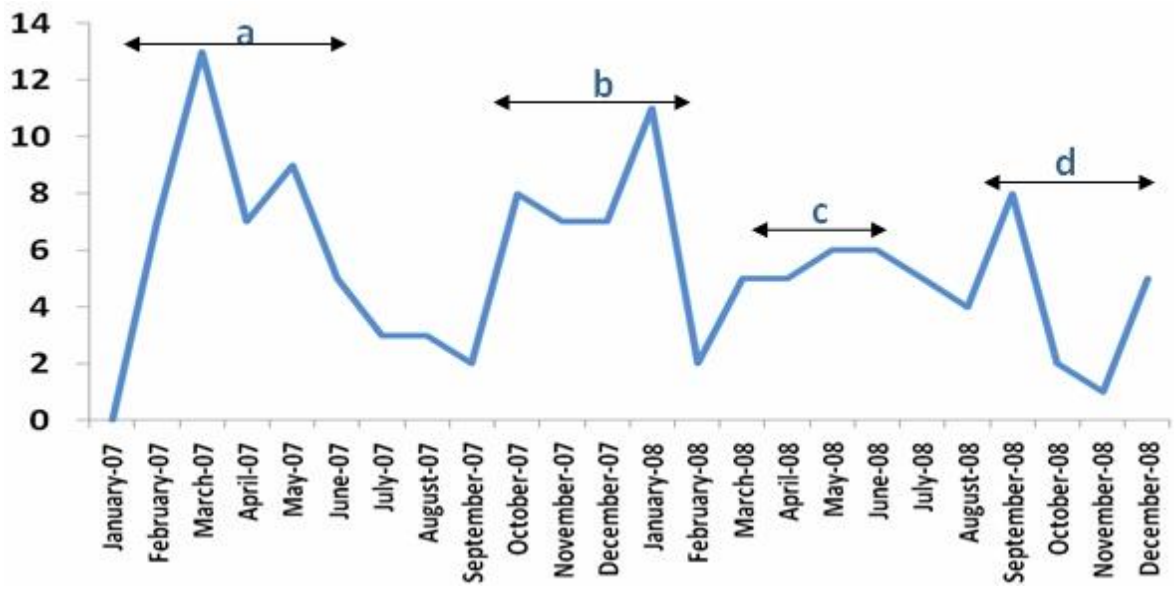

Figure 1. Isolation of ESBL-producing *K. pneumoniae* during the study period. a, b, c and d : epidemic outbreaks.

showed some fluctuations (Figure 1). In 2007, the highest number of infection occurred between March and June (outbreak a) and October-December (outbreak b). In 2008, the highest number was reached between March and June (outbreak c) and September-December (outbreak d). Among the 131 isolates, 80 (61 %) were recovered from blood samples, 21 (16 %) from urine specimens and were associated with leukocytes number higher than 10.000 cells per ml in all the cases, 19 (14 %) from suppuration, 9 (7 %) from catheters tips and 2 (2 %) from cerebrospinal liquid.

Antibiotic susceptibilities

All isolates were resistant to all β-lactams except to cefoxitin and imipenem. All strains were susceptible to colistin. ESBL-producing isolates in this study were found to be concomitantly resistant to various antibiotics classes. The rate of resistance to non-β-lactam was indicated in Table 2.

Characterization of β-lactamase genes

All strains were positive for SHV β-lactamases. ESBL belonging to CTX-M-1 group were found in 75 (57%) isolates. TEM genes were detected in 28 isolates (21%). Strains co-producing different β-lactamases (TEM, SHV and CTX-M-1 group) were detected on 17 cases.

Sequencing of selected amplicons revealed the presence of bla CTX-M-15 with coding regions containing identi-cal nucleotide sequences. The DNA sequence of the PCR products exhibited 100 and 98% similarity to bla CTX-M-15 from GenBank.

Molecular typing

The 84 epidemic isolates were assigned to 14 pulsotypes and the cut-off value of 90% of similarity was indicated by a red line (Figure 2a and b). Attribution of pulsotypes identified by PFGE to a specific outbreak revealed a high degree of clonal diversity of isolates recovered between

Table 2. Rate of resistance of strains to non-β-lactam antibiotics.

