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# Molecular epidemiology of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* strains in a university hospital in Tunis, Tunisia, 1999–2005

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## Abstract

During a period of 6 years and 5 months (January 1999 to May 2005), 103 extended-spectrum $\beta$-lactamase (ESBL)-producing *Klebsiella pneumoniae* isolates, each from an individual patient or site, were collected at Mongi Slim University Hospital Centre, Tunis, Tunisia. The objectives of our work were the characterization of the *bla* genes encoding ESBLs, the investigation of clonal diversity of strains, and identification of the transmission modes of the resistance genes. We carried out detection by PCR and sequencing of the $bla_{SHV}$, $bla_{CTX-M}$ and $bla_{TEM}$ genes, transferability studies, plasmid replicon typing, and analysis by multilocus sequence typing (MLST) on selected isolates. Forty-seven isolates were found to be producers of CTX-M-type ESBLs, of which 43 were CTX-M-15, two CTX-M-14 and two CTX-M-27. Fifty-eight isolates were producers of SHV-12, and three were producers of SHV-2a. More than one ESBL was detected in seven isolates, as five produced both CTX-M-15 and SHV-12, and two produced both CTX-M-27 and SHV-12. By a PCR-based replicon typing method, the plasmids carrying the $bla_{SHV-2a}$ or $bla_{CTX-M-15}$ genes were assigned to IncFII or, more rarely, to IncL/M types. Of 12 plasmids carrying the $bla_{SHV-12}$ gene, only one could be typed: it was positive for the HI2 replicon. The MLST results showed large genetic background diversity in the SHV-12-producing isolates and dissemination of specific clones of the CTX-M-15-producing isolates within the same ward and among wards, and suggested endemicity with horizontal dissemination of the $bla_{CTX-M-15}$ and the $bla_{SHV-12}$ genes.

**Keywords:** CTX-M-14, CTX-M-15, CTX-M-27, IncFII, IncHI2, IncL/M, *Klebsiella pneumoniae*, MLST, replicon typing, SHV-12, SHV-2a
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## Introduction

Extended-spectrum $\beta$-lactamases (ESBLs) have increased dramatically in prevalence among clinical isolates of *Enterobacteriaceae* during these last three decades. These enzymes are the major source of resistance to penicillins and oxyimino-cephalosporins in *Enterobacteriaceae*. ESBLs are most often derivatives of the TEM or SHV families, although other unrelated enzyme groups, such as CTX-M, PER, GES, VEB and OXA, have also been described [1]. ESBLs are most commonly detected in *Klebsiella pneumoniae*, a cause of significant community-acquired and hospital-acquired infections [1,2]. 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 [3–5]. The detected prevalence of this multidrug-resistant strain in Tunisian hospitals varies from 10% to 32.4%, with a high incidence (87.5%) in paediatric intensive-care units [4,6,7]. ESBLs have also been found in other species of *Enterobacteriaceae* causing nosocomial outbreaks in Tunisia [8,9]. The SHV-2 enzyme was the first ESBL to be isolated, in 1984 from a *K. pneumoniae* strain in Tunisia [10]. Recently, SHV-12, SHV-2a, CTX-M-27, CTX-M-28, CTX-M-15 and CTX-M-16 have been described in

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Tunisian hospitals [3,6,8,9,11]. Moreover, ESBLs have also been reported in *Escherichia coli* strains of food origin in Tunisia, including CTX-M-1, CTX-M-8, CTX-M-14 and SHV-5 [12].

The aims of the present study were the characterization of the *bla* genes encoding ESBLs, the investigation of clonal diversity in the isolates and the identification of the transmission modes of the resistance genes among ESBL-producing *K. pneumoniae* isolates in Mongi Slim University Hospital Centre, in the northern part of Tunisia.

## Materials and Methods

### Hospital setting

Mongi Slim University Hospital Centre is a general hospital with 220 beds, situated in the northern region of Tunis, Tunisia. It comprises different wards: Paediatric (with a neonatal unit), Gynaecology–Obstetric, General Surgery, Intensive-care Unit, Cardiology, Internal Medicine, and Rheumatology–Acupuncture.

