Molecular epidemiology of an outbreak due to IRT-2 β-lactamase-producing strains of *Klebsiella pneumoniae* in a geriatric department

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In February 1998, 195 patients in the geriatric department of a French hospital were screened for the presence of co-amoxiclav-resistant *Klebsiella pneumoniae*. Eleven co-amoxiclav-resistant isolates obtained all produced an identical IRT-2 β-lactamase. These *K. pneumoniae* isolates were clonally related and harboured a c. 55 kb non-conjugative plasmid encoding a non-class-1 integron-located *bla*\(_{\text{IRT-2}}\) gene. This study underlines that geriatric departments may be a reservoir for antibiotic-resistant strains and that IRT β-lactamase-producing strains may be nosocomial pathogens.

**Introduction**

*Klebsiella pneumoniae* is naturally resistant to amoxicillin and clavulanic acid. Co-amoxiclav resistance in *K. pneumoniae* may result from overproduction of either SHV-1 or plasmid-encoded TEM-1/TEM-2 β-lactamase or biosynthesis of a clavulanic acid-resistant β-lactamase. 2–8

Although clavulanic acid-resistant oxacillinases have been reported in Enterobacteriaceae (mainly *Escherichia coli*), \(^9\,10\) only three *K. pneumoniae* strains have been shown to date to produce inhibitor-resistant TEM (IRT) β-lactamase derived from TEM-1. \(^2\,6\)

In our French university hospital, several co-amoxiclav-resistant *K. pneumoniae* isolates were obtained from the same geriatric department. The first aim of the study was to investigate the molecular mechanisms involved in this β-lactam resistance phenotype. Additionally, an epidemiological study was conducted to evaluate the prevalence of such *K. pneumoniae* strains in this unit, since there is substantial concern about the possible role of geriatric departments as a reservoir for antibiotic-resistant strains. \(^12\)

**Material and methods**

**Bacterial isolates**

Rectal swabs and urine specimens were collected in February 1998 from 195 patients hospitalized in the geriatric department of Bicêtre Hospital, located in a suburb of Paris. Non-repetitive specimens (one per patient) were inoculated onto Drigalski agar plates containing 0.1 mg/L amoxicillin. All isolates were identified using the API 20E test (bioMérieux SA, Marcy l’Etoile, France). Co-amoxiclav-resistant *K. pneumoniae* was detected using a disc diffusion assay. Electrocompetent *E. coli* DH10B (GIBCO BRL, Life Technologies, Cergy Pontoise, France) was used as recipient in transformation experiments. Laboratory-obtained rifampicin-resistant *E. coli* C600 was used as host in conjugation experiments. *E. coli* NCTC 50192 harbouring 154, 66, 38 and 7 kb plasmids was used as a plasmid-containing reference strain. \(^13\)

**Susceptibility testing**

The susceptibility of *K. pneumoniae* isolates and their *E. coli* electroporants was initially determined by the disc diffusion method on Mueller–Hinton agar (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France). The
following antibiotics were tested: amikacin, amoxycillin, cefamandole, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, co-amoxiclav, gentamicin, imipenem, kanamycin, nalidixic acid, pefloxacin, piperacillin, piperacillin/tazobactam, spectinomycin, streptomycin, sulphonamides, tetracycline, ticarcillin, tobramycin and trimethoprim/sulphamethoxazole.

MICs were subsequently determined for selected β-lactams by an agar dilution technique on Mueller–Hinton agar with an inoculum of 10⁴ cfu/spot. All plates were incubated at 37°C for 18 h. MICs of β-lactams were determined alone or in combination with a fixed concentration of either clavulanic acid (2 mg/L) or tazobactam (4 mg/L). MICs were determined for the following β-lactams: amoxycillin and ticarcillin (SmithKline-Beecham, Nanterre, France); cefamandole and cephalothin (Eli Lilly, Saint-Cloud, France); piperacillin and tazobactam (Lederle, Oullins, France); cefotaxime (Hoechst-Roussel, Paris, France); cefoxitin and imipenem (Merck Sharp & Dohme-Chibret, Paris, France). MIC results were interpreted according to NCCLS guidelines.14

### Plasmid content, mating-out assays and electroporation

Plasmid DNA of *K. pneumoniae* isolates was extracted using the Plasmid Midi kit (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions. Plasmid DNA was analysed by electrophoresis on 0.8% agarose gels containing 0.5 mg/L ethidium bromide, using plasmids from *E. coli* 5019213 and the Kilobase DNA Marker (Pharmacia Biotech, Orsay, France) as molecular weight markers. The extracted plasmids were electroporated into *E. coli DH10B* (Gene Pulser II, BioRad, Ivy/Seine, France) and recombinant strains were selected on amoxycillin-containing (100 mg/L) trypticase soy (TS) agar plates. Plasmids were extracted from these electroporants as described above, digested with the restriction enzyme *Sac*I and analysed by electrophoresis on a 1.5% agarose gel.