Antibiotic	Aminoglycoside					TE	SXT	Fluoroquinolone				
	T	K	G	AN	S			NA	PEF	OFX	CIP	LVX
Neonatology Ward	105 (100%)	104 (99%)	102 (97%)	80 (76%)	42 (40%)	86 (82%)	63 (60%)	14 (13%)	12 (11%)	16 (15%)	12 (11%)	9 (9%)
Pediatric Ward	26 (100%)	26 (100%)	25 (96%)	20 (77%)	11 (42%)	23 (88%)	18 (69%)	7 (27%)	7 (27%)	7 (27%)	7 (27%)	4 (15%)
Total (131)	131 (100%)	130 (99%)	127 (97%)	100 (76%)	53 (40%)	109 (83%)	81 (62%)	21 (16%)	19 (15%)	23 (18%)	19 (15%)	13 (10%)

T, Tobramycin; K , kanamycin; G, gentamicin; AN, amikacin; S, streptomycin; TE, tetracycline; SXT, co-trimoxazole; NA, nalidixic acid; PEF, pefloxacin; OFX, ofloxacin; CIP, ciprofloxacin; LVX, levofloxacin.

2007 and 2008 and illustrated a complexity of outbreaks that involve more than two epidemics pulsotypes. Table 3 showed the distribution of pulsotypes illustrated by PFGE method in the several outbreaks that occurred in wards hospitals: P2; P3 and P5 were attributed to outbreak a. P8 was restricted to outbreak b. P11; P13 and P14 were identified in outbreak b and c. P7 appeared in outbreak a was encountered nearly one year later and was responsible with pulsotype P9 of severe cases of infections during outbreak d.

DISCUSSION

In Tunisia, ESBL-producing *K. pneumoniae* causes serious epidemic and endemic nosocomial infections and is one of the most important pathogens in hospital-acquired infections (Ben Jaballah et al., 2007). The prevalence of ESBL-producing *K. pneumoniae* varies according to countries, regions or even hospitals. In our study, *K. pneumoniae* has been the most frequently isolated ESBL-producing pathogen accounting for 70% of all isolated ESBL-producing *Enterobacteriaceae* and 39% of all the *Klebsiella*. This frequency is higher than 20.2% reported in a Tunisian care-teaching hospital of El Mehdia (Ben Haj Khalifa et al., 2010).

ESBL-producing *K. pneumoniae* proved to be the most common pathogen among patients in neonatal and pediatric intensive care units (Ben Jaballah et al., 2007; Szilagyi et al., 2010). In our study, ESBL-producing *K. pneumoniae* was isolated in 32 and 8% respectively, from neonatology and pediatric wards. This is in agreement with figures released by the “Outbreak Database” (the worldwide database for nosocomial outbreaks: http://www.outbreak-database.com), showing that *K. pneumoniae* is the most frequent causative agent in neonatal intensive care unit, representing 20.3% of all pathogens, 35% of which are ESBL-producers (Szilagyi et al., 2010). Effectively, under-staffing and overcrowding are real problems that are likely to raise colonization or infections rates in these wards (Bouallègue-Godet et al., 2005; Ruiz et al., 2010; Szilagyi et al., 2010).

Nosocomial pathogens evolve with selection pressures generated by changes in medical practice and antibiotic usage. Neonates are prone to invasive infections with Gram-negative organisms (Ben Jaballah et al., 2007) because of deficiency of immunity system, frequent use of broad-spectrum antibiotics that enhances the vulnerability to colonization by ESBL isolates in digestive flora which may be implicated in translocation and then bacteraemia (Biran et al., 2010). In Tunisia, *K. pneumoniae* was one of the leading pathogens involved in 19.5% of nosocomial bloodstream infections of which 87.5% were ESBL producers (Ben Jaballah et al., 2007). In our study, bloodstream infections proved to be the most frequent type of infection in neonatology with 61% of ESBL-producing *K. pneumoniae* being recovered from blood. These infections present a major therapeutic dilemma since the choice of antibiotics is restricted. Co-selection with other resistance especially those with aminoglycosides, co-trimoxazole and fluoroquinolones further limits therapeutic options (Messai et al., 2008). Indeed, the use of fluoroquinolones is not recommended for newborns (Pariente et al., 1998). In our study, fluoroquinolones still has activity against ESBL-producing *K. pneumoniae* and only 10% were resistant. In a European study that aims to monitor evolution of third generation cephalosporins resistance in *Enterobacteriaceae* from 2000 to 2008, incidence of fluorinated quinolone resistance association with ESBL production has increased from 51% in 2000 to become stable at 73% since 2003 (Mayoral et al., 2010).