### Bacterial strains

In total, 1280 *K. pneumoniae* isolates (841 from inpatients and 439 from outpatients) were collected at the clinical laboratory of Mongi Slim University Hospital Centre between January 1999 and May 2005. Among these, 103 ESBL-producing *K. pneumoniae* isolates, each from an individual patient or site, were detected. These consisted of 101 clinical isolates and two isolates from the hospital environment (aspiration reservoir and bed). Three of the 101 clinical ESBL-producing *K. pneumoniae* isolates were obtained from three outpatients. The incidence of ESBL producers was 7.89% (101/1280) of all isolates and 11.65% (98/841) of isolates from hospitalized patients.

### Identification

Identification was performed with the API-20E system (bioMérieux, Marcy L'Etoile, France) and was confirmed by sequence analysis of the gene (*rpoB*) coding for the $\beta$-subunit of RNA polymerase, as previously described [13].

### Antibiotic susceptibility tests

The susceptibilities to antimicrobial agents were determined by the disk diffusion method on Mueller–Hinton agar (Bio-Rad, Marnes La Coquette, France), according to the guidelines of the CLSI [14]. The following antibiotics were tested: amoxycillin, ticarcillin, cefalothin, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, streptomycin, gentamicin, tobramycin, kanamycin, amikacin, chloramphenicol, tetracycline, trimethoprim–sulphamethoxazole and ciprofloxacin. Production of ESBLs was detected by the double-disk synergy test with disks of amoxycillin–clavulanic acid surrounded at a radius of 20 mm by cefotaxime, ceftazidime and aztreonam.

### PCR amplification of antimicrobial resistance genes and sequence analysis

The resistance genes $bla_{TEM}$, $bla_{SHV}$ and $bla_{CTX-M}$ were amplified by PCR as described previously [8,15,16]. PrIS26-F and blaU4R primers were designed for specific detection of ESBL-encoding $bla_{SHV}$ genes [17,18]. PrIS26-F was located in the coding region of the transposase of IS*26*, which is upstream of the promoter region of the $bla_{SHV}$ gene in such structures [19,20].

Sequencing of PCR products was performed at the Institut Pasteur, Genotyping of Pathogens and Public Health Unit. The nucleotide sequences and the deduced protein sequences were analysed with EditSeq and Megalign software (Dnastar, Madison, WI, USA). The BLASTN program of NCBI was used for database searches (http://www.ncbi.nlm.nih.gov/BLAST/).

### Exploration of the upstream sequence of the $bla_{CTX-M}$ and $bla_{SHV}$ genes

The presence of IS*Ecp1* was investigated as previously described [21]. Exploration of the upstream sequence of the $bla_{SHV}$ gene was performed by PCR using PrIS26-F and SHV-INT-R (5′-GAT TTG CTG ATT TCG CTC GG-3′) primers [18]. PCR mixture and cycling conditions were as for the $bla_{CTX-M}$ amplification.

### Transfer of resistance

Conjugation experiments were performed on 48 isolates selected on the basis of year of isolation and type of resistance (Table 1) as previously described [15]. *E. coli* strain J53 $Rif^R$, *E. coli* strain C1A $Nal^R$ or *E. coli* strain K12 $Azi^R$ was used as recipient, as appropriate. Transconjugants were selected on Trypticase soy (Bio-Rad) agar plates containing ceftazidime (2 mg/L) or cefotaxime (4 mg/L) and either rifampicin (250 mg/L), nalidixic acid (64 mg/L) or sodium azide (500 mg/L). Transfer experiments using electroporation were performed for 12 non-conjugative plasmids. Plasmid DNA was extracted by a lysis alkaline procedure [22] and was transferred into electrocompetent *E. coli* DH10B by standard electroporation techniques, with a Micropulser electroporation apparatus (Bio-Rad). Transformants were selected on Mueller–Hinton agar containing cefotaxime (4 mg/L) or ceftazidime (2 mg/L). Transconjugants and transformants were submitted to bacterial identification with the API-20E system, antibiotic susceptibility

