Molecular epidemiology of an outbreak of multiresistant *Klebsiella pneumoniae* in a Tunisian neonatal ward

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During the first quarter of 1996, a major outbreak of clinical infection caused by multiresistant *Klebsiella pneumoniae* (MRKP) occurred in the neonatal ward of the 'Maternité Wassila Bourguiba' in Tunis, Tunisia. In total, 32 isolates of MRKP, comprising 23 clinical isolates and nine surveillance isolates, were recovered during this period and analysed for epidemiological relatedness. The isolates were compared with 17 other isolates of MRKP that were recovered during 1995. Macrorestriction profiles of total genomic DNA following *Xba*I restriction endonuclease digestion were analysed by PFGE; this typing classified 56 % of the 32 isolates recovered in 1996 into two major clusters. Cluster A included ten isolates from 1996 and three isolates recovered in 1995, whereas cluster B included eight isolates from the outbreak of 1996. The remaining isolates were genetically unrelated to those of clusters A and B; they constituted sporadic strains. The two major clusters were also evident using other molecular typing methods, such as random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC)-PCR, where isolates of clusters A and B could be identified on the basis of their discriminative patterns. This investigation showed the predominance of two epidemic strains, and illustrated the ease with which MRKP strains can disseminate and persist within a single ward.

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INTRODUCTION

Klebsiella pneumoniae is an important nosocomial pathogen that has the potential to cause severe morbidity and mortality, particularly in intensive care units and amongst paediatric patients, but also in medical and surgical wards (Branger *et al.*, 1998; Decré *et al.*, 1998; Podschun & Ullmann, 1998). In recent years, following extensive use of the expanded-spectrum cephalosporins, outbreaks of infection caused by extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) have been widely reported throughout the world (Branger *et al.*, 1998; Decré *et al.*, 1998; French *et al.*, 1996; Gniadkowski *et al.*, 1998; Nouvellon *et al.*, 1994; Peña *et al.*, 1998; Weller *et al.*, 1997).

Dissemination of ESBL-KP in a hospital may be a complex

event involving several modes of epidemic spread, such as dissemination of several epidemic strains or the propagation of a single clone from patient to patient (Bingen *et al.*, 1993; Branger *et al.*, 1997; Eisen *et al.*, 1995). Epidemiological studies on the spread of *K. pneumoniae* depend on the availability of sensitive and discriminative tests that permit differentiation between individual and epidemic strains. Since phenotypic methods lack reproducibility, discriminative power and typability, they are not always useful in epidemiological typing. Newer molecular typing techniques are employed most commonly, and have proven useful tools in epidemiological studies (Ahmad *et al.*, 1999; Asensio *et al.*, 2000; Bingen *et al.*, 1993; Branger *et al.*, 1997, 1998; Decré *et al.*, 1998; Eisen *et al.*, 1995; Gniadkowski *et al.*, 1998; Gori *et al.*, 1996; Gouby *et al.*, 1994; Peña *et al.*, 1998; Weller *et al.*, 1997).

During 1995 and the first quarter of 1996, nosocomial infections caused by multiresistant *Klebsiella pneumoniae* (MRKP) were observed with increasing frequency in the

Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; ESBL-KP, extended-spectrum β -lactamase-producing *Klebsiella pneumoniae*; MRKP, multiresistant *Klebsiella pneumoniae*; RAPD, random amplification of polymorphic DNA.

neonatal ward of the 'Maternité Wassila Bourguiba' (Tunis, Tunisia). This high incidence could be due to the dissemination and/or persistence of one or more epidemic strains. This study was designed to investigate the epidemiology of ESBL-KP in this ward, using molecular typing methods. It is the first genotypic description of a nosocomial outbreak of ESBL-KP infection in a Tunisian hospital.

METHODS

Bacterial isolates. A total of 49 isolates of *K. pneumoniae*, isolated between 1995 and 1996 in the neonatal ward of 'Maternité Wassila Bourguiba' in Tunis, were studied. Forty isolates were recovered from neonates, and nine environmental isolates were also recovered in the same ward. Of the 40 clinical isolates, 29 (72.5%) were recovered from blood cultures, ten from pus and one from cerebrospinal fluid (Table 1). All the isolates were identified by the API 20E system (bioMérieux).

