Occurrence of conjugative IncF-type plasmids harboring the *bla*$_{CTX-M-15}$ gene in Enterobacteriaceae isolates from newborns in Tunisia

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BACKGROUND: CTX-M-15 is the dominant type of extended-spectrum β-lactamase in clinical isolates. This enzyme constitutes the most widespread enzymes in Tunisia. In this study, we were interested to understand the causes of the evolutionary success of CTX-M-15 in a Tunisian university hospital.

METHODS: A total of of 72 cefotaxime-resistant Enterobacteriaceae were isolated from newborn patients at the hospital Taher sfar Mahdia in Tunisia and characterized their genetic support by means of molecular techniques.

RESULTS: Isolates were clustered into various clonal groups, although most isolates belonged to sequence types ST39 (*Klebsiella pneumoniae*) and ST131 (*Escherichia coli*). F replicons (FIA, FIB, and FII) were the most frequently detected replicon types in our collection (91.66%).

CONCLUSION: This is the first report of QnrB- and CTX-M-15-encoding large IncF-type conjugative plasmids in Tunisia.

Most countries have recently experienced the rapid dissemination of Enterobacteriaceae isolates producing extended spectrumBackground: β-lactamases (ESBLs) (1,2). During the past decade, CTX-M-type enzymes have represented the most rapidly growing group of ESBLs, and CTX-M-15 (a variant of CTX-M-3, resulting in a single Asp-240-Glu substitution) has recently emerged as the dominant type of plasmid-borne acquired cefotaximase in Gram-negative pathogens, which caused outbreaks in both nosocomial and community settings (3,4). Clonal outbreaks of CTX-M-15-producing Enterobacteriaceae have been reported in many countries, including France, Italy, Spain, Portugal, Austria, Norway, the United Kingdom, Tunisia, South Korea, and Canada, being *Escherichia coli* is the most frequently involved organism (5). Plasmid carrying the *bla*$_{CTX-M-15}$ gene are mostly incompatibility group FII plasmids (6,7). Furthermore, different genetic elements have been shown to be involved in the mobilization of *bla*$_{CTX-M-15}$ including ISEcp1-like insertion sequences, which are the most commonly reported (8).

In the present study, we report the emergence of CTX-M-15-producing isolates from a recent collection of Enterobacteriaceae strains from newborn and characterized their genetic support by means of molecular techniques to better understand the causes of the evolutionary success of this β-lactamase in Tunisia.

RESULTS

From January to July 2011, 72 multiresistant ESBL-producing clinical isolates of Enterobacteriaceae were collected from the Microbiology Laboratories at the University Hospital of Mahdia in Tunisia. The isolates were recovered from various pathological specimens (**Table 1**).

ESBL detection performed by the double-disk diffusion test revealed synergies between amoxicilin–clavulanic acid and oxyiminocephalosporins (cefotaxime, ceftazidime) containing disks with higher level of resistance to cefotaxime for all isolates, suggesting the production of a cefotaxime-type extended-spectrum β-lactamase. The most isolates showed similar susceptibility profiles, characterized by high minimum inhibitory concentration value (>1,024 μg/ml) for cefotaxime and ticarcillin, while being resistant to ceftazidime, cefepime, and aztreonam. Furthermore, some of the strains were also found to be resistant to ciprofloxacin (**Table 2**).

Polymerase chain reaction (PCR) analysis for β-lactamase genes of the family TEM, SHV, and CTX-M showed amplification products only for *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes. DNA sequence analysis of these PCR products revealed that all isolates carried both the *bla*$_{CTX-M-15}$ and the *bla*$_{TEM-1}$ genes class. Detection of qnrA, qnrB, and qnrS quinolone resistance determinants revealed that 63 of 72 isolates carried the qnrB1 gene, while qnrB4 was detected in a single isolate of *Klebsiella oxytoca* isolate (**Table 1**). The copresence of qnrB4 and CTX-M-15 genes was not, at our best knowledge, previously reported in Tunisia. PCR mapping of the genetic environment surrounding the *bla*$_{CTX-M-15}$ identified the same ISEcp1 elements in the upstream region of all resistant strains. The presence of the IS*Ecp1* element in the vicinity of *bla*$_{CTX-M}$ genes was analyzed as reported

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Table 1. Characteristics of CTX-M-15 producing the study isolates

Isolates	Source	Ward	PFGE type	Sequence type	ESBL	QNR	Replicon(s)
Klebsiella pneumoniae (*n* = 48)	Rectal	Pediatrics	A1	ST39 (*n* = 45)	CTX-M-15	B1	FIIAs
			B1	ST39 (*n* = 2)	CTX-M-15	—	L/M
			C1	ST4 (*n* = 1)	CTX-M-15	—	L/M
Escherichia coli (*n* = 18)	Rectal	Pediatrics	A2	ST131 (*n* = 16)	CTX-M-15	B1	FIIAs
			B2	ST69 (*n* = 2)	CTX-M-15	—	L/M
Serratia marcescens (*n* = 2)	Rectal	Pediatrics	A3	ND	CTX-M-15	B1	FIIAs
			B3	ND	CTX-M-15	—	FIA FIB
Citrobacter freundii (*n* = 1)	Rectal	Pediatrics	ND	ND	CTX-M-15	—	FIA FIB
Enterobacter cloacae (*n* = 1)	Rectal	Pediatrics	ND	ND	CTX-M-15	—	L/M
E. aeroginosa (*n* = 1)	Rectal	Pediatrics	ND	ND	CTX-M-15	B1	FIIAs
Klebsiella oxytoca (*n* = 1)	Rectal	Pediatrics	ND	ND	CTX-M-15	B4	FIC

ND, not determined.