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**TABLE 1. Characteristics of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates selected for multilocus sequence typing analysis and conjugation experiments**

| Isolate | Ward | Date of isolation (day/month/year) | Specimen | Non-β-lactam resistance phenotype | β-Lactamase(s) | Restriction plasmid profile group | Replicon typing[a] | Plasmid size (kb)[b] | Allelic profile | ST |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ICU | 16/01/1999 | PSB | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | E | Negative | 90–95 | 3-6-2-1-7-4-41 | 320 |
| 2 | PU | 22/04/1999 | Catheter | KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | E | | | | |
| 3a | ICU | 14/05/1999 | Urine | KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | E | | | | |
| 4 | PU | 11/11/1999 | Urine | KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | E | | | | |
| 5 | Cardiology | 16/01/2002 | Catheter | KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | E | | | | |
| 6 | PU | 08/11/1999 | Urine | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | G | Negative | 113 | | |
| 7 | PU | 15/06/2001 | CSF | KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | G | | | | |
| 8 | ICU | 09/01/2001 | Blood | KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | H | | | | |
| 9 | PU | 07/12/2004 | Urine | STR/KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | H | | | | |
| 10 | PU | 12/01/2005 | Urine | KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | H | | | | |
| 11 | PU | 02/12/2004 | Urine | STR/KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | H | Negative | 100 | 2-9-2-1-13-1-10 | 309 |
| 12 | PU | 12/02/2005 | Urine | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | S | | | 2-9-2-1-13-1-10 | 309 |
| 13 | ICU | 20/04/1999 | Pus | KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | S | | | | |
| 14b | PU | 09/03/2002 | Urine | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | S | Negative | 60, 110 | 2-1-2-1-10-1-19 | 35 |
| 15c | PUO | 30/01/2003 | Urine | STR/KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | S | | | | |
| 16 | PU | 24/06/2003 | Urine | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | S | | 120 | 2-5-2-2-7-1-10 | 48 |
| 17 | PU | 13/09/2003 | Urine | KAN/TOB/GEN/TET/SXT | SHV-12 | S | | | 12-1-1-2-5-1-36 | 133 |
| E3 | ICU | 09/02/1999 | Environment | STR/KAN/TOB/GEN/CHL/TET/SXT | SHV-12 | S | | | | |
| 18 | PU | 23/10/2001 | CSF | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | I | | | | |
| 19 | ICU | 09/05/2000 | Blood | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | I | Negative | 140 | 4-16-2-1-28-3-40 | 321 |
| 20 | ICU | 08/11/2000 | Blood | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | N | Negative | 120 | 4-16-2-1-28-3-40 | 321 |
| 21 | Cardiology | 14/09/2001 | Blood | STR/CHL | SHV-12 | Q | Negative | 110, 160 | | |
| 22 | ICU | 03/10/2001 | Blood | KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | SHV-12 | O | Negative | 120 | 2-1-1-1-9-1-93 | 323 |
| 23b | PUO | 10/10/2002 | Urine | STR/TET/SXT | SHV-12 | M | Negative | 200 | | |
| 24 | Obstetrics | 01/11/2003 | Urine | STR/TET | SHV-12 | P | Negative | 100 | | |
| E2 | ICU | 04/02/1999 | Environment | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | R | Negative | 180 | | |
| 25 | PU | 20/03/1999 | Urine | KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12 | – | | | 14-3-1-1-4-4-4 | 325 |
| 26 | PU | 25/05/2001 | Urine | KAN/TOB/GEN/AMK/TET/SXT | SHV-12 | – | | | 1-6-1-1-1-4-1 | 324 |
| 27 | ICU | 30/03/1999 | Urine | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT | SHV-12, TEM-1 | F | HI2 | 95 | | |
| 28 | PU | 03/09/2003 | Blood | KAN/TOB/GEN/AMK/TET/SXT | SHV-2a | K | FII | 70–80 | 2-1-5-1-26-4-12 | 322 |
| 29 | PU | 15/09/2003 | Pus | STR/KAN/TOB/GEN/AMK/TET | SHV-2a | L | L/M | 60–70 | 2-1-5-1-26-4-12 | 322 |
| 30 | PUO | 14/04/2004 | Urine | STR/KAN/TOB/GEN/AMK/TET/SXT | SHV-2a | L | | | | |
| 31 | PU | 19/06/2002 | Blood | KAN/TOB/GEN/AMK/TET/SXT | CTX-M-27, SHV-12 | J | Negative | 30 | 2-1-1-1-10-4-13 | 25 |
| 32c | PU | 26/06/2002 | Urine | KAN/TOB/GEN/AMK/TET/SXT | CTX-M-27, SHV-12 | J | | | | |
| 33d | ICU | 20/10/2003 | PSB | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15, TEM-1 | B | | | | |
| 34 | ICU | 22/12/2003 | Catheter | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, TEM-1 | B | | 60, 110 | | |
| 35 | ICU | 21/06/2003 | PSB | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, TEM-1 | B | | | | |
| 36 | ICU | 11/11/2004 | PSB | STR/KAN/TOB/GEN/AMK/TET/SXT/CIP | CTX-M-15, TEM-1 | B | | | | |
| 37 | ICU | 01/09/2003 | Catheter | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, TEM-1 | B | | | 2-6-1-5-4-1-6 | 101 |
| 38 | ICU | 12/01/2005 | PSB | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15, TEM-1 | B | Negative | 70 | 2-6-1-5-4-1-6 | 101 |
| 39 | ICU | 16/12/2004 | Blood | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15, TEM-1 | - | | | 2-6-1-5-4-1-6 | 101 |
| 40 | PU | 20/02/2004 | Urine | KAN/TOB/GEN/AMK/CHL/TET/CIP | CTX-M-15, SHV-12, TEM-1 | C | L/M | 65 | 12-1-1-2-5-1-36 | 133 |
| 41 | PU | 26/04/2004 | Urine | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M15, TEM-1 | C | | | | |
| 42 | ICU | 06/01/2004 | PSB | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, TEM-1 | A | FII, P | 80 | | |
| 43 | ICU | 19/01/2002 | Blood | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, SHV-12 | A | FII | 80 | 2-1-2-17-27-1-39 | 107 |
| 44 | ICU | 11/02/2002 | Blood | STR/KAN/TOB/GEN/AMK/CHL/TET/SXT/CIP | CTX-M-15, SHV-12 | A | | | 2-1-2-17-27-1-39 | 107 |
| 45 | ICU | 17/12/2004 | Nasal swab | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15 | A | FII | 80 | | |
| 46 | Surgery | 09/02/2005 | Blood | STR/KAN/TOB/GEN/TET/CIP | CTX-M-15 | A | | | 2-6-1-3-8-1-15 | 42 |
| 47 | ICU | 25/01/2005 | Rectal swab | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15 | A | | | | |
| 48 | Surgery | 18/02/2005 | Blood | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15 | A | | | | |
| 49 | PU | 20/05/2005 | Urine | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15 | D | FII | 120 | 4-16-2-1-28-3-40 | 321 |
| 50d | ICU | 30/09/2003 | Blood | KAN/TOB/GEN/CHL/SXT/CIP | CTX-M-15 | – | | | 3-4-6-1-7-4-38 | 147 |
| 51 | PU | 08/11/2004 | Nasal and rectal swab | STR/KAN/TOB/GEN/AMK/TET/CIP | CTX-M-15 | – | | | 3-4-6-1-7-4-38 | 147 |

