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Emergence and Dominance of CTX-M-15 Extended Spectrum Beta-Lactamase Among *Escherichia coli* Isolates from Children

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Of forty-seven extended-spectrum cephalosporin-resistant *Escherichia coli* isolates, collected from children at the Children's Hospital in 2006 (Tunis, Tunisia), we analyzed 32 isolates that were genotypically different by enterobacterial repetitive intergenic consensus -polymerase chain reaction. For all isolates, the double-disk diffusion test revealed synergy between clavulanate and cefotaxime and/or ceftazidime, suggesting the production of extended-spectrum beta-lactamases. Polymerase chain reaction experiments, performed on plasmid DNA, and sequencing revealed the presence of $bla_{\text{TEM-1B}}$ (26 isolates, 81%), $bla_{\text{TEM-34(IRT-6)}}$ (3 isolates, 9%), $bla_{\text{SHV-12}}$ (2 isolates, 6%), and $bla_{\text{CTX-M-15}}$ (31 isolates, 97%). Further, the insertion sequence IS*Ecp1* was found upstream from the $bla_{\text{CTX-M-15}}$ gene in 11 isolates. The *bla* genes were found alone or in various combinations in a single isolate. $bla_{\text{TEM-1B}}$ and $bla_{\text{CTX-M-15}}$ genes were detected in 26 out of the 32 isolates. Three isolates harbored both $bla_{\text{TEM-34(IRT-6)}}$ and $bla_{\text{CTX-M-15}}$. $bla_{\text{SHV-12}}$ was identified either alone or with $bla_{\text{CTX-M-15}}$ in a single isolate. Our investigation showed the dominance of CTX-M-type extended-spectrum beta-lactamases, with CTX-M-15 particularly common, and to our best knowledge, this is the first report of the coexistence of CTX-M-15 and IRT-6 in *E. coli* isolates from children in Tunisia.

Introduction

Extended-spectrum β-lactamases (ESBLs) have emerged as a significant mechanism of resistance to extended-spectrum cephalosporins and monobactams in Gram-negative bacteria.[12,18,22,32,38] They are primarily reported in members of Enterobacteriaceae, but they have been found in other families of bacteria. *Escherichia coli* and *Klebsiella pneumoniae* have been prominent among ESBL-producing Gram-negative bacilli.[5,38] After the detection of the first ESBL, SHV-2 in Germany in 1983,[23] many types of ESBLs, exhibiting high degrees of diversity in their structure and activity, have been reported. Until the beginning of the 21st century, various surveys showed the predominance of ESBLs that were structurally related to the narrow spectrum TEM- and SHV-type β-lactamases although other types of ESBLs have been documented.[12,32] Since the end of the 1990s, a new family of ESBLs, the CTX-M-type β-lactamases, emerged worldwide, mostly from *E. coli* and *K. pneumoniae*.[7,14,16,29] Based on amino acid sequence similarity, at least five different lineages of CTX-M-type enzymes have been identified, indicated as CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups.[7] Most of the CTX-M β-lactamases, unlike other ESBLs, hydrolyze cefotaxime more efficiently than ceftazidime. However, CTX-M enzymes displaying an increased catalytic activity toward ceftazidime were reported (*e.g.*, CTX-M-15).[34]

The ESBL-encoding gene are usually found in transferable plasmids, and some of them are associated with mobile elements, particularly insertion sequences (IS). IS*Ecp1* element, a member of the IS1380 family, is often located upstream from $bla_{\text{CTX-M}}$ genes, belonging to different CTX-M groups (*e.g.*, CTX-M-15, CTX-M-14, and CTX-M-40 ESBLs), and may be involved in gene transfer; moreover, it has been shown that IS*Ecp1* can provide the promoter and direct transcription of $bla_{\text{CTX-M}}$ gene (*e.g.*, $bla_{\text{CTX-M-17}}$ gene in *K. pneumoniae*).[13,14,20,22,27]

In Tunisia, studies on ESBLs were first described in the early 1990s.[3] Data on molecular characterization of ESBLs, especially CTX-M-type, responsible for several outbreaks caused by extended-spectrum cephalosporin-resistant enterobacteria, have been reported since the beginning of

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2000s.[2,8,9,10,26] However, very limited data regarding the molecular investigation of ESBLs are available on children. In a previous work,[36] we found ESBLs in 5 of 76 ampicillin-resistant *E. coli* isolates collected over a 2-month period in 2002 at the Children's Hospital (Tunis, Tunisia). Three of them (4%) had SHV-type ESBLs, and two (2.6%) expressed ESBLs that were suspected to be CTX-M-type enzymes. Subsequently, extended-spectrum cephalosporin-resistant *E. coli* strains from children were increasingly isolated. We, therefore, designed the present study that explored the prevalence of diverse ESBLs among *E. coli* isolates recovered during a 10-month period (in 2006) at the Children's Hospital. Part of this work was presented at the 29th Interdisciplinary Meeting of Anti-infectious Chemotherapy (Paris, France, 2009).

