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Full Length Research Paper

Molecular characterization of a transferable *bla*$_{CTX-M-28}$ gene in clinical isolates of *Enterobacter cloacae*

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The extended-spectrum beta-lactamase (ESBL) producing phenotype is frequent among *Enterobacter cloacae* isolates at the Military Hospital of Tunis, Tunisia. We identified the ESBLs of a collection of these strains showing a particular extended-spectrum cephalosporin resistance profile characterized by a higher level of resistance to cefotaxime. These isolates were analyzed by antibiotic susceptibility testing, pI determination, plasmid profiles, transconjugation test, enterobacterial repetitive consensus (ERIC)-PCR and DNA sequencing. All these isolates were found to contain the CTX-M-28 ESBL genes and were multiresistant, producing other β-lactamases type of TEM-1 and OXA-1. ERIC-PCR analysis and conjugation experiments revealed that the dissemination of CTX-M-28 was due to both strain spreading and plasmid diffusion. The detection of CTX-M-28 on a conjugative plasmid is a significant factor for the dissemination of this enzyme. We supposed that the CTX-M-28-encoding plasmid will become an eventual epidemiological problem, by horizontal transfer, in ICU (Intensive Care Unit) of Military hospital in Tunisia.

Key words: β-lactamase, *Enterobacter cloacae*, cefotaximase, CTX-M-28, enterobacterial repetitive consensus (ERIC)-PCR.

INTRODUCTION

Enterobacter species are a common cause of several human diseases and are predominantly associated with nosocomial infections. They are responsible for 5% of all nosocomial septicaemia cases in the United States and can be found as the third most common pathogens recovered from the respiratory tracts of patients in intensive care units (Stock et al., 2001). Among the variety of *Enterobacter* species, *Enterobacter cloacae* is a well-recognized nosocomial pathogen that causes significant infections, especially in the last recent years (Haertl et al., 1993). This microorganism is the most commonly isolated member of the *Enterobacteriaceae* that possess a chromosomally encoded AmpC β-lactamase that plays an important role in resistance to antibiotics (Liu et al., 2004). However, several reports have demonstrated that these species can acquire and express genes encoding extended-spectrum β-lactamase (ESBL) (Szabo et al., 2005). In fact, it has been reported that the wild-type strains of *E. cloacae* can express the Bush group 2f carbapenemases Nmc-A and IMI-1 (Mimoz et al., 2000; Rasmussen et al., 1996). The resistance to broad-spectrum penicillins and extended-spectrum cephalosporins via the acquisition of plasmids encoding TEM and SHV derivatives extended spectrum beta-lactamase (ESBL) has also been reported (Arpin et al., 2002). Various extended-spectrum β-lactamases not derived from TEM or SHV enzymes have been described in this species, such as SFO type in Japan (Matsumoto and Inoue, 1999), IBC-1 type in Greece (Giakkoupi et al., 2000), VEB-3 type in China (Jian et al., 2005) and CTX-M class that have been described more recently (Lee et al., 2005).

CTX-M β-lactamases are a class of β-lactamases which have been more recently recognized to preferentially hydrolyze cefotaxime and were initially

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Table 1. Characteristics of extended-spectrum β-lactamase-producing *E. cloacae* isolates.

Isolates	Wards	Date of isolation (day/month/year)	Sample origin	Antibiotype[a]	β-lactamases content	
					PI(s)	Enzymes (s)
E. cloacae S1	Intensive care unit	14/12/05	Axillaire	BLSE, GTNt, Te, SXT	8,6 ; 7,4	CTX-28 ; OXA-1
E. cloacae S2	intensive care unit	14//12/05	Rectal	BLSE, GTNt, Te, C	8,6 ; 5,4	CTX-28 ; TEM-1
E. cloacae S3	intensive care unit	14//12/05	Axillaire	BLSE, GTNt, Te, SXT,	8,6 ; 7,4	CTX-28 ; OXA-1
E. cloacae S4	intensive care unit	14//12/05	Rectal	BLSE, GTNt, Te, C, SXT,	8,6 ; 5,4	CTX-28 ; TEM-1
E. cloacae S5	intensive care unit	11//11/06	Hemoculture	BLSE, TNtA, SXT,	8,6 ;5,4 ; 7,4	CTX-28 ;TEM-1 ; OXA-1
E. cloacae S6	intensive care unit	27//05/07	Hemoculture	BLSE, GTNt, Te, SXT,	8,6 ; 7,4	CTX-28 ; OXA-1
E. cloacae S7	Hygiene	06//02/07	PTP	BLSE, GTNt	8,6 ; 7,4	CTX-28 ; OXA-1
E. cloacae S8	Neonatal	05//12/06	Hemoculture	BLSE, Te, C, SXT,	8,6 ; 5,4	CTX-28; TEM-1