CSF, cerebrospinal fluid; PU, Paediatric Unit; PUO, Paediatric Unit Outpatient; ICU, Intensive-care Unit; PSB, fibreoptic protected specimen brush; ST, sequence type; –, conjugation experiments were not performed for this isolate; AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SXT, trimethoprim-sulphamethoxazole; TET, tetracycline; TOB, tobramycin.
[a]*gapA–infB–mdh–pgi–phoE–rpoB–tonB*. [b]Plasmid extracted from transconjugants and electroporants.

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testing and PCR amplification of the antimicrobial resistance genes mentioned above.

#### Plasmid analyses, Southern blotting and hybridization

Plasmid DNA from *K. pneumoniae* strains and their corresponding *E. coli* transconjugants or transformants extracted by an alkaline lysis method was analysed by electrophoresis in 0.8% agarose gels as previously described [22,23]. Plasmid DNA was digested with either *Eco*RI or *Pst*I endonuclease (Roche, Meylan, France), and the fragments were analysed as previously described [16]. Southern blotting using *bla*$_{SHV}$ (1060 bp) and *bla*$_{CTX-M-15}$ (840 bp) PCR-generated probes was performed as previously described [23], using ECL direct nucleic acid labelling and detection systems (Amersham Biosciences, Little Chalfont, UK). The blaU5F and blaU4R primers were used to generate the *bla*$_{SHV-12}$ probe [17]. The content and size of plasmids were determined as described by Barton *et al.* [24].

#### Plasmid replicon type determination

PCR-based replicon typing was performed on 21 conjugative and non-conjugative plasmids (including three of group A), as described by Carattoli *et al.* [25]. The 18 primer pairs targeting FIA, FIB, FIC, HI1, HI2, I1-I$\gamma$, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FII replicons were used in separate PCR reactions.