Direct transfer of the amoxycillin resistance marker from *K. pneumoniae* isolates into rifampicin-resistant *E. coli* C600 strain was attempted using liquid and solid mating-out assays at 37°C.15,16 Transconjugants were selected on to TS agar plates containing rifampicin (500 mg/L, Sigma, Saint-Quentin Falavier, France) and amoxycillin (100 mg/L).

### Isoelectric focusing and β-lactamase assays

Cultures of co-amoxiclav-resistant *K. pneumoniae* isolates and the *E. coli* electroporants were grown overnight at 37°C in 10 mL of TS broth containing amoxycillin (100 mg/L). Then, 1 mL of each overnight culture was further grown for 3 h at 37°C in 10 mL of TS broth with amoxycillin. After centrifugation (5 min, 4°C, 5000 g), bacterial pellets were resuspended in 200 μL of 50 mM sodium phosphate buffer (pH 7.0). The bacterial cells were disrupted by sonication at 20 Hz for two 30 s periods (Phospholysar Vibra Cell 300, Bioblock, Illkirch, France) and centrifuged for 1 h at 48 000 g and 4°C. The residual nucleic acids in the supernatant were precipitated by 7% of spermine 0.2 M (Sigma) for 2 h at 4°C. This suspension was ultracentrifuged at 100 000 g for 1 h at 4°C. The supernatants containing the enzyme extracts were subjected to analytical isoelectric focusing (IEF) using a mini IEF 111 (BioRad) with a polyacrylamide gel containing a gradient made up of polyampholites with a pH range of 3–10 (BioRad). Migration was performed at three consecutive voltages (100 V for 15 min, 200 V for 15 min and 450 V for 1 h). The focused β-lactamas were detected by overlaying the gel with 1 mM nitrocefin (Oxoid, Paris, France). The pI values were determined by comparison with those of known β-lactamas (PER-1, pI 5.3; TEM-1, pI 5.4; TEM-2, pI 5.6; SHV-3, pI 7; SHV-2, pI 7.6) and with IEF Standards (BioRad). The 50% inhibitory concentrations (IC₅₀) for clavulanic acid, sulbactam and tazobactam were calculated as described17 for the β-lactamas extracted from *E. coli* electroporants. The *E. coli* β-lactamase extracts were preincubated for 3 min at 30°C with different concentrations of clavulanic acid, tazobactam or sulbactam ranging from 0 to 100 μM and the assay was initiated by the rapid addition of benzylpenicillin at a final concentration of 100 μM. Hydrolysis of this substrate was measured at 232 nm.

β-Lactamase-specific activity from cultures of the *E. coli* electroporants and of *E. coli DH10B* harbouring the plasmid pBR322 which encodes TEM-118 was measured by monitoring the rate of hydrolysis of benzylpenicillin. One unit of enzyme activity was defined as the activity that hydrolysed 1 μmol of benzylpenicillin per min per mg of protein. The total protein content was measured using the BioRad DC Protein assay kit (BioRad).

### PCR for detection of blaTEM derivatives and class 1 integrons, and sequencing

For each reaction 0.5 μg of plasmid DNA from the *E. coli* electroporants was used. The PCR amplification for detection of *blaTEM* derivatives used laboratory-designed primers TEM-1A (5’-GTATGGATCCTCAAACATTTCCGTTGC-3’) and TEM-1B (5’-ACCAAGCATTAA-TCAGTGGAGCA-3’); for detection of class 1 integrons primers 5’CS (5’-GCGATCAAAGCGAAG-3’) and 3’CS (5’-AAGCAGACTTGACCTGA-3’) were used.19 The 5’CS and 3’CS primers were complementary to nucleotides located in the 5’ conserved region of the integrase gene and the 3’ conserved region of the disinfectant-resistance gene qacEΔ1, respectively. The PCR fragments obtained were purified using Qiaquick spin columns (Qia-gen) and sequenced on both strands, using an Applied Biosystem, sequencer (ABI 373; Cybergene, Evry, France).
Random amplified polymorphic DNA (RAPD)