Antibiotic susceptibility testing. Susceptibility to antibiotics was determined by the disc-diffusion method using Mueller-Hinton agar (Chabbert, 1982), and interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (Communiqué de l'Antibiogramme de la Société Française de Microbiologie, 1994). The antibiotic discs (Diagnostics Pasteur) used were amoxicillin (25 µg), ticarcillin (75 µg), amoxicillin plus clavulanic acid (20/10 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), imipenem (10 µg), streptomycin (10 IU), kanamycin (30 IU), tobramycin (10 µg), gentamicin (15 µg), amikacin (30 µg), tetracycline (30 IU) and chloramphenicol (30 µg). All test isolates were screened for the production of extended-spectrum β -lactamases (ESBLs) using the double-disc test (Jarlier *et al.*, 1988).

Genomic fingerprinting by PFGE. Genomic DNA was prepared by a modification of the procedure described by Poh *et al.* (1993). *K. pneumoniae* strains were grown overnight in Luria-Bertani broth at 37 °C. Cells were harvested, washed with 10 mM Tris/HCl, pH 7.6, 1 M NaCl, and resuspended in an adequate volume of the same buffer to obtain a bacterial cell density equivalent to 1.5×10^9 c.f.u. ml⁻¹. The resulting bacterial suspension was mixed with an equal volume of 2% low-melting-point agarose (Gibco-BRL) in TE buffer (pH 7.6) at 50 °C. The bacterial agarose suspension was introduced into a plug mould (Bio-Rad) and allowed to solidify at 4 °C for 20 min. The agarose plugs were incubated overnight at 37 °C with gentle shaking in lysis buffer (6 mM Tris/HCl, pH 7.6; 1 M NaCl; 100 mM EDTA, pH 8; 0.5% Brij 58; 0.2% sodium deoxycholate; 0.5% N-lauryl sarcosine; 1 mg lysozyme ml⁻¹; and 20 µg RNase ml⁻¹). The agarose plugs were then washed in a buffer composed of 0.5 M EDTA (pH 9.5), 0.5% N-lauryl sarcosine and 50 µg proteinase K ml⁻¹ for 48 h at 55 °C. The plugs were washed again, once for 1 h at 37 °C and once for 1 h at room temperature, in a solution of 1 mM PMSF in TE buffer, and then three times (for 30 min each time) in TE buffer at room temperature. Restriction endonuclease digestion of the chromosomal DNA was then performed using 10 U *Xba*I (New England BioLabs) at 37 °C for 24 h. Restriction fragments of DNA were separated by PFGE using 1% agarose gels in 0.5× TBE buffer with CHEF-DRII apparatus (Bio-Rad). Electrophoresis conditions were 14 °C at 200 V for 35 h, with pulse time ranging from 2 to 30 s. Gels were stained with ethidium bromide and photographed under UV light.

Analysis of DNA relatedness. Only *Xba*I fragments exceeding 48.5 kb were taken into account when PFGE patterns were compared visually. Clustering correlation coefficients were calculated by UPGMA, determined with the program STATISTICA (StatSoft). Pearson's correlation coefficient was used to estimate the inter-isolate relatedness.

Genomic fingerprinting by random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC)-PCR. Genomic DNA for epidemiological typing was extracted from bacterial cells as described by Chen & Kuo (1993), and DNA concentrations were estimated on agarose gels. RAPD analysis and ERIC-PCR were performed using oligonucleotides RAPD7 and ERIC2, respectively (van Belkum *et al.*, 1995; Versalovic *et al.*, 1991). The PCR mixture contained 200 µM each dNTP, 200 ng primer, approximately 10 ng genomic DNA, 5 µl DyNAzyme EXT buffer (10 mM Tris/HCl, pH 8.8; 1.5 mM MgCl₂; 50 mM KCl; 0.1% Triton X-100) and 2 U DyNAzyme EXT DNA polymerase (Finnzymes) in a total volume of 50 µl. The amplification protocol, performed in a TRIO-thermoblock PCR machine (Biometra), consisted of the following steps: initial denaturation at 96 °C for 5 min, followed by 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 35 or 50 °C for RAPD7 and ERIC2, respectively), extension (72 °C for 3 or 10 min with RAPD7 and ERIC2, respectively), and a final extension step (72 °C for 10 min). Amplified PCR products were separated using 1% agarose gels and visualized by UV transillumination. DNA fingerprints were compared by visual inspection.