In the last decade, a new trend distribution of ESBL was observed with the emergence and rise of certain type of ESBL named CTX-M. Unlike former TEM or SHV–derived ESBL, CTX-M has massively spread over community environments. The steadily increasing prevalence in the community led to an original situation in creating an influx of CTX-M carriers infected patients from the community to healthcare structures (Ruppé, 2010). Since 2003, a temporal shift in prevalence of ESBL types was noted in Tunisia with predominance of CTX-M instead of the SHV genotypes (Elhani et al., 2010). In another study carried out at Charles Nicole hospital, the detection rates of the ESBL-producing

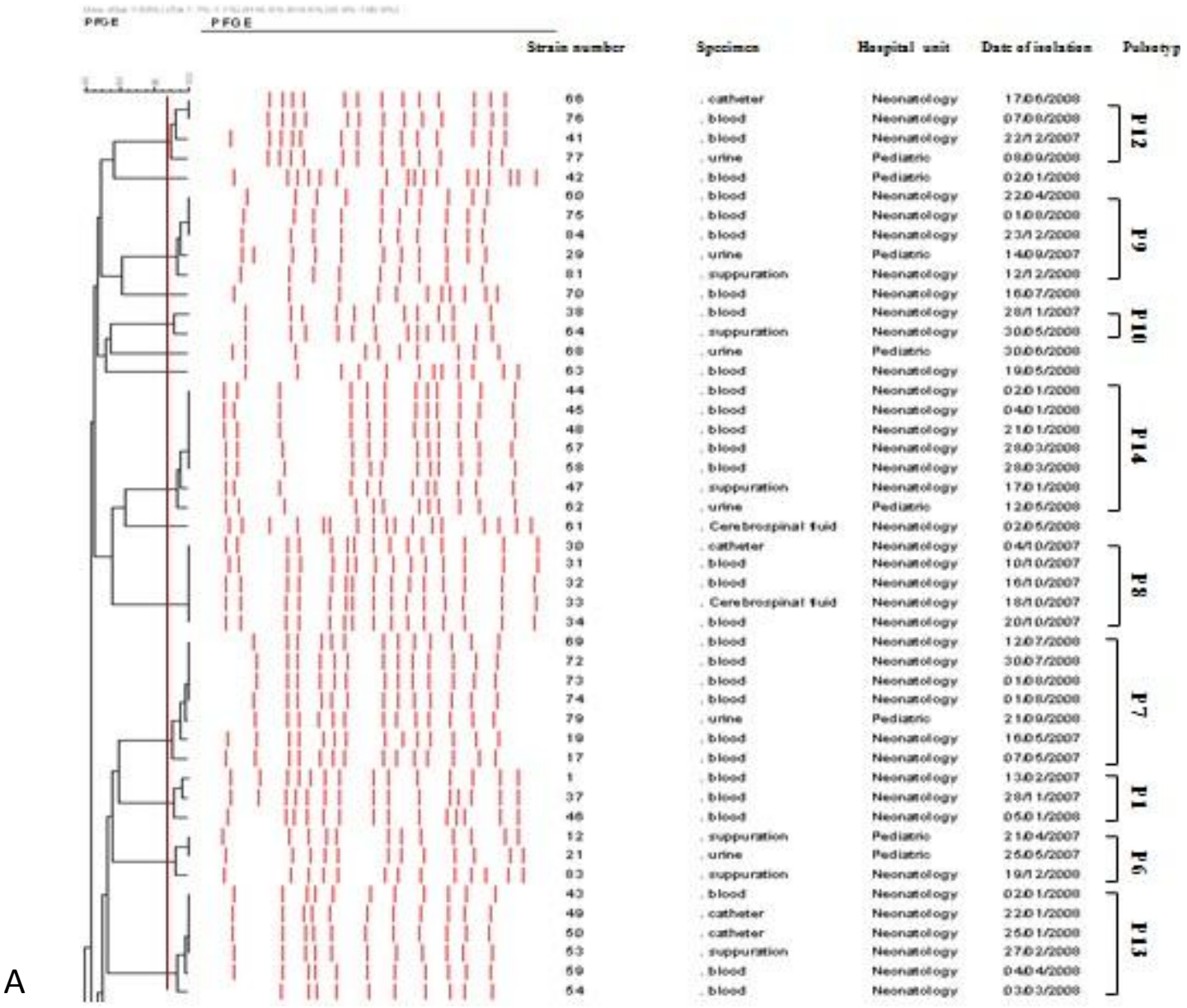

Figure 2a and b. Dendrogram of PFGE profile similarity of 84 ESBL-producing *K. pneumoniae* selected during the four outbreaks.