#### Multilocus sequence typing (MLST)

Analysis by MLST was performed as described previously [13,26] (http://www.pasteur/fr/mlst) on 24 isolates selected on the basis of year of isolation and type of ESBL (Table 1).

## Results

#### Bacterial identification

The *rpoB* sequences showed that 101 *K. pneumoniae* isolates belonged to phylogroup Kp1 [27], and two ESBL-producing isolates belonged to Kp2 and Kp6, respectively [17].

#### Antibiotic susceptibility tests

The disk diffusion method showed that all isolates were resistant to all $\beta$-lactams used except for cefoxitin, and imipenem. Clinical isolates manifested high-level resistance to the aminoglycosides kanamycin (94%), tobramycin (93%), gentamicin (92%), amikacin (77%), and streptomycin (73%), to tetracycline (94%), and to trimethoprim–sulphamethoxazole (72%). Resistance of the strains to fluoroquinolones was also remarkable (ciprofloxacin, 48.5%). The double-disk synergy test was positive for the 103 isolates studied (data not shown).

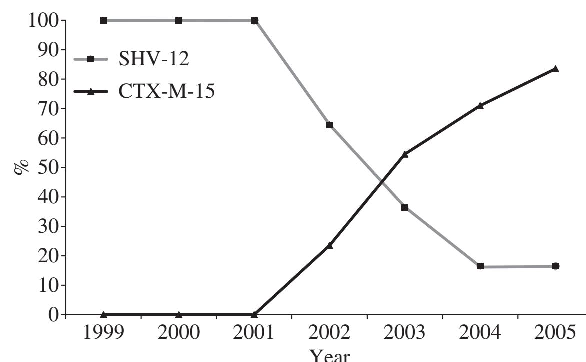


**FIG. 1.** Evolution of CTX-M-15 and SHV-12 incidence during the study period.

#### $\beta$-Lactamase gene characterization

PCR and sequence analysis revealed that 47 of 101 clinical isolates had a *bla*$_{CTX-M}$ gene; of these, 43 of 47 had the *bla*$_{CTX-M-15}$ gene, two of 47 the *bla*$_{CTX-M-14}$ gene, and two of 47 the *bla*$_{CTX-M-27}$ gene. Analysis of SHV PCR products showed that 58 of 101 clinical isolates had the *bla*$_{SHV-12}$ gene, and three of 101 the *bla*$_{SHV-2a}$ gene. Five of the 101 isolates were found to carry the *bla*$_{CTX-M-15}$ and *bla*$_{SHV-12}$ genes (isolates 40, 43, 44 and two others not listed in Table 1), and two of 101 isolates had the *bla*$_{CTX-M-27}$ and *bla*$_{SHV-12}$ genes (isolates 31 and 32). The *bla*$_{TEM-1}$ gene was detected in 22 of 43 isolates (51%) containing the *bla*$_{CTX-M-15}$ gene, and in 11 of 58 isolates (19%) containing the *bla*$_{SHV-12}$ gene. The occurrence of the *bla*$_{SHV-12}$ gene decreased over time, whereas the *bla*$_{CTX-M-15}$ gene became predominant from 2003 to 2005 (Fig. 1). Two isolates from the hospital environment were found to contain the *bla*$_{SHV-12}$ gene.

#### Exploration of the upstream sequence of the *bla*$_{CTX-M}$ and *bla*$_{SHV}$ genes

The insertion sequence IS*Ecp1* was identified upstream of the *bla*$_{CTX-M}$ gene in all but one of the isolates. This isolate had the *bla*$_{CTX-M-15}$ gene. The insertion sequence IS*26* was localized 73 bp upstream of all the *bla*$_{SHV}$ genes in the opposite direction.

#### Transfer of resistance

Cefotaxime and ceftazidime resistance was successfully transferred by conjugation from 36 of the 48 isolates (75%). For the 12 non-conjugative plasmids, the transfer of resistance to the above antibiotics was obtained by electroporation of plasmid DNA. We also observed co-transfer, following conjugation or electroporation, of resistance to other antibiotics, such as i.e. streptomycin (51.6%), gentamicin (60%), tobramycin (89%), kanamycin (89%), amikacin (50%),

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chloramphenicol (23%), tetracycline (75%) and trimethoprim–sulphamethoxazole (75.6%). Production of ESBLs was detected in all transconjugants and transformants by the double-disk synergy test. The presence of the ESBL gene was confirmed by PCR. Only the $bla_{CTX-M}$ gene was detected in transconjugants corresponding to *K. pneumoniae* isolates in which more than one ESBL was present.