RESULTS

Antibiotic resistance patterns

As determined by disc-diffusion antibiotic susceptibility testing, the isolates, including those from environmental sites, exhibited the same pattern of resistance to β -lactam agents, demonstrating resistance to penicillins (amoxicillin and ticarcillin), cephalothin and extended-spectrum cephalosporins (ceftriaxone and cefotaxime). All isolates also demonstrated a strong synergy between clavulanic acid and cefotaxime, indicating ESBL production. In addition, all isolates exhibited resistance to kanamycin and tobramycin. Based on the susceptibility to other aminoglycosides (gentamicin, amikacin and streptomycin), tetracycline and chloramphenicol, 13 antibiotypes (A1–A13) were defined for the 49 isolates (Table 1). Eleven isolates belonged to resistance pattern A8, which was characterized by resistance to gentamicin, amikacin and tetracycline. The antibiotype A7 (12 isolates) differed from A8 by susceptibility to amikacin and resistance to chloramphenicol. All isolates tested remained susceptible to cefoxitin and imipenem.

Chromosomal DNA analysis by PFGE

The *Xba*I macrorestriction profiles of DNA from all isolates tested are shown in Fig. 1. *Xba*I digestion was found to produce 9–19 fragments ranging in size from 20 to > 380 kb. Among the 49 isolates, 25 PFGE types (P1–P25) were identified by UPMGA analysis (Fig. 2 and Table 1). Twenty-one isolates belonged to two major clusters, A and B, which correspond to patterns P6 and P11, respectively.

Ten isolates from 1996 and three isolates recovered in 1995 (isolates 34, 46 and 48) formed a major cluster, A. They showed the most prevalent PFGE type, P6, including 12 subtypes (P6a–m), with a genetic similarity of about 80%. Eight isolates from 1996 belonged to a second major cluster, B (P11), including six subtypes (P11a–f), with a genetic similarity of about 77%. Clusters A and B showed less than

Table 1. Characteristics of MRKP isolates

Strains 1–32 were isolated during the outbreak of 1996; strains 33–49 were isolated in 1995. CSF, Cerebrospinal fluid; S, streptomycin; G, gentamicin; A, amikacin; C, chloramphenicol; Tet, tetracycline.

Isolate	Source of isolate	Resistance phenotype*	Antibiotype	PFGE pattern
1	Environment	G–C–Tet	A7	P10
2	Environment	G–C–Tet	A7	P6m
3	Environment	G–C–Tet	A7	P6h
4	Environment	G–A–Tet	A8	P6k
5	Environment	C–Tet	A12	P8
6	Environment	G–C–Tet	A7	P6c
7	Environment	C–Tet	A12	P8
8	Environment	C–Tet	A12	P6c
9	Pus	S–G–A–C–Tet	A1	P17
10	Blood	G–A–C–Tet	A4	P6b
11	Blood	G–A–Tet	A8	P6e
12	Pus	G–C–Tet	A7	P6i
13	Pus	C–Tet	A12	P17
14	Blood	C–Tet	A12	P6f
15	Pus	G–A–Tet	A8	P12
16	Pus	S–G–C	A6	P11b
17	Blood	G–A–Tet	A8	P11d
18	Pus	G–A–Tet	A8	P11e
19	Blood	G–A–C–Tet	A4	P6g
20	Blood	S–G–A–Tet	A3	P11a
21	Blood	G–C–Tet	A7	P9
22	Blood	G–A–Tet	A8	P19
23	Blood	G–A–Tet	A8	P13
24	Blood	G–A	A11	P25a
25	Blood	Tet	A13	P11e
26	Blood	S–G	A10	P25b
27	Blood	G–A–Tet	A8	P11f
28	Blood	S–G–A–Tet	A3	P11e
29	Blood	S–G–A	A5	P4
30	Blood	G–A–Tet	A8	P3b
31	Blood	G–A–Tet	A8	P3a
32	Environment	S–G–A–C–Tet	A1	P11c
33	CSF	G–A–C–Tet	A4	P14
34	Pus	G–C–Tet	A7	P6d
35	Pus	G–A	A11	P5
36	Pus	S–G–A–C–Tet	A1	P7
37	Pus	G–A–C–Tet	A4	P18b
38	Blood	G–A–C–Tet	A4	P20
39	Blood	G–A–C–Tet	A4	P1
40	Blood	S–G–A–C	A2	P16
41	Blood	G–A–C–Tet	A4	P21
42	Blood	G–C–Tet	A7	P18a
43	Blood	G–C–Tet	A7	P15
44	Blood	G–C–Tet	A7	P2
45	Blood	G–C–Tet	A7	P24
46	Blood	S–G–A–C–Tet	A1	P6a
47	Blood	G–A–Tet	A8	P22
48	Blood	G–C–Tet	A7	P6j
49	Blood	G–A–C	A9	P23

