Molecular biology of extended-spectrum \( \beta \)-lactamase-producing Enterobacteriaceae responsible for digestive tract colonization

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Extended spectrum \( \beta \)-lactamase; Digestive tract colonization

Summary Twenty-nine extended-spectrum \( \beta \)-lactamase (ESBL)-producing Enterobacteriaceae strains (14 *Klebsiella pneumoniae*, 10 *Escherichia coli* and five *Citrobacter diversus*) isolated from April to July 1996 from faecal carriers in a surgical intensive care unit at the university hospital of Casablanca (Morocco) were studied. Plasmid content and DNA macrorestriction polymorphism determined by pulsed-field gel electrophoresis (PFGE) were used to compare the strains. Restriction profiles of total genomic DNAs cleaved by \textit{XbaI} and compared by PFGE revealed nine, four and two clones in *K. pneumoniae*, *E. coli* and *C. diversus*, respectively. Plasmid profile analysis of ESBL-producing strains of *K. pneumoniae* showed that only seven of 14 isolates had a plasmid; four different plasmid profiles were observed. Three different plasmid profiles were observed in *E. coli* and two in *C. diversus*. Plasmids responsible for ESBL production could be transferred by conjugation to *E. coli* K\textsubscript{12} J53-2 from all *E. coli* isolates and from four of seven *K. pneumoniae* strains. No plasmid transfer could be obtained from *C. diversus* strains. Restriction enzyme digests of plasmids from transconjugants (four transconjugants of *K. pneumoniae* and five transconjugants of *E. coli*) showed different patterns. In the surgical intensive care unit where the survey was conducted, the dissemination of ESBLs was due to a mix of strain spread and strain diversity rather than to plasmid dissemination.

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**Introduction**
Extended-spectrum \( \beta \)-lactamase-producing Enterobacteriaceae (ESBLPE) are important agents of...
nosocomial infections and are responsible for out-
breaks, which occur mainly in intensive care
units.1–3 The dissemination of ESBL is due to clonal
spread,2,4,5 to plasmid dissemination among6,7 or
between species,8 or to both mechanisms.3,9–11

Genes encoding for ESBL may also be found on
transposons.1,11

Digestive tract colonization is a prerequisite for
infection by ESBLPE.2,12 In Casablanca a prospec-
tive study conducted in 1996 in an intensive care unit
of the IbnRochd University Hospital13 revealed that
digestive tract colonization with ESBLPE was fre-
cquent, occurred shortly after admission to the unit,
and was transient. Bacteria responsible for this
colonization belonged to five species; Klebsiella
pneumoniae was the most frequent (42.4%) fol-
lowed by Escherichia coli (30.3%) and Citrobacter
diversus (15.2%).

To analyse the mode of spread of ESBLs (plasmid
and/or clone spread) in the intensive care unit, we
studied the ESBLPE isolates by macrorestriction
polymorphism of DNA determined by pulsed-field
gel electrophoresis (PFGE) and plasmid DNA
analysis.

Materials and methods

Bacterial strains

The most prevalent species of the 33 ESBLPE
isolated in the colonization survey were studied:
K. pneumoniae (N = 14), E. coli (N = 10) and
C. diversus (N = 5). Three of the 10 E. coli
isolates were isolated sequentially from the same patient.
Four patients were simultaneously colonized by two
species: K. pneumoniae + E. coli (two patients),
K. pneumoniae + Klebsiella oxytoca (one patient),
K. pneumoniae + C. diversus (one patient).

Genomic fingerprinting by PFGE

Agarose plugs containing chromosomal DNA were
prepared by a method similar to that described by
Yuan et al.5 The chromosomal DNA was digested
overnight with 30 U of XbaI (Boehringer, Mannheim,
Germany). The PFGE was run in a CHEF-DRIII
apparatus (Bio-Rad, Richmond, CA, USA) at
6 V/cm, 13.5 °C during 26 h for E. coli and 28 h for
K. pneumoniae and C. diversus. The pulse times
were 5–40 s. The banding patterns were analysed
with the Biogene software (Vilbert-Lourmat,
Marne-la-Vallée, France) and were interpreted
according to the criteria of Tenover et al.14

The PFGE experiments were performed by one of the authors (NM) at the microbiology laboratory
of the Ramon y Cajal Hospital of Madrid, Spain
(Dr F. Baquero).

Plasmid isolation and resistance transfer

Plasmids were extracted by the method of Kado and
Liu,15 the alkaline lysis method as described by
Sambrook et al.16 and by the High Pure Plasmid
Isolation Kit (Boehringer Mannheim, Germany). Transfer of plasmids by conjugation was performed
as described previously.17 A rifampicin-resistant
strain of E. coli K12 J53-2, was used as recipient.
Transconjugants were selected on MacConkey agar
containing rifampicin (250 mg/L) and amoxicillin
(100 mg/L).