RAPD was performed as described by Williams et al. with some modifications. The PCR mixture comprised buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM MgCl₂, 0.001% gelatin), four deoxynucleotide triphosphates each at a concentration of 400 μM (Boehringer-Mannheim, Meylan, France), 150 pmol of primer, about 1 μg of genomic DNA and 2 U of Taq DNA polymerase (Perkin Elmer Cetus) in a total volume of 50 μL. The primers ERIC-2 (5'-AAGTAAAGTGACTGGGTGAGCG-3') and A3 (5'-AGTCAGCCAC-3') were used. Each sample was subjected to the first cycle of amplification (4 min at 94°C, 1 min at 36°C and 2 min at 72°C) in a DNA thermal cycler 9600 (Perkin Elmer Cetus). Each of the 35 subsequent cycles consisted of denaturing at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 3 min (for the last cycle, extension at 72°C was increased to 10 min). Amplified products were separated by electrophoresis in a 1.5% agarose gel and visualized by UV transillumination following ethidium bromide staining. A kilobase DNA ladder (Pharmacia Biotech) was used as molecular size standard. The fingerprints were compared visually and patterns were considered different when they differed by at least one amplification band, regardless of band intensity.

Pulsed-field gel electrophoresis (PFGE)

Plug preparation was done according to BioRad instructions. Genomic DNA from K. pneumoniae isolates was digested with XbaI at 37°C overnight. Electrophoresis through a 1% agarose gel in 0.5× TBE (Tris-Borate-EDTA) buffer was performed using a CHEF DRII apparatus (BioRad). Migration conditions were 14°C, 6 V/cm, 120° switch angle and run time of 20 h with a linear switch ramp of 2–30 s. The ethidium bromide-stained gel was photographed (Polaroid under UV illumination). Lambda DNA ladder (BioRad) was used as DNA molecular weight marker. The chromosomal fingerprints were compared by eye and assigned to PFGE types and subtypes.

Hybridizations

DNA–DNA hybridizations were performed as described by Sambrook et al. with an agarose gel containing plasmid DNA from E. coli electroporants. The probe consisted of a labelled 720 bp PCR fragment generated from recombinant plasmid pBR322 encoding blaTEM-1. Labelling of the probe and signal detection were carried out using a non-radioactive labelling and detection kit according to the manufacturer’s instructions (Amersham, Les Ulis, France). PFGE gels containing XbaI-restricted DNA from K. pneumoniae isolates were also submitted to hybridization with a blaTEM-1 probe.

Results and discussion

Epidemiological data

A total of 31 K. pneumoniae isolates (six urinary specimens, 24 rectal swab specimens and one foot biopsy specimen) were collected from 195 hospitalized patients in the geriatric department of Bicêtre Hospital in February 1998. The patients were not submitted to any restriction in making social contact whenever possible. Eleven of the isolates appeared resistant to co-amoxiclav. All the patients from whom these isolates were obtained were previously treated with co-amoxiclav. Co-amoxiclav-resistant K. pneumoniae did not cause infection in any other unit of the hospital during this time, but rectal swab screening was not performed in these units.

The 11 isolates were from separate patients and were from the following sites: four from rectal swabs, six from urinary tract infections and one from a foot biopsy.

Phenotypic analysis and antimicrobial susceptibility

Disc diffusion susceptibility assays indicated that the 11 co-amoxiclav-resistant K. pneumoniae isolates had the same β-lactam resistance profile, characterized by resistance to amoxycillin, ticarcillin and piperacillin, reduced susceptibility to co-amoxiclav and ticarcillin/clavulanic acid, and susceptibility to cephalothin. This resistance profile was confirmed by determination of MICs (Table). These isolates were also resistant to chloramphenicol, nalidixic acid, spectinomycin, streptomycin, sulphonamides, tetracycline, trimethoprim and trimethoprim/sulphamethoxazole and were of intermediate susceptibility to pefloxacin. Six out of the 11 isolates exhibited additional resistance to kanamycin (K. pneumoniae strains 5, 7–11).

