Comparison of Pulsed-Field Gel Electrophoresis and Randomly Amplified DNA Polymorphism Analysis for Typing Extended-Spectrum-β-Lactamase-Producing *Klebsiella pneumoniae*

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The incidence and transmission patterns of extended-spectrum-β-lactamase (ESBL)-producing *Klebsiella pneumoniae* in patients admitted to the intensive care unit (ICU) of a university hospital were investigated over a 3-year period. *K. pneumoniae* isolates were characterized by antibiotic susceptibility, capsular serotyping, plasmid profiles, and pulsed-field gel electrophoresis (PFGE) of genome macrorestriction patterns with *XbaI*, and the results were compared with those obtained by typing with the randomly amplified polymorphic DNA (RAPD) patterns. The discriminatory power of RAPD typing was evaluated for three primers. The incidence of isolation of ESBL-producing *K. pneumoniae* was 2.5 cases per 1,000 admissions to the ICU versus 0.35 cases per 1,000 admissions to other units (relative risk, 7.03; 95% confidence interval, 3.89 to 12.69). Infection developed in 53% of evaluable patients. Thirty-six percent of the cases were possibly acquired in other institutions. Isolates from ICU patients were subdivided into six capsular serotypes and into four clonal groups based on antibiotic, plasmid content, and PFGE and RAPD patterns. Two clones were associated with clusters of cross-infection, involving 5 and 12 patients, respectively. Following implementation of contact isolation precautions, the incidence of nosocomial acquisition of ESBL-producing *K. pneumoniae* decreased from 0.55 to 0.26 cases per 1,000 admissions (P = 0.03). PFGE and RAPD analysis showed concordant results and comparable discrimination for differentiation between groups of epidemiologically related strains of ESBL-producing *K. pneumoniae*. More subclonal variants were determined among epidemic clones by PFGE analysis than by RAPD analysis. Both methods are useful for typing *K. pneumoniae* strains in epidemiological investigations, although RAPD analysis is more efficient.

Resistance to broad-spectrum cephalosporins has emerged in strains of members of the family Enterobacteriaceae following frequent use of these drugs in the hospital setting (13, 18). Endemic and epidemic nosocomial infections caused by extended-spectrum-β-lactamase (ESBL)-producing *Klebsiella pneumoniae* cells represent a persistent problem in many parts of the world, especially in intensive care units (ICU) (2, 4, 5, 12, 17–19). Epidemic strains of cephalosporin-resistant *K. pneumoniae* have been associated with increased morbidity and mortality in hospitalized patients (7, 18). Since 1983, nosocomial outbreaks of ESBL-producing *K. pneumoniae* infections in various species of Enterobacteriaceae have been described (12). In France, 10 to 30% of *K. pneumoniae* strains are reported to produce plasmid-mediated ESBL of the TEM or SHV families (12).

Understanding the epidemiology of ESBL-producing *K. pneumoniae* requires the use of accurate epidemiological markers able to differentiate between the spread of resistance plasmids and strain dissemination. Initially, *K. pneumoniae* strains were characterized by capsular serotyping and plasmid analysis (2, 21, 23). Subsequently, pulsed-field gel electrophoresis (PFGE) analysis of genome macrorestriction fragments was shown to be a more discriminating typing technique (1, 22). However, PFGE is a technically demanding and time-consuming technique that requires specific equipment. PCR-based typing techniques, such as randomly amplified polymorphic DNA (RAPD) analysis, are faster and easier to perform (24). In recent studies, RAPD analysis has been successfully used to type a diversity of microorganisms (2, 10, 15–17, 24).

The aims of the present study were to determine the incidence and origin of acquisition of ESBL-producing *K. pneumoniae* strains isolated from ICU patients admitted to a Belgian university hospital and to delineate the clonal diversity and transmission patterns of these strains by comparing results of capsular serotyping, plasmid profile, genome macrorestriction analysis (PFGE), and RAPD patterns. The respective values of the latter two genomic typing methods for epidemiologic typing of *K. pneumoniae* were evaluated in more detail. (This work was presented in part at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy, Anaheim, Calif., 11 to 14 October 1992.)

MATERIALS AND METHODS

Epidemiological investigation. Erasme hospital is a 900-bed university hospital that admits approximately 27,000 patients annually. In four adult ICU with a total of 30 beds, 240 patients are admitted monthly. The hospital antibiotic policy is based on a drug formulary, written local guidelines for empiric and documented therapy, and daily consultancy with infectious disease specialists and clinical microbiologists for management of serious infections and use of reserved drugs, including extended-spectrum cephalosporins.