*Only additional resistance markers allowing individualization of phenotypes are indicated.

Fig. 1. PFGE fingerprinting of *Xba*I-digested DNA from multiresistant *K. pneumoniae*. M, Lambda ladder PFG marker (New England Biolabs).

40 % genetic similarity (Fig. 2). Four minor clusters (P3, P8, P17 and P25) were identified among isolates recovered in 1996 and a further cluster, P18, was identified among isolates recovered in 1995. A large number of isolates ($n = 18$) demonstrated individual PFGE patterns.

When PFGE types were compared with antibiotic resistance profiles, six isolates from cluster A exhibited the A7 anti-biotype and three isolates from cluster B displayed anti-biotype A8. In most cases, isolates with indistinguishable antibiotypes had distinct PFGE patterns.

Genomic fingerprinting by RAPD analysis in comparison with that by PFGE

Forty isolates were analysed by RAPD typing, allowing the identification of distinct banding patterns. Seventeen profiles were obtained, each comprising 6–9 fragments with molecular sizes ranging between 0.5 and 5.6 kb (Fig. 3).

Visual inspection readily distinguished two patterns, R_A and R_B , that differed by the fragments in the size range of 0.8–0.6 kb and 5.6–3.6 kb. Eleven isolates belonging to cluster A exhibited the R_A pattern, and all five cluster B isolates subjected to RAPD analysis were assigned to pattern R_B . Of 24 sporadic isolates tested by RAPD, only three (numbers 7, 21 and 36) had a similar pattern to R_A , and three others (numbers 15, 23 and 44) exhibited DNA fragments characterizing the R_B pattern. The remaining sporadic isolates exhibited individual RAPD patterns.

Relationship between PFGE type and ERIC analysis

Ten isolates from cluster A, the eight isolates from cluster B and 11 isolates selected to represent sporadic strains were tested by ERIC-PCR for genomic fingerprinting. Discriminatory profiles are shown in Fig. 4. A distinctive ERIC pattern, E_A , characterized by the presence of three amplified fragments (4.0, 3.6 and 3.3 kb), was obtained for the isolates belonging to cluster A. The isolates of cluster B shared one

PCR fragment of 5.6 kb that was specific for a distinct ERIC pattern, namely E_B . Among the sporadic isolates tested, one (no. 21) had a profile similar to E_A and the other (no. 23) exhibited a profile similar to E_B .

DISCUSSION

The ability of *K. pneumoniae* to spread rapidly amongst patients often leads to nosocomial outbreaks of infection, especially in neonatal units (Hart, 1993; Shannon *et al.*, 1998). During the first quarter of 1996, the neonatal ward of the 'Maternité Wassila Bourguiba' experienced an outbreak of nosocomial infections caused by MRKP. To investigate the outbreak in the ward, 32 ESBL-KP isolates collected over this period were examined and compared with 17 other strains of ESBL-KP involved in previous outbreaks in the same ward, using phenotypic and genotypic epidemiological fingerprinting methods.

Nosocomial outbreaks caused by ESBL-producing *Enterobacteriaceae* have been described mostly in intensive care units (Branger *et al.*, 1998; Decré *et al.*, 1998; Jarlier *et al.*, 1988). In Tunisia, these organisms were identified mainly among paediatric isolates of *K. pneumoniae*, *Salmonella* species and *Escherichia coli* and, as observed in another Tunisian hospital, the majority of our isolates were recovered from blood (Ben Hassen *et al.*, 1990; Hammami *et al.*, 1991; Philippon *et al.*, 1989).