reported in the second half of the 1980s. Theses enzymes (cefotaximases) are a relatively novel family of plasmid-mediated extended-spectrum cephalosporins and have been classified under Ambler class A (Kingsley et al., 2008). On the basis of their amino acid sequences, the phylogenic study reveals 5 major groups of acquired CTX-M enzymes: the CTX-M-1 group, the CTX-M-2 group, the CTX-M-8, group the CTX-M-9 group and the CTX-M-25 group. Despite their structural diversity, most CTX-M β-lactamases have similar hydrolysis profiles (Novais et al., 2008).

Despite the increasing number of reports that studied the ESBL-producing strains throughout the world, few reports of the *E. cloacae* existing in Tunisia have been published. In this work, we analysed the production of extended spectrum β-lactamase in a multiresistant strains of *E. cloacae* isolated from different wards in the Military hospital of Tunis in Tunisia.

MATERIALS AND METHODS

Bacterial strains

A total of 8 cefotaxime-resistant *E. cloacae* strains are included in this study. These strains were recovered from different wards of the Military Hospital of Tunis from 2005 to 2007, exclusively from the intensive care unit (Table 1). They were identified by using the API 20 E identification system (bioMérieux, Marcy l'Etoile, France).

Susceptibility testing and extended-spectrum β-lactamase detection

The antibiotics susceptibilities of the *E. cloacae* strains were determined on Muelluer-Hinton agar by the standard disk diffusion procedure as described by the Antibiogram Committee of the French Society for Microbiology (www.sfm.asso.fr). The following antibiotics were tested: ampicillin, ticarcillin, cefalotin, cefoxitin, gentamicin, cefepim, cefuroxim, tobramycin, amikacin, cefotaxim, colistin, netilmicin, ceftazidim, imipinem, tetracycline, cloramphenicol, fosfamycin, nalidixic acid, ofloxacin, cotrimoxazol, rifampicin, ticarcilin/ clavulanic acid and amoxicillin/clavulanic acid (Biorad, Marnes-la-Coquette, France).

The ESBL detection was based on the double-disk synergy test (DDST) as described previously (Chouchani et al., 2006). DDST was performed as follows: the surface of a Muelleur-Hinton (MH) agar plate was inoculated with an overnight culture suspension of clinical isolate. After inoculation, disks containing 30µg of CAZ, CTX, CRO, CEP, and amoxicillin-clavulanic acid (20/10 µg) were placed at distances of 20 mm (centre to centre).

Enhancement of the inhibition zone between the disks containing clavulanic acid and CAZ, CTX, CRO or CEP, indicated the presence of ESBL production.

Polymerase chain reaction (PCR) amplification

Detection of gene sequences coding for β-lactamase enzymes was performed with total deoxyribonucleic acid (DNA). Briefly, bacteria were removed from a plate with an inoculation loop and suspended in 100 µl of sterile deionised water. The cells were lased by heating to 100°C for 10 min and cellular debris was removed by centrifugation for 5 min at 14,000 rpm. The supernatant was used as the source of the template for amplification (Dubois et al., 2002). PCR amplification was carried out on a DNA 2720 thermal cycler (AB applied Biosystems) using the primers listed in Table 2. Cycling conditions were: 34 cycles at 94, 50 and 72°C for 1 min respectively, with a final extension period at 72°C for 10 min. the PCR products were separated in 1.2% agarose gel and visualised with UV. The PCR products were purified using microcolumns of the Microspin Sephacryl S-400 purification system (Amersham Biosciences) and sequenced on both strands with the same primers (Eurogentec, France), an automated fluorescent method based on dye terminator chemistry (Ampli Taq DNA polymerase FS Dye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems Division, Perkin-Elmer, France), and the ABI-Prism 310 sequencer (Applied Biosystems Division, Perkin-Elmer).

Table 2. Primers used in this study.