During the period from January 1990 to December 1992, patients with *K. pneumoniae* isolates resistant to extended-spectrum cephalosporins were identified by a daily computer search in the microbiology database and were investigated clinically. Resistant isolates were stored by freezing at –70°C. A cluster of
cases was defined as two or more cases of resistant *K. pneumoniae* isolation among patients who stayed at the same time in the same unit of the hospital.

To prevent nosocomial transmission of ESBL-producing *K. pneumoniae* strains, colonized patients were placed in contact isolation starting in January 1991. Patients colonized at the time of hospital admission were placed in patient care procedures, which were followed by alcohol-based disinfection of hands.

**Antimicrobial susceptibility.** Susceptibility to antimicrobial agents was tested by the disk diffusion method on Mueller-Hinton agar (Biomerieux, Marcy l'Etoile, France) as described in the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie. The production of clavulanic acid-susceptible ESBL was detected by using the double-disk synergy test as described previously by Jarlier et al. (13). Additional, inhibition zone diameter tests with various β-lactams, including aztreonam and imipenem, amoxicillin, amoxicillin–clavulanic acid, chloramphenicol, and other antibiotics, were measured. The MICs of ceftazidime and ceftriaxone were determined by using the E-test method (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar, with and without clavulanic acid (4 μg/ml) added in agar, for quantification of inhibition of β-lactamase activity.

**Capsular serotyping.** The capsular (K) antigen was determined by counter-immunoelectrophoresis at the Laboratory for Hospital Infections of the Public Health Laboratory Service, Colindale, London, United Kingdom (24).

**Plasmid profile analysis.** Plasmid DNA was extracted by a rapid procedure described previously. (22). Plasmid DNA was analysed by electrophoresis in 1% agarose gels and stained with ethidium bromide and soft tissues (21.9%), and the urinary tract (19.1%). All patients who had a positive culture for ESBL-producing *K. pneumoniae* (median, 22 days).

Clinical data were recorded from 17 evaluable patients with ESBL-producing *K. pneumoniae* in 1991 to 1992, 7 of whom were cared for in the ICU. Infection occurred in 9 of 17 (52.9%) of these patients. The crude fatality rate was 11 of 45 (24.4%) patients with isolation of ESBL-positive strains. Among clinically evaluable patients, death occurred in 1 of 8 colonized patients, compared with 5 deaths in 9 infected patients.

**Use of extended-spectrum cephalosporins did not vary substantially during the study period.** The yearly consumption of ceftazidime, expressed in standard daily doses, was 2,200 in 1989, 2,417 in 1990, 2,167 in 1991, and 2,483 in 1992, while that of ceftriaxone was approximately 1,000 in 1989, 750 in 1990, 850 in 1991, and 900 in 1992. During the 2-year evaluation period of contact isolation precautions for patients with a positive culture for ESBL-producing *K. pneumoniae*, the rate of nosocomial acquisition of this organism was 14 of 54,302 (0.26 per 1,000) admissions, compared with a rate of 15 of 27,038 (0.55 per 1,000) admissions during the year before introduction of control measures (relative risk, 0.46; 95% confidence interval, 0.22 to 0.96; \( P = 0.03 \) [chi-square test]). Likewise, the occurrence of clusters decreased from an initial rate of 1.85 per 1,000 admissions to 0.74 per 1,000 admissions (rate ratio, 0.40).

The distribution in time and place of patients positive for ESBL-producing *K. pneumoniae* revealed the occurrence of three clusters involving, respectively, seven, five, and five patients. The first cluster started in ICU no. 4 (five patients) and continued, after transfer of the fifth patient, in ICU no. 2 (two patients) over a 93-day period. The second cluster occurred 3 months after the first episode in ICU no. 4 (five patients) over a 63-day period. The third cluster began 9 months later in ICU no. 2 (two patients) and continued, after transfer of the second patient, in ICU no. 4 (three patients) over a period of 55 days. The other patients colonized with ESBL-producing *K. pneumoniae* isolates presented a sporadic occurrence.

**Antimicrobial susceptibility.** *K. pneumoniae* isolates from 19 patients from the ICU were distributed into four different antibiotype (Table 1). All strains exhibited a β-lactam susceptibility profile consistent with the production of ESBL: (i) decreased susceptibility or resistance to amoxicillin, piperacillin, cephalothin, cefamandole, extended-spectrum cephalosporins and aztreonam; (ii) full susceptibility to imipenem, temocillin, and cephapencillin; and (iii) a positive disk synergy test with extended-spectrum cephalosporins. The MICs of ceftazidime (2 to 256 mg/ml) and ceftriaxone (12 to 24 mg/ml) showed a reduction of more than fourfold in the presence of clavulanic acid. The antibiotype all shared resistance to sulfonamides, trimethoprim, cotrimoxazole, and chloramphenicol. They were distinguished by different susceptibilities to fluoroquinolones, aminoglycosides, and tetracycline. Resistance to amoxicillin and tetracycline, but resistance to both was associated in only one (Table 1).
Serotype. The strains were subdivided into six serotypes by K antisera, of which five serotypes were found in multiple cases (Table 1). Multiple serotypes were found to subdivide strains with a given antibiotic type.