Table 2. Antibiotic susceptibility of study isolates

Isolate and/or PFGE type	MIC(s) (μg/ml) of antimicrobials							
	TIC	CTX	CAZ	FEP	FOX	AZT	IMP	CIP
Klebsiella pneumoniae								
Type A1	≥1,024	≥1,024	256	512	4	512	0.125	32
Type B1	≥1,024	≥1,024	128	256	2	128	0.5	1
Type C1	≥1,024	≥1,024	128	256	2	128	0.5	1
Escherichia coli								
Type A2	≥1,024	≥1,024	256	512	4	512	0.125	32
Type B2	≥1,024	≥1,024	128	256	2	128	0.5	1
Serratia marcescens								
Type A3	≥1,024	≥1,024	256	512	4	512	0.125	32
Type B3	≥1,024	≥1,024	512	512	2	512	0.006	2
C. bruki	≥1,024	≥1,024	512	512	2	512	0.006	2
Enterobacter cloacae	≥1,024	≥1,024	128	256	2	128	0.5	1
E. aerogenes	≥1,024	≥1,024	256	512	4	512	0.125	32
Klebsiella oxytoca	≥1,024	≥1,024	128	512	4	256	0.5	16

ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; TIC, ticarcillin.

previously (9). It was located 48 bp upstream from $bla_{CTX\text{-}M\text{-}15}$ demonstrating that this gene was present in widespread IS*Ecp1* transposition modules (10).

PFGE analysis of the 72 CTX-M-15-producing isolates showed two different types of two *E. coli*, three clone of *Klebsiella pneumoniae*, and two clone of *Serratia marcescens* isolates. Isolates belonging to the same clonal group were recovered from the same ward, indicating the local simultaneous dissemination of several enterobacterial strains/clones rather than the spread of a single clone. Isolates of all PFGE types were subjected to multilocus sequence typing (**Table 1**). The *K. pneumoniae* isolates represented sequence types ST39 and ST4, which are identified the first time in Tunisia. The *E. coli* isolates represented STs belonging to international clones from humans, ST131 and the ST69, which have been identified as an important cause of infection outbreaks in different communities (11–13) and the most prevalent clonal groups among CTX-M-15-producing *E. coli* isolates in Europe, Asia, North America, and Africa (11,14).

Cefotaxime and ceftazidime resistance was successfully transferred by conjugation from all CTX-M-15-producing strains representative of the different clones. Production of ESBLs was detected in all transconjugants by the double-disk synergy test and the presence of the CTX-M-15 was confirmed by sequence analysis of PCR product from these transconjugants. These findings indicate that the CTX-M-15 was located in conjugative plasmids and confer resistance to cefotaxime and ceftazidime. Southern hybridization analysis revealed that each clinical isolate carried a single plasmid encoding $bla_{CTX\text{-}M\text{-}15}$ with size of 100 kb and over (data not shown). Classification based on plasmid incompatibility showed that the F replicons (FIA, FIB, FIC, and FII) were the most frequently detected replicon types in our collection (91.66%). These results are in accordance with those of other authors (15,16).

DISCUSSION

This study demonstrates the predominant presence of CTX-M-15-producing Enterobacteriaceae among ESBL-positive isolates recovered from hospitalized newborns in Hospital Tahar Sfar in Tunisia. The most of the plasmids transferred from the clonal strains studied, belonged to the incompatibility (Inc) group. This, in addition to the presence on the same DNA fragment of the particular set of resistance genes described above, including $bla_{CTX\text{-}M\text{-}15}$, suggested the presence of a multidrug resistance region similar to that described for plasmid pC15-1a by Lavollay *et al.* (17). Plasmid pC15-1a is a circular molecule of 92,353 bp consisting of two distinct regions. The first is a 64-kb region that is essentially homologous to the non-R-determinant region of plasmid R100 except for several point mutations, a few small