Primer (orientation)*	Sequence (5'→3')	Annealing temperatures (°C)	Amplicon Sizes (bp)
bla$_{CTX-M}$			
CTX-MA	CGCTTTGCGATGTGCAG	54	539
CTX-MB	ACCGCGATATCGTTGGT		
CTX-M-1- group			
CTX-1F	ATGGTTAAAAAATCACTGCGTC	60	864
CTX-1R	TTGGTGACGATTTTAGCCGC		
bla $_{TEM}$			932
TEM-A2	GTATCCGCTCATGAGACAAT	54	
TEM-ext	GTATATATGAGTAAACTTGGTCTG		
bla $_{OXA}$			
OXA1F	CACAATACATATCAACTTCGC	54	793
OXA1R	GTGTGTTTAGAATGGTGATCGC		
ERIC1	ATGTAAGCTCCTGGGGATTCAC	56	
ERIC2	AAGTAAGTGACTGGGGTGAGCG		

*Orientation of each primer: A, A2 and F forward; B, ext and R, reverse.

Conjugation transfer experiments and plasmid analysis

Conjugation assays were carried out by the filter-mating procedure using the *E. coli* K-12 Nal^r Rif^r mutant as the recipient. Transconjugants were selected on MH agar containing nalidixic acid (100 µg/ml) and Ticarcillin (100 µg/ml) plus cefotaxime (2 µg/ml). Transconjugants growing on the selection plates were subjected to DDST and IEF to confirm the presence of ESBL phenotype. Samples from the donor and recipient were used as controls. Plasmid DNA was extracted by an alkaline lysis method and analyzed by electrophoresis on 0.8% weight per volume (wt/vol) agarose gels (Promega, France) (Arpin et al., 2005).

Analytical isoelectric focusing (IEF)

IEF was performed in nondenaturing polyacrylamide gel containing ampholines pH range of 3 to 10 at 4°C in an 111Mini IEF cell (Bio-Rad). TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-3 (pI 6.3), OXA-1 (pI 7.4), and CTX-M-15 (pI 8.6) were used as pI markers. The β-lactamase activity was detected by the iodine procedure in gel by using benzylpenicillin (30 µg/ml) as the substrate.

RAPD and ERIC-PCR typing

The epidemiological relationships between the 8 strains were analyzed by random amplified polymorphic DNA (RAPD) analysis with primer AP12h and Enterobacterial repetitive intergenic consensus (ERIC) PCR (ERIC-PCR) with primers ERIC2 and ERIC1. The amplification conditions were as follows: 94°C for 5 min and 45 subsequent cycles of 1 min at 94, 37, and 72°C respectively, with a final step at 72°C for 10 min. The amplification products were analyzed by electrophoresis of 20 µl samples on 1.5% agarose gels in the presence of ethidium bromide.

RESULTS

Description of clinical isolates

Eight *E. coloacae* strains resistant to cefotaxime were recovered between June, 2005 and August, 2007. All isolates were isolated at the Military hospital of Tunis. The strains were collected from differents wards of the Hospital and were associated with various infections (Table 1).

Antibiotic susceptibility testing revealed that all eight isolates were resistant to ticarcillin, ampicillin, cefoxitin, cefalotin, cefuroxim, ceftazidime, cefotaxime, amoxicillin/clavulanic acid, gentamicin (except S5), tobramycin, and netilmycin and trimethoprim/sulfamethoxazole. The isolates remained susceptible to imipenem. The disk diffusion method showed synergy between ceftazidime, cefotaxime, cefepime, and clavulanic acid against all the strains and their transconjugants.

ESBL characterization

All strains included in this study produced an ESBL as detected by the disk synergy test (Table 3). Transconjugants (Tc) producing ESBLs were obtained for all strains except for *E. cloacae* S2 and S4 that were isolated from the same origin. The β-lactamase contents of the clinical strains and their transconjugants were first analyzed by isoelectric focusing. All isolates showed a

Table 3. Antibiotics susceptibility of *E. cloacae* strains and their trasconjugants.

Isolates	Zone diameter (mm)																	
	AM	TIC	TCC	AMC	FEP	CTX	CAZ	CXM	FOX	CF	IPM	C	TE	AN	GM	TM	NET	SXT
*E.cloacae*S1	6	6	17	12	14	8	14	6	6	6	28	25	20	19	22	10	18	6
Trc S1	6	6	25	12	19	6	17	6	26	6	30	25	6	20	11	10	18	28
*E.cloacae*S2	6	6	21	6	14	14	22	6	6	6	25	6	6	18	12	12	22	6
*E.cloacae*S3	6	6	12	6	8	6	8	6	6	6	24	21	6	16	6	6	13	16
Trc S3	6	6	17	12	20	6	18	6	28	6	30	20	6	19	10	10	21	28
*E.cloacae*S4	6	6	14	6	8	6	10	6	6	6	25	17	6	18	6	6	14	6
*E.cloacaeS*5	6	6	17	10	9	6	10	6	6	6	26	22	6	18	6	6	14	6
Trc S5	6	6	14	16	14	8	14	6	28	6	28	23	23	20	22	8	24	6
*E.cloacae*S6	6	6	12	6	8	6	8	6	6	6	24	21	6	16	6	16	13	16
Trc S6	6	6	12	12	22	20	12	20	28	8	30	24	18	18	20	9	25	6
*E.cloacaeS*7	6	6	18	8	13	6	16	6	6	6	26	24	22	18	6	18	14	26
Trc S7	6	6	14	16	14	8	12	6	29	6	32	25	17	18	15	9	25	6
*E.cloacae*S8	6	6	22	20	22	14	6	6	6	6	28	6	6	17	6	17	10	6
Trc S8	6	6	10	18	14	6	12	6	38	6	32	30	6	24	20	14	26	30