ESBL production is frequently accompanied by multiresistance to antibiotics (Podschun & Ullmann, 1998). All the *K. pneumoniae* isolates in this study demonstrated ESBL-mediated resistance, were also resistant to kanamycin and tobramycin and most of them to gentamicin, amikacin, tetracycline and chloramphenicol. Outbreaks of infection caused by *K. pneumoniae* strains that are resistant to extended-spectrum cephalosporins and aminoglycosides have been observed previously in neonatal wards, and appeared to be associated with the increased use of anti-

Fig. 2. Dendrogram, as generated by UPGMA, illustrating the relatedness of the 25 PFGE types. Isolate number, corresponding PFGE type and the major clusters A and B are indicated.

biotics (Bingen *et al.*, 1993; Podschun & Ullmann, 1998; Shannon *et al.*, 1998). In this study, antibiotic susceptibility patterns did not show enough strain-to-strain variation to be sufficiently discriminative. The determination of a stable chromosomal marker thus appeared to be necessary to identify relatedness of the *K. pneumoniae* isolates. Macrorestriction analysis by PFGE has been proposed as a suitable epidemiological tool, being considered useful in the investigation of the source, transmission and spread of nosocomial infections caused by various species including ESBL-KP (Branger *et al.*, 1997; Gouby *et al.*, 1994; Poh *et al.*, 1993).

PFGE typing of MRKP isolates revealed that at least two

Fig. 3. Ethidium bromide-stained agarose gel showing RAPD patterns of representative isolates of multiresistant *K. pneumoniae*. Patterns of isolates from cluster A (pattern R_A), cluster B (pattern R_B) and of sporadic isolates are shown. M, Molecular size marker (Raoul, Appligene).

Fig. 4. Ethidium bromide-stained agarose gel showing ERIC2-PCR patterns of representative isolates of multiresistant *K. pneumoniae*. Patterns of isolates from cluster A (pattern E_A), cluster B (pattern E_B) and of sporadic isolates are shown. Position of molecular size marker (Raoul, Appligene) is indicated.

distinct clonal patterns, P6 and P11, were involved in the outbreak. Ten isolates from the outbreak in 1996 and three isolates recovered in 1995 clustered in pattern P6 (cluster A). This suggests that these isolates may represent genotypic variants of the same clone, which may have persisted endemically within the ward. This is also supported by the finding that environmental isolates (numbers 2, 3, 4, 6 and 8) recovered in 1996 exhibited the same PFGE type, P6. The second major pattern, P11 (cluster B), exhibited by eight isolates recovered during the outbreak period, revealed the dissemination of an epidemic strain genetically unrelated to the former clone. A high number of genetically unrelated or sporadic strains were also recovered during the outbreak. PFGE typing results showed the complexity of the outbreak.

No direct correlation was found between PFGE profiles and

antibiotic susceptibility patterns. Isolates with identical antibiotic types frequently belonged to different PFGE types, and many isolates that were unrelated on the basis of susceptibility had identical PFGE patterns. PFGE typing and both the RAPD and ERIC typing methods demonstrated concordant results and comparable discrimination between groups of epidemiologically related strains. All the isolates belonging to cluster A with PFGE type P6 were characterized by the unique RAPD and ERIC patterns R_A and E_A , respectively, while those isolates of cluster B with PFGE type P11 exhibited unique R_B and E_B patterns. However, some disagreement between the results of RAPD and ERIC typing was apparent for two sporadic strains. Indeed, isolate no. 21 (characterized by the pattern R_A , E_A), and isolate no. 23 (R_B , E_B) fell into different PFGE types, P9 and P13, respectively.

In this study, PFGE analysis, combined with RAPD typing and ERIC-PCR, showed that the increasing number of MRKP isolates in the neonatal ward was the result of the spread of at least two epidemic strains and the endemic persistence of one of them within the ward. A concomitant emergence of sporadic isolates was also involved in the outbreak. PFGE- and PCR-based methods showed concordant results and comparable discrimination and differentiation between clusters of epidemiologically related strains. ERIC- and RAPD-PCR offer the advantages of simplicity and rapidity, but the availability of guidelines for interpretation of DNA macrorestriction patterns generated by PFGE makes this method more easily understood and accessible as a typing tool (Tenover *et al.*, 1995).

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