Restriction endonuclease analysis

Plasmid DNA from the transconjugants was digested
with EcoRI (Sigma Bio-Sciences, St Louis, MO, USA)
and HindIII (Roche Molecular Biochemicals, Mann-
heim, Germany) according to the manufacturer’s
recommendations.

Antibiotic susceptibility testing

The antibiotic susceptibilities were determined by
disk diffusion on Mueller-Hinton agar. The following
agents were tested: gentamicin, tobramycin, netil-
micin, amikacin, trimethoprim-sulfamethoxazole,
tetracycline, chloramphenicol and imipenem. The
minimal inhibitory concentrations (MICs) of cefo-
taxime and ceftazidime alone and combined with
clavulanic acid (4 mg/L) were determined by agar
dilution with an inoculum of 10^6 cfu/spot.18 E. coli
ATCC 25922 and E. coli ATCC 35218 were used as
controls.

Results

Macrorestriction polymorphism of DNA determined
by PFGE distinguished nine clones among the 14
K. pneumoniae strains (Figure 1). The 10 E. coli and
five C. diversus isolates divided into four and two
clones, respectively (Table I). In each of the three
species, some pulsotypes were detected only once
whereas others were shared by two or three
patients.

For each of the three species, patients carrying
the same clone were analysed in an attempt to
determine the links between them. Cases carrying a
given clone were either imported from another
ward or due to the presence in the ICU of a patient

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already colonized. In some instances no link could be established between cases with the same clone.

During the study five new clones were introduced in the ICU by patients transferred from three different hospital wards. Three clones detected in patients on admission to the unit were already present in it.

Plasmid DNA analysis of ESBL producing isolates of *K. pneumoniae* showed that only seven of 14 isolates had a plasmid and four plasmid profiles were found. Three clones (C, F, I) had the same plasmid profile, but the restriction enzyme studies performed in clones C and F revealed different patterns. Three different plasmid profiles were observed in *E. coli* and two in *C. diversus*. Three clones of *E. coli* (J, K, and M) shared a common plasmid pattern, but the enzyme restriction profiles were different (Table I).

Plasmid transfer by conjugation to *E. coli* K12 J53-2 occurred with all the *E. coli* isolates. This transfer was observed for only four of seven *K. pneumoniae* isolates. Plasmid restriction analysis from the transconjugants obtained with four *K. pneumoniae* and five *E. coli* strains showed three and five patterns, respectively (Figure 2). No plasmids could be transferred from the *C. diversus* isolates.

Different antibiotic resistance profiles were noted in isolates belonging to the same pulsotype (pulsotype L of *E. coli* and pulsotype A of *K. pneumoniae*). The two isolates of *E. coli* pulsotype L had a different plasmid restriction pattern.

Among the 29 ESBLPE isolates, 10 phenotypes of resistance were detected. Most isolates, except one of *C. diversus*, were multiresistant (Table I). The antibiotics most frequently involved in resistance were gentamicin, tobramycin, cotrimoxazole, tetracycline and chloramphenicol. The MICs of ceftazidime were frequently higher than those of cefotaxime suggesting a ceftazidimase activity of the ESBLs.19 Clavulanic acid at a concentration of 4 mg/L substantially reduced the MICs of both ceftazidime and cefotaxime (Table I), indicating the presence of an ESBL.

**Discussion**

Molecular biology methods have improved the understanding of ESBL epidemiology.20–22 Specific identification of the enzyme subtype has revealed the considerable diversity of ESBLs,21 their spread between bacterial species,20,21 the coexistence and turnover of ESBLs in the same centre20 and their variable distribution according to geographic area.20,22 Precise identification of ESBL variant is very challenging21,22 as the reference method is nucleotide sequencing.21 For the investigation of strain relatedness, PFGE is usually used.2,5,7,12,24

The analysis of ESBLPE responsible for digestive tract colonization by DNA macrorestriction polymorphism determined by PFGE and plasmid analysis
<table>
<thead>
<tr>
<th>Species</th>
<th>Number of isolates</th>
<th>PFGE type</th>
<th>Plasmid profile</th>
<th>Plasmid digestion pattern</th>
<th>MIC (mg/L) phenotype of resistance</th>
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CTX, cefotaxime; CTX-CLA, cefotaxime + clavulanic acid; CAZ, ceftazidime; CAZ-CLA, ceftazidime + clavulanic acid; Ge, gentamicin; To, tobramycin; Ne, netilmicin; Ak, amikacin; Ts, trimethoprim-sulfamethoxazole; Ch, chloramphenicol; Te, tetracycline; NPD, no plasmid detected; ND, not determined.
allowed the study of the mode of spread of the ESBLs in an intensive care unit during the four-month study, in which seven of 29 isolates had no detectable plasmid and eight isolates failed to transfer their plasmids by conjugation. Although only a limited number of plasmids could be analyzed, the prevalent mechanism of ESBL dissemination appeared to be the existence of multiple clones and the limited spread of a few clones rather than a plasmid spread as shown by the different plasmid digestion profiles observed for each species. Outbreaks caused by ESBPLE have been reported as due to the dissemination of a single strain,2 to horizontal transfer of plasmid7 or to concurrent dissemination of plasmids and strains.9,11