Conjugation, electroporation and β-lactamase levels

Conjugation experiments to transfer co-amoxiclav resistance were repeated four times using both liquid and solid media were unsuccessful. However, E. coli electroporants with reduced susceptibility to co-amoxiclav were obtained from all 11 K. pneumoniae isolates (Table). Evaluation of β-lactamase activity was carried out with the E. coli electroporants. The electroporants had low activity compared with strains overproducing β-lactamase or with E. coli DH10B expressing TEM-1 from pBR322 (a 65-fold lower activity; 370 and 24,050 mU for E. coli electroporants and E. coli harbouring pBR322, respectively). Thus, β-lactamase overproduction did not account for the observed resistance phenotype.

Identification of IRT-2 β-lactamase

IEF analysis showed that the 11 K. pneumoniae isolates produced two β-lactamases with pIs of 7.6 and 5.2 (data not shown); the pI 7.6 β-lactamase probably corresponds to an
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Table. MIC_{90} of $\beta$-lactams for 11 *K. pneumoniae* clinical isolates, their electroporants in *E. coli DH10B* and the reference strain *E. coli DH10B*

<table>
<thead>
<tr>
<th>$\beta$-Lactams</th>
<th><em>K. pneumoniae</em> Kp 1–11</th>
<th><em>E. coli</em> electroporants Tr 1–11</th>
<th><em>E. coli</em> DH10B</th>
</tr>
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<tbody>
<tr>
<td>Amoxycillin</td>
<td>&gt;512</td>
<td>512</td>
<td>8</td>
</tr>
<tr>
<td>Amoxycillin/clavulanate$^a$</td>
<td>&gt;512</td>
<td>128</td>
<td>2</td>
</tr>
<tr>
<td>Amoxycillin/tazobactam$^b$</td>
<td>&gt;512</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>&gt;512</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Ticarcillin/clavulanate</td>
<td>256</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Ticarcillin/tazobactam</td>
<td>256–512</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>256–512</td>
<td>8</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Piperacillin/clavulanate</td>
<td>16–32</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>16–32</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>4–8</td>
<td>4–8</td>
<td>4</td>
</tr>
<tr>
<td>Cephalothin/clavulanate</td>
<td>4–8</td>
<td>4–8</td>
<td>4</td>
</tr>
<tr>
<td>Cephalothin/tazobactam</td>
<td>4–8</td>
<td>4–8</td>
<td>4</td>
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<tr>
<td>Cefamandole</td>
<td>1–4</td>
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<td>Cefamandole/clavulanate</td>
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<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefamandole/tazobactam</td>
<td>1–2</td>
<td>0.25–1</td>
<td>0.5</td>
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<tr>
<td>Cefoxitin</td>
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<td>4</td>
<td>4</td>
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<td>0.06</td>
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</tr>
<tr>
<td>Imipenem</td>
<td>0.12–0.25</td>
<td>0.12</td>
<td>0.12</td>
</tr>
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</table>

$^a$Clavulanate at fixed concentration of 2 mg/L.

$^b$Tazobactam at fixed concentration of 4 mg/L.

SHV-1-like $\beta$-lactamase. *E. coli* electroporants from *K. pneumoniae* produced a similar $\beta$-lactamase with a pI of 5.2. Inhibition studies performed with $\beta$-lactamase extracts from all *E. coli* electroporants indicated that the $\beta$-lactamase activity had high IC_{50} values for clavulanate (6 $\mu$M), tazobactam (0.78 $\mu$M) and sulbactam (65 $\mu$M) compared with TEM-1, which had IC_{50} values of 0.13, 0.07 and 10 $\mu$M for the same inhibitors, respectively. This result raised the possibility that production of an IRT $\beta$-lactamase or an oxacillinase might explain the $\beta$-lactam resistance phenotype observed in *K. pneumoniae*.

Using bla_{TEM-1}-specific primers, a 720 bp PCR fragment was obtained from plasmid extracts of all *E. coli* electroporants. Direct sequencing of the PCR products revealed 100% identity with bla_{IRT-2}. This gene is identical to the bla_{TEM-1} gene, differing only by a point mutation leading to the amino acid change Arg to Ser at Ambler position 244, producing IRT-2 (TEM-30). This $\beta$-lactamase was previously found in another *K. pneumoniae* isolate and is encountered among IRT $\beta$-lactamase-producing *E. coli* strains.