Plasmid profiles. Three different plasmid profiles, each including one to four plasmids with apparent molecular sizes ranging from 2 to >70 kb, were found in the 18 strains analyzed (Table 1). The plasmid profiles were consistently associated with the antibiotic type.

Genome macrorestriction analysis by PFGE. Chromosomal DNA digested with XbaI produced an average of 20 fragments ranging in size from less than 36 kb to approximately 700 kb (Fig. 1). Four major groups of related macrorestriction genomic patterns (A to D) were recognized, with 13 subclonal variants distinguished by one to two fragment differences (Table 1). The temporal distribution of episodes of colonization among patients admitted to the ICU according to the classification of isolates into major macrorestriction types showed the occurrence of two consecutive clusters associated with clone A and of one cluster associated with clone C (Fig. 2).

RAPD analysis. For each of the primers used, RAPD analysis yielded four groups of closely related fingerprints (major types A through D), each exhibited by 12, 1, 5, and 1 strain(s), respectively (Table 1). For group A, two subtypes obtained with primer R108 and three subtypes obtained with primer HLW74 were distinguished by single-band variations of the core pattern. As shown in Fig. 3, primer HLW74 produced four clearly different groups of fingerprints based on bands with high intensity. Within group A, this primer allowed distinction of three variant fingerprints (Fig. 3, lanes 1, 2, and 3). Besides several common bands, one subtype (a1) showed an

![FIG. 1. PFGE separation of XbaI macrorestriction fragments of genomic DNA of ESBL-producing K. pneumoniae isolates from ICU patients at Erasme Hospital between 1990 and 1992. Four major genotypes are shown: type A (lanes 2 to 15), type B (lane 17), type C (lanes 18 to 23), and type D (lane 24). Lanes 1, 6, 11, 16, 21, and 25, molecular size markers (Smal chromosomal digest of S. aureus NCTC 8325).](http://jcm.asm.org/)

![FIG. 2. Temporal distribution of ESBL-producing K. pneumoniae isolation in the ICU of Erasme Hospital, 1990 to 1992, according to major macrorestriction types.](http://jcm.asm.org/)

### Table 1. Phenotypic and genomic markers of ESBL-producing K. pneumoniae strains from 19 patients admitted to the Erasme hospital ICU

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotype</th>
<th>K serotype</th>
<th>Plasmid profile</th>
<th>Macrorestriction profile</th>
<th>RAPD pattern with</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>ESBL/TETRA/CMP/SULF/TMP/SXT</td>
<td>6,19</td>
<td>1</td>
<td>A8</td>
<td>II 1 a1 A1</td>
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<tr>
<td>2</td>
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<td>1</td>
<td>A1</td>
<td>II 1 a2 A2</td>
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<tr>
<td>3</td>
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<td>1</td>
<td>A1</td>
<td>II 1 a3 A3</td>
</tr>
<tr>
<td>4</td>
<td>ESBL/TETRA/CMP/SULF/TMP/SXT</td>
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<td>1</td>
<td>A1</td>
<td>II 1 a3 A3</td>
</tr>
<tr>
<td>5</td>
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<td>1</td>
<td>A2</td>
<td>II 1 a3 A3</td>
</tr>
<tr>
<td>6</td>
<td>ESBL/TETRA/CMP/SULF/TMP/SXT</td>
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<td>1</td>
<td>A3</td>
<td>II 1 a3 A3</td>
</tr>
<tr>
<td>7</td>
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<td>1</td>
<td>A3</td>
<td>II 1 a3 A3</td>
</tr>
<tr>
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<tr>
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<td>1</td>
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<td>I1 1 a3 A3</td>
</tr>
<tr>
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<td>1</td>
<td>A7</td>
<td>I2 1 a3 A5</td>
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<tr>
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<td>ESBL/KTNA/CMP/SULF/TMP/SXT/PEF</td>
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<td>2</td>
<td>B</td>
<td>II 2 b B</td>
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<td>3</td>
<td>C2</td>
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<td>3</td>
<td>C3</td>
<td>III 3 c C</td>
</tr>
<tr>
<td>19</td>
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<td>25</td>
<td>ND</td>
<td>D</td>
<td>IV 4 d D</td>
</tr>
</tbody>
</table>

* Abbreviations: TETRA, tetracycline; CMP, chloramphenicol; SULF, sulfonamide; TMP, trimethoprim; SXT, cotrimoxazole; PEF, pefloxacin; K, kanamycin; T, tobramycin; N, netilmicin; A, amikacin.