The frequencies, origin and the temporal distribution of the observed clones were studied. The high number of clones suggests that ESBL production has become endemic rather than epidemic. For each species studied some clones were observed only once whereas a few were isolated from two or three patients. This finding may be explained by differences in isolation procedures among colonized patients and by the fact that the colonization was transient.13 Similar studies of ESBPLE responsible for digestive tract colonization revealed different results depending on the epidemiology in the unit. During an outbreak the same strain was responsible for most infections and for digestive tract colonization.12 After an outbreak of K. oxytoca infections in an intensive care unit,11 a screening programme of gastrointestinal colonization identified by PFGE a predominant clone and 15 unrelated isolates. Cukier et al.23 reported that the ESBL-producing E. coli strain responsible for an outbreak of urinary tract infections in a geriatric ward had a unique ribotype, whereas after the outbreak ESBL-producing E. coli strains responsible for colonization belonged to four different ribotypes.

The number of clones detected in this study was higher in K. pneumoniae (nine clones in 14 isolates) than in E. coli (four clones in 10 isolates) and in C. diversus (two clones in five isolates). This finding confirms that the spread of ESBL production in K. pneumoniae is due to multiple clones rather than to the spread of a single epidemic clone. A multicentre study of K. pneumoniae with ESBLs conducted in European intensive care units5 reported 85 distinct strains in 220 isolates. Major variations in antibiotic resistance and plasmid patterns were noticed within strains, with some intra-strain variations in β-lactamase subtype.5 This result is also supported by the important variety of plasmids and types of ESBL reported in K. pneumoniae.5,6,24,25

This circulation of multiple clones at one time was found by Bingen et al.,9 Nouvellon et al.10 and Essack et al.24 who reported on multiple strains prevalent simultaneously in single ICUs.

Colonization by ESBLE could be due to clones already present in the unit, or to imported clones from other hospital wards. ESBL production may have appeared de novo in the unit, although their pre-existence cannot be ruled out as screening for ESBLE was not performed in the patients who were present at the beginning of the survey. Five new clones were introduced in the ICU by patients transferred from three separate wards, demonstrating the spread of ESBLE in our hospital. This finding confirms the observations that the problem of ESBLE is usually of hospital-wide importance19 and that spread occurs through transfer of patients between wards.26 These results support the recommendations for screening of patients from at risk areas on admission26 and documenting the carriage of multi-resistant bacteria in case of transfer of patients between wards.23

Temporal analysis of cases due to the same clone was used to identify the link between patients. In some cases no link could be found between the cases raising the question of persistence of the agents within the unit. The sensitivity of the method used for the detection of the ESBLE may have been insufficient and may explain the failure to detect a colonized patient who could have served as a reservoir. Although the environment is not considered a major reservoir for ESBLE, one outbreak strain was isolated from equipment.3 Transmission from asymptomatic colonized staff is unlikely.26

Isolates belonging to the same clone may have a different plasmid digestion profile (e.g. clone L of E. coli), which can be explained by the possible existence of transposons or integrons or an unstable plasmid that may change easily.27 Conversely, isolates of the same clone may have different antibiotic resistance phenotypes,10 which may be due to gain, or loss of plasmids or fragments of plasmids.5 This finding confirms that the antibiotic resistance profile is not a valuable marker.

In this study, despite using three methods of extraction, plasmids could be detected in only seven of 14 K. pneumoniae strains, and of these, only four transferred their plasmids by conjugation. In these isolates, the conjugative plasmid was responsible for ESBL production. The absence of plasmid transfer by conjugation has been reported.6,19,27 although in other studies all isolates tested had detectable plasmids5,26 or could transfer their plasmids.9,25

The absence of plasmids in
ESBLPE may be explained by the fact that genes coding for ESBL may be on transposons.11 Transfer of plasmids responsible for ESBL production between two species present simultaneously in the same patient is one mode of spread of ESBLs.8,11 In the four patients who harboured two ESBLPE simultaneously this transfer could not be demonstrated because, in each case, one of the species did not have a detectable plasmid or did not transfer its plasmid.

Macrorestriction polymorphism of DNA determined by PFGE and plasmid analysis are useful tools for the study of ESBLPE responsible for digestive tract colonization in a hospital unit. The mix of strain diversity and strain dissemination, the existence of multiple different plasmids, the importation of ESBLPE from different wards of the hospital and antibiotic multi-resistance all confirm the complexity of the epidemiology of ESBLPE.5,9,24 Consideration of these factors is important in making recommendations for prevention of spread of ESBLPE. Current recommendations include screening on admission to the Unit,26 improvement of infection control measures22 and rational use of antibiotics.22

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