insertions and deletions, and the absence of Tn*10*. The second is a 28.4-kb multidrug resistance region that has replaced the R-determinant region of the R100 progenitor and consists mostly of transposons or partial transposons and five copies of the insertion element IS*26* (18). In fact, the majority of the *bla*$_{CTX-M-15}$ genes were mobilized on various multiresistance IncF-type plasmids, harboring multiple addiction systems that presumptively contribute to their maintenance (19). These addiction models such as toxin–antitoxin systems modules (*pemK–pemI*, *hok–sok*, or *ccdA–ccdB*) have been described for the first time in Tunisia by mnif *et al.* (20). Curiously, the L/M replicon was detected in six of the strains (8.33%), although this replicon has been considered to be rare in Enterobacteriaceae isolates from humans (21). The spacer describes above between the ISEcp1 and the start of the gene *bla*$_{CTX-M-15}$ carried by L/M plasmid is 48 bp and not 127 bp as decribed for CTX-M-3 and CTX-M-15 in Algeria (22). This group of plasmids was initially responsible for the spread of CTX-M-3 in Poland since common plasmid scaffolds were identified in eight species in 15 hospitals (23). The representative plasmid of that family was pCTX-M-3, first observed in 1996 in *C. freundii* isolates in which CTX-M-3 had been originally identified (24). IncL/M plasmids carrying the *bla*$_{CTX-M-3}$ gene were also reported in Eastern countries, France, Belgium, and South Korea and very often encoded, in addition to CTX-M-3 ESBL, the aminoglycoside resistance gene *armA*. IncL/M family is also responsible for the spread of the class D carbapenemase OXA-48 that has been identified in many clonally unrelated strains and different enterobacterial species from distantly located geographic areas (25).

This is the first report of a CTX-M-15 occurrence in Tunisia associated with large conjugative plasmids with exclusive IncF replicon-type. IncF plasmids have been reported to be of low copy number with more than one replicon promoting the initiation of replication. The multireplicon status has been described to be one means by which plasmids with a narrow host range can accomplish broad host range replication (26), which explains why they are widely diffused in clinically relevant Enterobacteriaceae, representing one of the most frequently encountered plasmid types. Addiction systems encoded by these plasmids also contributed to the promotion of plasmid spread and adaptation to the host (19). Thus, The IncF plasmid family is clearly playing a major role in the dissemination of antimicrobial resistance in Enterobacteriaceae. Based on these findings, the high prevalence of CTX-M-15 is not only due to the spread of a single clone, mainly the pandemic ST131 clone, but is also due to horizontal transfer of multiresistance IncF, which remains widely scattered across Tunisia.

METHODS

Bacterial Isolates

A total of 72 consecutive, nonredundant multidrug-resistant clinical isolates of Enterobacteriaceae were recovered from rectal swabs of newborn hospitalized patients at the University Hospital of Tunisia in the period January–June 2011. All isolates were identified using the VITEK 2 system (bioMérieux, La Balme-les-Grottes, France) and the API 20E system (bioMérieux). The collection consisted of 48 *K. pneumoniae*, 18 *E. coli*, 2 *Serratia marcescens*, 1 *Enterobacter aerogenes*, 1 *Citrobacter freundii*, 1 *Enterobacter cloacae*, and 1 *K. oxytoca*.

Antimicrobial Susceptibility Testing

All the study isolates were phenotypically screened for the production of ESBLs using the double-disk synergy test (27). The minimum inhibitory concentration was determined by a dilution technique on Mueller-Hinton liquid medium with inoculums of 10^6 colony-forming units (CFU). The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (28).

Characterization of β-Lactamase and qnr-Encoding Genes

The detection of ESBL-encoding genes was performed by PCR using primers specific for the amplification of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ genes (29). Detection of *qnrA*, *qnrB*, and *qnrS* quinolone resistance determinants was performed similarly by multiplex PCR (30).

Genetic Environment of *bla*$_{CTX-M}$ Genes

The genetic organization of *bla*$_{CTX-M}$ was investigated by PCR and by sequencing the regions surrounding these genes. Primers used to investigate the surrounding regions of the *bla*$_{CTX-M}$ are performed according to ref. (31).

Molecular Epidemiology

The clonal relation between the different clinical isolates was studied by pulsed-field gel electrophoresis using the XbaI restriction enzyme for 4 h at 37 °C to generate the macrorestriction fragments prior to electrophoretic separation (1% agarose gel). All the different clones were characterized by multilocus sequence typing according to the protocol described previously (32,33).

Transfer of Resistance

Transfer of the gene was studied by conjugation experiments with rifampicin-resistant *E. coli* MKD-135 recipient strain, as described previously (34). The transconjugants were selected with 2 μg/ml cefotaxime and 400 μg/ml rifampicin and they are subjected to an ESBL screening test and PCR to confirm the possible acquisition of *bla*$_{CTX-M}$.

Hybridization

Plasmid DNA extraction was performed by using the Kieser method (35) followed by a Southern transfer of an agarose gel containing plasmid DNA of CTX-M-positive isolates onto a nylon membrane (Hybond N$^+$; GE Healthcare, Orsay, France). Plasmid DNA hybridization was performed as described by Sambrook *et al.* (36). The probes specific for the CTX-M genes consisted of PCR products generated from respective *CTX-M*-positive isolates. Labeling of those probes and signal detection were carried out using the electrochemiluminescence nonradioactive labeling and detection kit according to the manufacturer's instructions (GE Healthcare).

Identification of Plasmids by PCR-Based Replicon Typing

PCR-based replicon typing was applied to type the resistance plasmids using the major plasmid incompatibility groups among Enterobacteriaceae (37). Eighteen pairs of primers were designed to perform five multiplex- and three simplex-PCRs, recognizing FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA.

Ethical Information

The study was approved by the Military Hospital of Tunis, Tunisia. Parental informed consent was obtained.

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