AM: ampicillin, TIC: ticarcillin, TCC: ticarcilin/ clavulanic acid, AMC: amoxicillin/clavulanic acid, CF: cefalotin , FOX: cefoxitin, GM: gentamicin, FEP: cefepim, CXM: cefuroxim, TM: tobramycin, AN: amikacin, CTX: cefotaxim, NET: netilmicin, CAZ: ceftazidim, IPM: imipinem, TE: tetracycline, C: cloramphenicol, SXT: cotrimoxazol.

band with a pI value of 8.6. Also, they presented additional bands consistent with TEM-1 penicillinase with a pI value 5.4 in S2, S4, S5 and S8 or OXA-1 penicillinase with a pI value of 7.4 in S1, S3, S5, S6 and S7.

According to the isoelectric points, PCR experiments were performed using crude DNA extracts from the clinical strains and primers specific for them, CTX-M-type, OXA-type, TEM- or SHV-encoding genes, and then the amplicons were directly sequenced. Sequence analysis demonstrated that all strains of *E. cloacae* contained an identical $bla_{\text{CTX-M-28}}$ gene, differing from $bla_{\text{CTX-M-15}}$ by the change of an Asp to Asn at positions 288. The strains S2, S4, S5 and S8 had the same nucleotide sequence of the $bla_{\text{TEM-1}}$ gene. Finally, S1, S3, S5, S6 and S7 expressing the ESBL with a pI value of 7.4 exhibited an identical $bla_{\text{OXA-1}}$ gene. Alignment of the DNA sequence and of the amino acid sequence of these β-lactamases against that of CTX-M-28, TEM-1 and OXA-1 was performed (http://prodes.toulouse.inra.fr/multalin/multalin.html) and the blast of the amino acid sequences of these β- lactamases were performed (http://www.ncbi.nlm.nih.gov/).

Epidemiological analysis

The strains were first compared with regard to their antibiotic resistance phenotypes. These strains were then typed by AP-PCR and ERIC-PCR using five different primers. Results obtained showed that the eight isolates gave different restriction patterns and can be clustered in four clones (Figure 1). The four *E. cloacae* strains elaborating the CTX-M-28, TEM-1 and OXA-1 enzymes yielded a strictly identical pattern (clone E1). These isolates were recovered in the Intensive care unit and hygiene unit. Two isolates that produced CTX-M-28 and OXA-1 have the same profile and were isolated in the same ward (clone E2). The strain S6 and S8 yielded two distinct Enterobacterial repetitive intergeric consensus sequences PCR patterns.

DISCUSSION

At the Military hospital of Tunis, 8 *E. cloacae* strains were found to be highly resistant to penicillins, the extended spectru cephalosporines (cefotaxime, ceftazidime, ceftriaxone and