#### Plasmid analyses, Southern blotting and hybridization

The analysis of transconjugants and transformants revealed the presence of large plasmids (30–120 kb for CTX-M type; 60–200 kb for SHV type). Digestion with *Eco*RI of plasmid DNA from transconjugants and the transformants producing SHV-12-type $\beta$-lactamases classified them into 12 different restriction profile groups (Table 1; Fig. 2). Plasmids isolated from transconjugants producing SHV-2a yielded two *Eco*RI restriction profiles (Table 1 and data not shown). Hybridization with a $bla_{SHV-12}$ probe showed different plasmid *Eco*RI-generated fragments (Fig. 2).

*Pst*I digestion of plasmid DNA from transconjugants containing $bla_{CTX-M-27}$ yielded an identical restriction profile (Table 1 and data not shown). Plasmid fingerprinting of transconjugants and transformants of isolates producing CTX-M-15 using *Pst*I classified them in four groups (Fig. 3; Table 1).

#### Plasmid replicon type determination

The replicons could not be determined for the $bla_{SHV-12}$-carrying plasmids, except for the plasmid showing the restriction profile F and associated with the IncHI2 replicon (Table 1). The plasmids carrying $bla_{SHV-2a}$ or $bla_{CTX-M-15}$ were assigned to the FII and L/M replicon types (Table 1). The $bla_{CTX-M-15}$-carrying plasmid representing the restriction profile B was negative for all of the replicons tested, as well as the $bla_{CTX-M-27}$-carrying plasmid (Table 1).

#### MLST

When compared by MLST analysis, the 24 selected isolates showed 15 different sequence types (STs) (Table 1). This analysis showed the dissemination of specific CTX-M-15-producing *K. pneumoniae* clones: ST101 and ST107 in the Intensive-care Unit, and ST147 between the Intensive-care Unit and the Paediatric Unit (Table 1).

On the other hand, the MLST analysis showed diversity among the SHV-12-producing isolates, as the 15 tested isolates showed 11 different STs: ST25, ST35, ST48, ST107, ST133, ST309, ST320, ST321, ST323, ST324 and ST325 (Table 1). However, MLST analysis also showed dissemination of the specific clone ST321 between the Intensive-care Unit and the Paediatric Unit and of the specific clone ST309 in the Paediatric Unit (Table 1). We noted an evolution of resistance for some clones over time. For example, the SHV-12-producing isolates 19 and 20, obtained in May and November 2000, respectively, as well as CTX-M-15-producing isolate 49, isolated in May 2005, showed the same ST (ST321). The SHV-2a-producing isolates 28 and 29, whose plasmids showed different *Eco*RI restriction profiles and different replicons, manifested an indistinguishable ST (ST322).