* ND, not determined.
additional fragment of high intensity (approximate molecular size of 0.7 kb) and the disappearance of a low-intensity fragment with a size <0.7 kb. For another subtype (a2), the modification consisted of an additional fragment of low intensity with a molecular size of approximately 0.6 kb.

**DISCUSSION**

Plasmid-mediated ESBLs are found more frequently in *K. pneumoniae* strains than in other Enterobacteriaceae species (8, 12, 13). Nosocomial outbreaks caused by ESBL-producing *K. pneumoniae* have been reported in Europe and in the United States (2, 4, 5, 8, 11, 14, 18–20). European surveys of nosocomial isolates of *K. pneumoniae* show that approximately 15% produce ESBLs (1). Infections caused by these strains, particularly in adult patients admitted to ICUs as observed in our study, have been described elsewhere (2, 5, 14, 18). Colonization of the gastrointestinal tract appears common in patients hospitalized for prolonged periods (5). Selection pressure from widespread hospital use of late-generation cephalosporins can apparently be responsible for increasing occurrence of colonization of the digestive or respiratory tracts of patients (6). In our hospital, small outbreaks occurred that were apparently not associated with a significant increase in the use of extended-spectrum cephalosporins.

Since 1990, ESBL-producing *K. pneumoniae* isolates were screened in our hospital by routine use of a synergy test with disk diffusion susceptibility testing. Between 1990 and 1992, 5% of *K. pneumoniae* clinical isolates produced ESBLs, a rate similar to that of a previous report from Belgium (25). With an overall incidence of 0.6 cases per 1,000 admissions, isolation of ESBL-producing strains was uncommon in this hospital population. Nevertheless, it was observed sevenfold more frequently in patients admitted to ICU than in those admitted to other units. In the ICU, 53% of the colonized patients developed an infection, further indicating that ESBL-producing *K. pneumoniae* can be associated with significant morbidity in this setting.

Space and time patterns of isolation suggest that the patients hospitalized for long periods in the ICU acted as a reservoir for cross-infection. This hypothesis was supported by the fact that before contact precautions were introduced, epidemic strains were responsible for several secondary cases, whereas only sporadic cases were observed subsequently.

In this study, we compared the results of typing ESBL-producing *K. pneumoniae* strains by a combination of phenotypic and genomic markers. Various antibiotic resistance patterns were observed, including resistance to aminoglycosides and fluoroquinolones. No modification in the resistance pattern was noted for a given clone, as defined by the combined typing results, during the course of infection and transmission. Serotyping of capsular antigen displayed six serotypes. Although this classical method is valued for its reproducibility and stability, its usefulness was limited in some studies by a high proportion of nontypeable strains (23). In our experience, this method correlated poorly with molecular typing techniques; isolates concordantly grouped in the same clone by all genomic methods displayed as many as three distinct serotypes, indicating antigenic instability.

Plasmid profile analysis has been widely used to differentiate *K. pneumoniae* strains in epidemiological studies. This method is of limited value because genetically unrelated strains may harbor the same plasmids (2, 4) and related strains may lose unstable plasmids (22, 23). In our study, all ESBL-producing *K. pneumoniae* isolates tested had detectable plasmids, allowing their classification into three different plasmid profiles that were in agreement with clusters defined by chromosomal fingerprints. Other workers also found good agreement between plasmid profile and RAPD patterns of ESBL-producing *K. pneumoniae* (9). Nevertheless, the epidemiology of nosocomial outbreaks can be more complex when the spread of ESBL-encoding plasmid occurs concomitantly with patient-to-patient transmission of epidemic strains (4).

Macrorestriction polymorphisms resolved by PFGE clearly delineated the genomic relatedness of ESBL-producing strains of *K. pneumoniae*, as previously reported for this species (1, 11, 22). PFGE has been shown to be more discriminatory than other genotypic techniques for typing *Klebsiella* species and other bacteria (11, 15, 22, 24). PFGE analysis can detect chromosomal rearrangements, even within closely related lineages. The present study confirms the usefulness of this technique. *K. pneumoniae* strains clustered into highly related groups, which correlated well with epidemiological data, and further revealed subtype variants displaying one to two fragment differences. *K. pneumoniae* clones recovered during the course of the small outbreaks examined here exhibited minor genomic differences, as was found in studies of other bacterial species (15, 24). This variability may reflect the genetic flexibility of bacterial pathogens in the hospital environment. On the other hand, PFGE requires specialized equipment, costly reagents, and long DNA preparation and electrophoresis times.