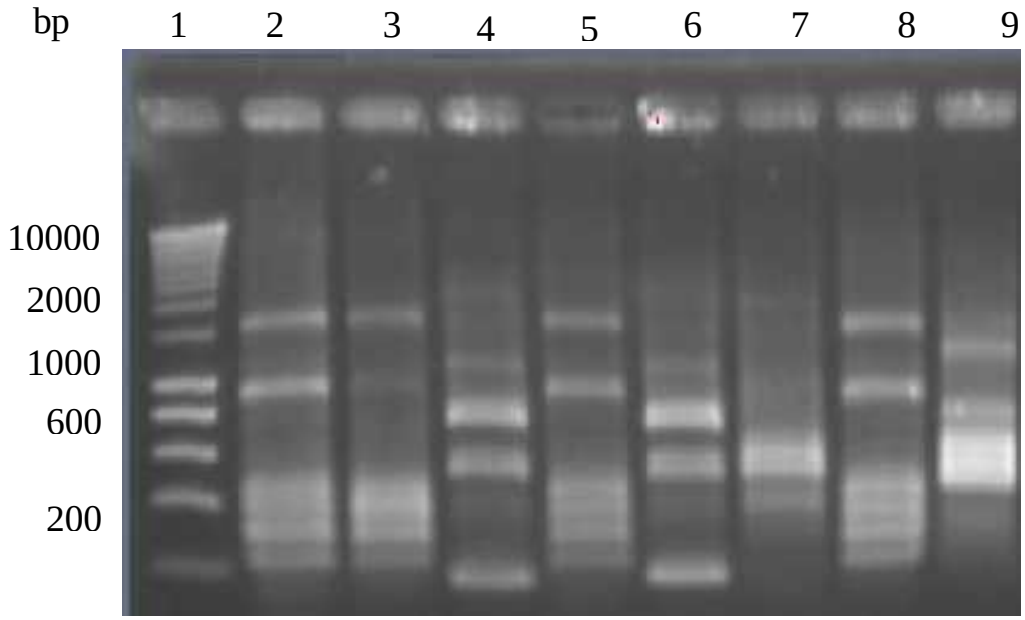

Figure 1. ERIC-PCR profiles obtained with the primer ERIC-2 from *E.cloacae* S1 (lane 2), S2 (lane 3), S3 (lane 4), S4 (Lane 5), S5 (lane 6), S6 (lane 7), S7 (lane 8) and S8 (lane 9). Lane 1 show band patterns of DNA marker fragments (sizes in bp are indicated on the edge of the gel).

cefpirome) and to the association amoxicilline-clavulanic acid. Resistance to others antibiotic of these strains was also demonstrated. Analysis of these strains by the disk diffusion test revealed synergies between clavulanic acid and ceftazidime, cefotaxime which suggested the production of an extended-spectrum β-lactamases. By polymerase chain reaction and sequencing, these isolates were found to produce an extended-spectrum β-lactamase, CTX-M-28. All of these strains were multiresistant, producing other β-lactamases type of TEM-1 and OXA-1. Many strains that express CTX-M β-lactamases were multidrug resistant. Genes conferring resistance to aminoglycosides and tetracycline and other bla genes have been found on the same plasmids as the bla_{CTX-M} genes. Genes conferring plasmid-mediated quinolone resistance have also been associated with blaCTX-M genes (Mendonça et al., 2007).

These strains (producing CTX-M-28) were isolated from differents wards. On the basis of the antibiotype and the ERIC-PCR results, these isolates differ according to their spicemen. Indeed, six of them were isolated in the Intensive care unit from different origin. Three isolates producing CTX-M-28 and OXA-1 were epidimiologically related. In the same ward, two isolates producing CTX-M-28 and TEM-1 showed the same profil by ERIC-PCR, suggesting probable clonally spread. The strains from the neonatal and hygiene wards were unrelated. However, the CTX-M-28 and OXA-1-producing strains were found to carry very similar *ECOR*I and *Hind*III restriction plasmid patterns, thus indicating the diffusion of the same plasmid. The same result was found for the CTX-M-28 and TEM-1-producing strains. Epidemiological studies showed that the dissemination of CTX-M-28 could be the consequence of both strain spreading and plasmid diffusion.

Among the different groups of described ESBLs, nowadays, the production of CTX-M enzymes is an emerging phenomenon that has been called 'the CTX-M pandemic' (Abbassi et al., 2008). All our strains harboured the $bla_{CTX-M-28}$ gene of which the first detection was reported by Li et al. (2007). This enzyme differs from $bla_{CTX-M-3}$ by a single nucleotide (A750G) within the PCR amplicon (Kingsley and Verghese, 2008). Compared to other enzymes of the CTX-M-1 group, different acid substitutions were found, among them the Asp240Gly substitution. From molecular modeling studies of class A ESBLs, amino acid substitutions at residue 240 have been associated with expansion of activity towards ceftazidime, most probably because of its position in a key β-strand of the catalytic site of class A β-lactamases (Matagne et al., 1998). These results also appear to represent the in vivo evolution of CTX-M-type β-lactamase genes (Jeong et al., 2005).

In Tunisia, CTX-M-28 enzyme was recently reported in a clinical strain of *Klebsiella pneumoniae* (Achour et al., 2008). According to this study, the CTX-M-28-producing *K. pneumoniae* was obtained from the intensive care unit of the Military hospital in Tunisia. Since, our strains (*E. cloacae*) were recovered from the same ward and in the same hospital; it is possible to suppose the dissemination of the CTX-M-28-encoding plasmid by horizontal transference.

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