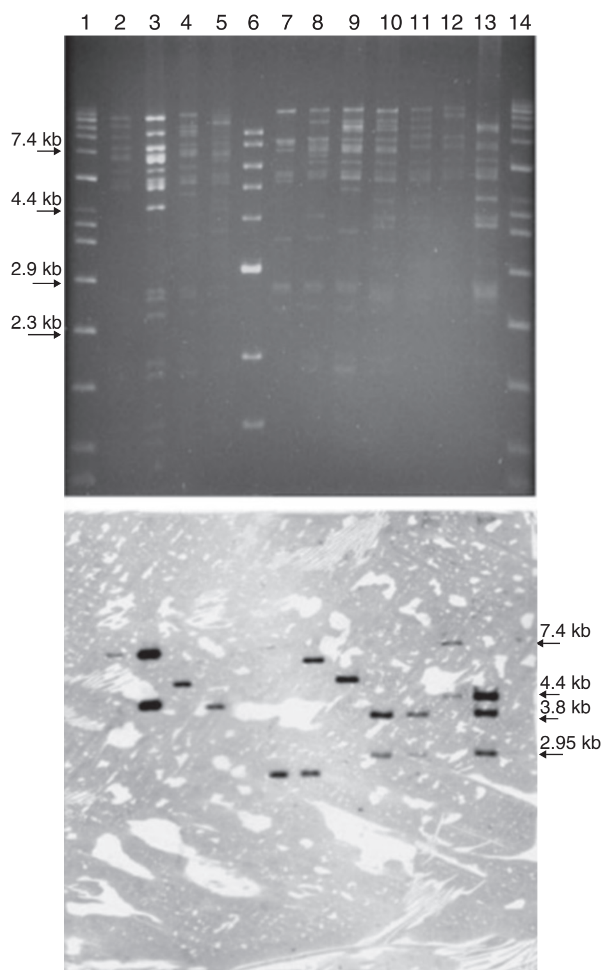


**FIG. 2.** *Eco*RI restriction profiles of plasmids from transconjugants producing SHV-12 enzymes and Southern hybridization with the $bla_{SHV-12}$ probe. Lanes: 1, Raoul marker (QBiogene, Illkirch, France); 2, Tc-17 (profile S4); 3, Tc-16 (profile S3); 4, Tc-18 (profile I1); 5, Tc-21 (profile Q1); 6, 1-kb DNA ladder; 7, Tc-8 (profile H4); 8, Tc-20 (profile N1); 9, Tc-19 (profile I1); 10, Tc-4 (profile E3); 11, Tc-2 (profile E2); 12, Tc-13 (profile S1); 13, Tc-1 (profile E1); 14, Raoul marker (QBiogene). For Tc-1, Tc-2, Tc-4, Tc-20 and Tc-16, the presence of multiple positive hybridization signals of the $bla_{SHV-12}$ gene is due to the presence of several copies of this gene in the same plasmid (Table 1).

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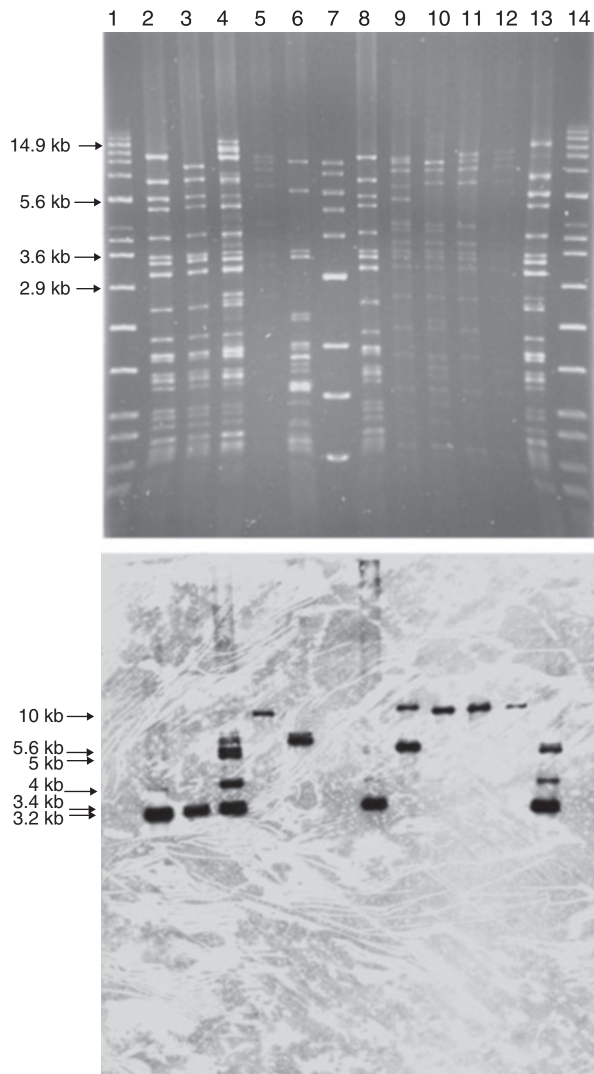


**FIG. 3.** *Pst*I restriction profiles of plasmids from transconjugants or electroporants producing CTX-M-15 enzymes and Southern hybridization with the $bla_{CTX-M-15}$ probe. Lanes: 1, Raoul marker (QBiogene); 2, Tc-48 (profile A3); 3, Tc-46 (profile A1); 4, Tc-45 (profile A4); 5, Ep-36 (profile B5); 6, Tc-41 (profile C1); 7, 1-kb DNA ladder; 8, Tc-42 (profile A3); 9, Ep-34 (profile B4); 10, Ep-33 (profile B3); 11, Ep-37 (profile B2); 12, Ep-35 (profile B1); 13, Tc-43 (profile A2); 14, Raoul marker (QBiogene). The presence of multiple positive hybridization signals of the $bla_{CTX-M-15}$ gene is probably due to the partial digestion of the plasmid carrying the $bla_{CTX-M15}$ gene (Tc-43 and Tc-45) or due to the presence of different plasmids in the same preparation (Ep-34) (Table 1).