**Random PCR, PFGE and hybridization**

Analysis of the 11 *K. pneumoniae* isolates by random PCR gave identical electrophoresis patterns with the primer ERIC-2. Only one strain (*K. pneumoniae* 9) gave a distinguishable pattern by simultaneous amplification with B3 and 628 primers (data not shown).

PFGE analysis of XbaI-restricted DNA of the *K. pneumoniae* isolates revealed that they were identical or highly related (Figure 1). Only *K. pneumoniae* isolates 5 and 9 appeared distinct owing to the absence of two bands of 48.5 and 170 kb, the presence of an additional band of 200 kb and the replacement of a 400 kb band with a 380 kb band. As described by Tenover et al., patterns that differ by two or three fragment differences were considered to be subtypes of the outbreak pattern. Hybridization of these DNA digests with the internal probe for bla_{TEM-1} revealed a unique hybridizing fragment of c. 55 kb in size, thus indicating the presence of only one copy of a TEM-1/TEM-2-derivative gene in these *K. pneumoniae* isolates.

**Plasmid characterization and integron detection**

All *K. pneumoniae* isolates and their *E. coli* electroporants harboured a single large plasmid of c. 55 kb—apart from *K. pneumoniae* 5, which harboured an additional small plasmid (c. 3.5 kb). The co transferred resistance markers were chloramphenicol, sulphonamides, tetracycline and trimethoprim/sulphamethoxazole. Plasmid DNA from the *E. coli* electroporants digested with restriction endo-
IRT-2 in *K. pneumoniae*

Figure 1. PFGE of *XbaI*-restricted DNA from *K. pneumoniae* isolates. Lanes 1–11, *K. pneumoniae* strains 1–11; lane 12, unrelated *K. pneumoniae* strain. M corresponds to the Lambda DNA ladder used as a molecular weight marker (kb).

nuclease *SacI* gave one restriction profile (Figure 2). Hybridization with the internal probe for *bla*TEM-1 gave a positive result corresponding to an 8 kb fragment (Figure 2). *XbaI* restriction of the plasmid followed by its hybridization with the internal probe for *bla*TEM-1 gave a positive result with a 55 kb fragment similar in size to the fragment found after hybridization of *XbaI*-restricted PFGE fragments from *K. pneumoniae* strains. Interestingly, repeated electroporation of a plasmid preparation from a single *K. pneumoniae* isolate led to isolation of *E. coli* containing plasmids of varying size and structure but sharing common DNA fragments after restriction digestions (data not shown). Electroporation and/or plasmid extraction may favour breaking of large plasmid molecules, which may explain, for example, the isolation of similar but not identical plasmids from an extended-spectrum *β*-lactamase-producing *Enterobacter aerogenes* isolate from an outbreak.29

Using plasmid DNA from *E. coli* electroporants as templates, detection of class 1 integrons revealed integron-located streptomycin/spectinomycin and trimethoprim genes on the c. 55 kb plasmid (data not shown). However, sequencing of the integron-specific PCR products did not show the presence of the *bla*IRT-2 gene, therefore excluding its integron location. As is well known and as found in the present study, co-resistance in *K. pneumoniae* is commonly plasmid mediated.30 To the best of our knowledge, TEM-derived genes have not yet been identified as integron located. Only a few Ambler class A *β*-lactamases are known to possess integron-located genes such as the carbenicillinase PSE-1 (CARB-2)31 and the extended-spectrum *β*-lactamase VEB-1.32

Our study reports an outbreak of identical *K. pneumoniae* isolates harbouring a c. 55 kb plasmid encoding production of an IRT-β-lactamase. We believe this is the first reported outbreak involving a co-amoxiclav-resistant strain. Interestingly, all the patients from whom IRT-producing organisms were isolated had been treated with co-amoxiclav. This finding agrees with the *in vitro* selection of IRT-β-lactamases by co-amoxiclav.33 The clinical significance of isolation of IRT *β*-lactamase-producing strains remains unknown, since failure of co-amoxiclav treatment for curing systemic infections due to these strains has not yet been reported in the literature. Finally, this report underlines the role of geriatric departments as a reservoir for nosocomial antibiotic-resistant strains.3,34,35

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References


IRT-2 in *K. pneumoniae*


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