isolates sharply increased from 2.7% in 2000 and reached 30% in 2003 (Mamlouk et al., 2006). In our study, CTX-M-1 group β-lactamases accounted for more than 50% of *K. pneumonia*e isolates. Specific genetic groups of CTX-M have been characterized in different geographic areas. Therefore, it is noteworthy that CTX-M-1 group was mainly found in Tunisia with abundance of CTX-M-15 (Dahmen et al., 2010). Dissemination of specific CTX-M β-lactamases, which is probably driven by the frequent use of newer β-lactam antibiotics, may mainly be attributed to very efficient horizontal transfer of transposable genetic elements and spread of related strains (Vranic-Ladavac et al., 2010). Indeed, CTX-M genes might be associated with insertion-sequence or integrons involved in their expression and mobilization (Abbassi et al., 2008; Eckert et al., 2004).

PFGE analysis has proved to be a useful and adaptable method to study the epidemiology of nosocomial ESBL *K. pneumoniae* populations (Dahmen et al., 2010; Vranic-Ladavac et al., 2010). As previously reported (Ben Hamouda et al., 2003), PFGE typing in this study revealed a clonal diversity and epidemiologic complexity of *K. pneumonia*e infections that involve several epidemic pulsotypes. It has also illustrated the persistence of sporadic strains and their capacity to evolve endemically in neonatology ward. However, sporadic isolates showing distinct PFGE fingerprints were found in pediatric ward. Detection of CTX-M-15 in each representative isolate of