## Discussion

We noted a temporal shift in the prevalence of ESBL types (Fig. 1). Thus, the CTX-M-type ESBLs have clearly been predominant during this last decade, as has been recently described worldwide [28]. Some dominant SHV types continue to be important, especially SHV-12 [29]. SHV-12 and SHV-2a were found in the isolates investigated. These enzymes seem to be common not only in Tunisia but also in many countries in north and central Africa [3,23].

MLST is well suited to characterizing the genetic relationships between the organisms of bacterial species, including *Klebsiella* spp. [13,26]. By this method, 375 STs are currently distinguished among *K. pneumoniae* isolates (http://www.pasteur.fr/mlst/Kpneumoniae.html). This is the first report using MLST analysis for typing of *K. pneumoniae* isolates in Tunisia. MLST analysis showed that two isolates containing the $bla_{SHV-2a}$ gene were clonally related but possessed different SHV-2a-carrying plasmids, whereas the isolates containing the $bla_{SHV-12}$ gene displayed chromosomal and plasmid diversity, confirming the results of our first investigation [7]. Only one of the $bla_{SHV-12}$-carrying plasmid could be characterized by replicon typing. Further research is necessary to provide more information about these $bla_{SHV-12}$-carrying plasmids.

The $bla_{CTX-M-15}$ gene represented the most abundant CTX-M allele (43 of 47 strains producing CTX-M) detected in our study. The first CTX-M-15 was described in India in 1999 [30], but this enzyme has now emerged in different parts of the world, and seems to be the most recently described ESBL type [16,28,31,32]. In Tunisia, dissemination of CTX-M-15 has been reported in *K. pneumoniae* and *E. coli* strains [6,9,33]. Recent reports indicate that worldwide dissemination of CTX-M-15 is mediated by clonally related *E. coli* strains, particularly specific clones of phylogroup B2, ST131, and phylogroup D, ST405) [31,33,34]. The successful spread of CTX-M-15 seems to be also related to IncFII plasmids [31,33]. A few reports have characterized the Inc type of the $bla_{CTX-M-15}$-carrying plasmids disseminated in *K. pneumoniae* strains, and detected IncFII, IncFI or IncL/M types [35,36]. The $bla_{CTX-M-15}$-carrying plasmids studied here were also assigned to incompatibility groups IncFII and IncL/M, suggesting mobilization of this gene among different plasmid scaffolds. All but one of the $bla_{CTX-M}$ genes studied here were flanked by the insertion sequence IS*Ecp1* on their 5′-side. IS*Ecp1* was identified in the vicinity of many $bla_{CTX-M}$ genes, and it has been shown that this insertion sequence mobilizes various $\beta$-lactamase genes, including the $bla_{CTX-M-15}$ gene, to transfer between different replicons [21,30,37].

MLST analysis of our CTX-M-15-producing *K. pneumoniae* isolates showed that dissemination of these resistant strains is due to the spread of specific clones of ST42, ST107, ST101, ST147, ST133 and ST321. Interestingly, a countrywide dissemination of an ST147 ciprofloxacin-resistant CTX-M-15-producing *K. pneumoniae* clone was described during a

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national survey in Hungary in 2005 [32]. In our study, the first isolation of this specific clone was made in 2003.

We report here for the first time the presence of the $bla_{CTX-M-14}$ gene in clinical isolates in Tunisia. Our study also revealed that two isolates had the $bla_{CTX-M-27}$ gene. This gene had already been identified in isolates of *Salmonella enterica* during a nosocomial neonatal outbreak in Tunisia [8]. The appearance of CTX-M-producing *K. pneumoniae* strains at Mongi Slim University Hospital is probably due to the importation of these multidrug-resistant strains, as their occurrence has been described in other hospitals in Tunisia [6,8,9,33].

In conclusion, the present article documents the emergence of CTX-M $\beta$-lactamase-producing *K. pneumoniae* strains in Tunisia and the decline of the SHV type. Tunisian laboratories should have appropriate methods for detection of this ESBL and of strains harbouring these enzymes.

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## Transparency Declaration

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