RAPD fingerprinting is another powerful typing method (26) which has been used for studying the molecular epidemiology of ESBL-producing *K. pneumoniae* strains in a previous investigation (9) but had not previously been compared with other high-resolution methods of genomic typing. The discriminatory power of RAPD was good in the present study, since all epidemiologically unrelated strains that were found markedly different by PFGE also exhibited distinct RAPD types. Reproducible patterns were obtained in independent experiments (data not shown). RAPD and PFGE data showed complete agreement in delineating closely related from less related isolates, thereby further validating the reliability of PCR fingerprinting. However, PFGE with XhoI appeared more discriminatory than the combined PCR fingerprints obtained with three primers, since it identified more subclonal variants than did RAPD analysis. Similar observations were made with other gram-negative pathogens (15, 16, 24). For example, typing of *Legionella pneumophila* by RAPD analysis with two 22-mer primers showed good agreement with other genomic markers while displaying a lower level of discrimination than PFGE of genomic DNA digested with three infrequently cleaving endonucleases (24). Likewise, RAPD analysis with two primers showed a lower level of discrimination than macrorestriction by two endonucleases for subtyping *Pseudomonas aeruginosa*.
Strains from cystic fibrosis patients but provided complete agreement with the latter method in delineating clonal groups of strains (15).

The discriminatory ability of PFGE typing depends on the number and type of restriction enzymes used, whereas that of RAPD analysis is related to the number and type of primers used, as well as PCR cycling parameters (15, 16, 24, 26). Thus, extensive evaluation and selection of optimally discriminating primer sets and PCR conditions can lead to RAPD schemes that exhibit resolution superior to that of PFGE analysis, as shown by typing *Salmonella enteritidis* (16). Discrepancies between the results of RAPD and PFGE typing might be explained by the fact that these methods explore different levels of DNA polymorphism. PFGE is based on restriction fragment length polymorphism and can resolve the whole chromosomal DNA. In contrast, RAPD probes local sequence polymorphisms associated with repeat motifs occurring within genomic DNA and also detects short-range length polymorphism of the amplified segments.

In theory, plasmid DNA in whole-cell lysates may contribute to particular RAPD patterns. Elaichouni et al. found no influence of plasmid DNA on the RAPD results in *E. coli* and concluded that the excess amount of chromosomal DNA is sufficient to inhibit observable amplification of plasmid DNA (10). In our study, the correlations between plasmid profile and PFGE patterns of *K. pneumoniae* isolates were 100, 90, and 75%, respectively, when typed with primer AP4, primer HLW74, and primer R108, thereby suggesting a possible contribution of plasmid DNA to the PFGE patterns. If this were true, the inferior discriminatory power of RAPD compared with that of PFGE could be related to amplification of plasmid rather than chromosomal target DNA. Therefore, additional studies should be performed with epidemiologically unrelated isolates lacking ESBL-encoding plasmids before firm conclusions can be drawn regarding the superior discrimination of PFGE for typing *K. pneumoniae*.

Compared with PFGE, RAPD analysis is significantly simpler to perform and produces results more rapidly. Typing was done directly on genomic DNA released by a rapid heat lysis of bacterial cells, without the need for any additional DNA extraction or purification procedure. Therefore, RAPD typing is more suitable for first-pass screening of related isolates during investigation of outbreaks. Nevertheless, interassay reproducibility may be a problem with RAPD typing because of minor variation in PCR reagents or cycling parameters (15). This was not a problem in the present study, in which all isolates could be processed within single assays.

In conclusion, although nosocomial acquisition of ESBL-producing *K. pneumoniae* was uncommon in this hospital population, it was associated with significant morbidity in ICU patients. In agreement with epidemiological data, the results of PFGE and PCR typing indicated that several clones of ESBL-producing *K. pneumoniae* were associated with either sporadic infection or cross-infection. Both PFGE and RAPD analysis appeared to be useful and discriminatory techniques for typing ESBL-producing *K. pneumoniae* as a complement to antimicrobial susceptibility profiles. Although PFGE was more discriminating, RAPD provided more efficient screening of clonally related isolates. We recommend the use of RAPD analysis as the initial method for delineating groups of closely related isolates and the use of PFGE typing for confirmation.

Acknowledgments

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