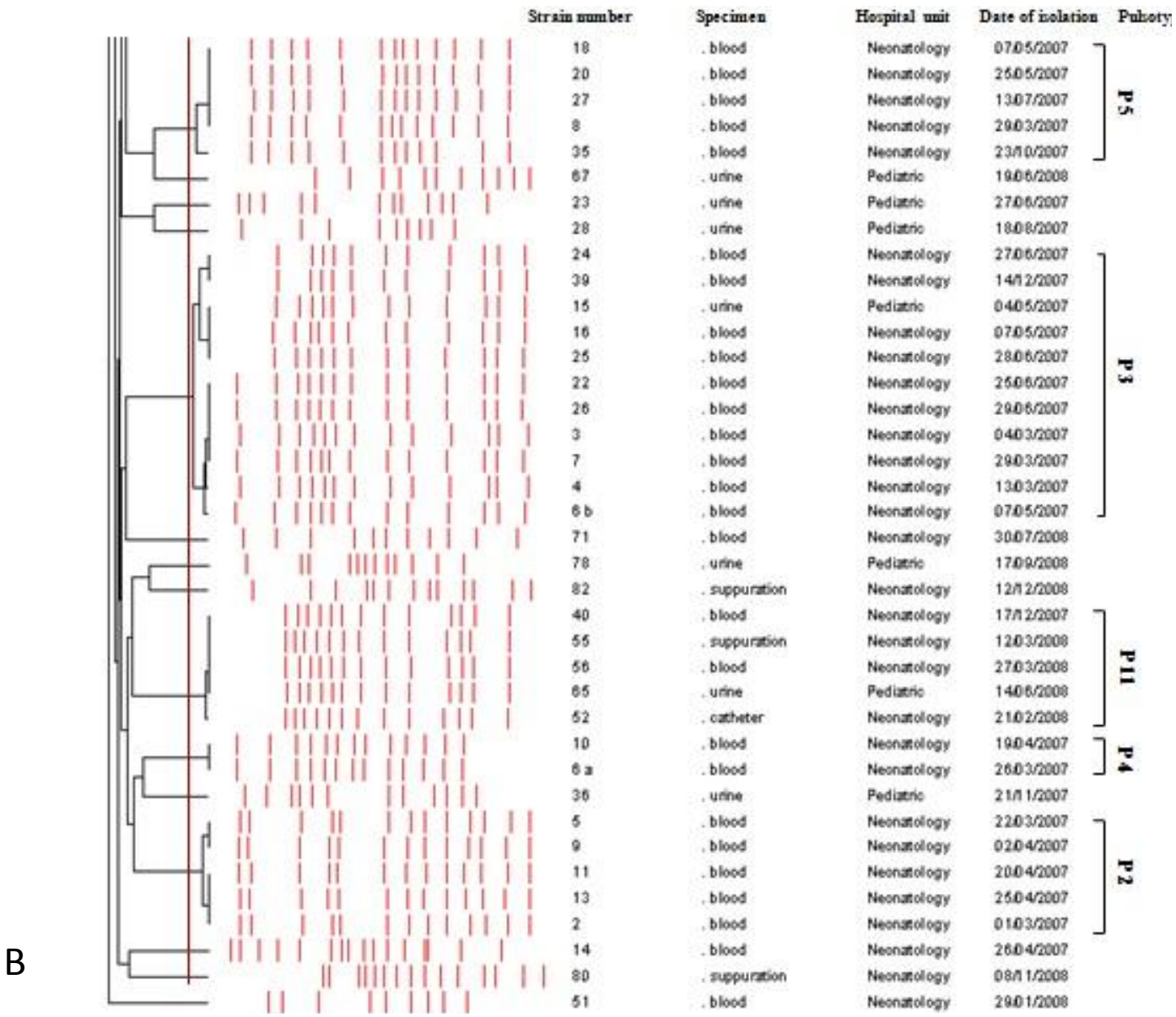

Figure 2. Contd.

epidemic group might contribute to the proliferation of CTX-M-15 in our hospital. Indeed, CTX-M-15 ESBLs accounted for the overwhelming majority of ESBL types among *Enterobacteriaceae* in Tunisia (Dahmen et al., 2010). Furthermore, several reports have referred to alarming outbreaks of CTX-M-15 producing *K. pneumonia*e in Russia, France, Hungary and Sweden and highlighted the epidemic potential of this species allowing the successful dissemination of this type of ESBL (Carrër and Nordmann, 2009).

Bacterial populations may alter in genetic structure due to the stress of survival in hostile environments (Aucken et al., 2000). Thus, the selection pressure, very important in neonatology (Doit et al., 2010), may explain partially the genetic diversity of isolates in our study. It may be attributed to hospital antibiotic policy results in prior consumption of third-generation cephalosporins and aminoglycosides used in treatment of the suspicions of primitive infection and expected to exert selection that enhance colonization of digestive flora by resistant ESBL-organisms (Doit et al., 2010). The significant prevalence of CTX-M β-lactamases producing *K. pneumoniae* in newborns could be associated with the worldwide emergence of CTX-M ESBL enzymes and their ability to cause increasingly community-urinary infections in pregnant women implicated in materno-foetal infections (Mayoral et al., 2010).

In conclusion, this study showed the complexity of outbreaks which were associated with a large genetic diversity of multidrug resistant isolates and indicated that

Table 3. Characteristics of ESBL-producing clinical isolates of *K. pneumoniae*.

Number of Isolate	Outbreak	Ward	PFGE type	β-lactamases produced		
				CTX-M-1 group	SHV	TEM
3	a ; b	Neonatal		+	+	-
5	a	Neonatal	P 2	+ (CTX-M-15)	+	-
11	a ; b	Neonatal	P 3	+ (CTX-M-15)	+	-
	a	Pediatric				
2	a	Neonatal	P 4	+	+	-
5	a ; b	Neonatal	P 5	-	+	+
3	a	Pediatric	P 6	+	+	+
	d	Neonatal				
	a*	Neonatal				
7	a ; d	Neonatal	P 7	-	+	-
	d	Pediatric				
5	b	Neonatal	P 8	-	+	-
	b	Pediatric	P 9	+ (CTX-M-15)	+	+
5	c ; d	Neonatal				
2	b ; c	Neonatal	P 10	+	+	+
5	b; c	Neonatal	P 11	-	+	-
	c	Pediatric				
	b**	Neonatal				
4	c; d		P 12	+	+	-
	d	Pediatric				
6	b; c	Neonatal	P 13	+ (CTX-M-15)	+	-
	b***					
7	b ; c	Neonatal	P 14	-	+	-
	c	Pediatric				

a*, Strain that co-produce TEM; b**, strain that produce exclusively SHV; b***, strain that co-produce CTX-M-15.

proliferation of ESBLs in neonatology ward was quite dramatic even that strains have a great invasive and epidemic potential. So, this situation emphasizes the necessity of continuous epidemiological monitoring with rapid molecular diagnosis followed by immediate intervention to prevent severe nosocomial infections especially in critical care patients.

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