Materials and Methods

Bacterial strains

Forty-seven extended-spectrum cephalosporin (cefotaxime or ceftazidime)-resistant *E. coli* isolates were collected in 2006 at the Microbiology Laboratory of the Children's Hospital (Tunis, Tunisia). Only one isolate per patient was investigated in the study.

Phenotypic screening for ESBLs

Double-disk synergy test was performed as a standard disk diffusion assay on Mueller-Hinton agar. Disks containing amoxicillin, cefotaxime, and ceftazidime were placed around an amoxicillin-clavulanate disk (Bio-Rad). Susceptibility breakpoints were those recommended by the French Society for Microbiology Guidelines (www.sfm.asso.fr). Enhancement of the inhibition zone toward the amoxicillin-clavulanate disk was taken as presumptive evidence of ESBL production. The E-test screening, based on the recognition of a reduction in the cefotaxime and/or ceftazidime minimum inhibitory concentrations (MICs) in the presence of clavulanic acid at 4 μg/ml, was subsequently assayed (AB, Biodisk).

Enterobacterial repetitive intergenic consensus-polymerase chain reaction

Genomic DNA of *E. coli* isolates was extracted with Illustra Bacteria Genomic Prep Mini-Kit (G.E. Healthcare). Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) of genomic DNA (approximately 100 ng) was performed with ERIC-1 (CACTTAGGGG TCCTCAATGTA) and ERIC-2 (AAGTAAGTGACTGGGG TGAGCG) primers by using the following program: initial denaturation at 95°C for 5 min, annealing at 50°C for 1 min, and elongation at 72°C for 7 min in Analytik Jena AG Thermal Cycler (Analytik Jena AG). The resulting products were resolved in 1.5% agarose gels, stained with SYBR- Safe (Invitrogen), and visualized using a UV trans-illuminator. DNA molecular weight marker (O'Generuler™ 1Kb DNA ladder; Fermentas) was used as a molecular size standard.

Patterns were considered different when the profiles differed by at least one band.

PCR amplification and sequencing of bla *genes*

Plasmid DNA was purified with Gene JET plasmid miniprep Kit (Fermentas). PCR detection of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{PER-1}}$, and $bla_{\text{PER-2}}$ genes was performed with specific primers (Table 1) using 3 μl plasmid DNA. Primers ISEcp1F, designed from the *tnpA* gene of IS*Ecp1*, and CTX-M-1interR, an internal $bla_{\text{CTX-M-1}}$ group gene primer, were used to screen for the presence of IS*Ecp1* upstream from $bla_{\text{CTX-M-1}}$ group gene. PCR amplification was carried out as previously reported.[36] Cycling conditions were as follows: (i) an initial denaturation step of 5 min at 95°C; (ii) 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and (iii) a final extension step of 72°C for 10 min. PCR products were purified with Illustra™ GFX PCR DNA and gel band puri-

Table 1. Primers Used for Polymerase Chain Reaction Amplification and Sequencing of *bla* Genes and IS*Ecp1* Element

Target	*Primer*	*Primer sequence (5′-3′)*	*Position*	*GenBank accession number*
bla_{TEM}	TEMF	ATAAAATTCTTGAAGACGAAA	1–21	AB282997
	TEMR	GACAGTTACCAATGCTTAATCA	1059–1080	AB282997
bla_{SHV}	SHVF	ATGCGTTATATTCGCCTGTG	6–25	AF148850
	SHVR	TTAGCGTTGCCAGTGCTC	849–866	AF148850
$bla_{\text{PER-1}}$	PER1F	ATGAATGTCATTATAAAAGC	1409–1428	AY779042
	PER1R	AATTTGGGCTTAGGGCAGAA	2314–2333	AY779042
$bla_{\text{CTX-M-1}}$ group	CTXM1F	ATGGTTAAAAAATCACTGCGC	202–222	AY960984
	CTXM1intR	GACGTGCTTTTCCGCAATCG	2011–2030	AM003904
	CTXM1R	TTACAAACCGTCGGTGACGATTTAGC	1051–1077	AY960984
IS*Ecp1* element	ISECP1F	GCAGGTCTTTTTCTGCTCC	168–186	AM003904
	TNPAF	CTCACAAGCAACGAATTCATC	845–865	AM003904
$bla_{\text{CTX-M-2}}$ group	CTXM2F	TTAATGATGACTCAGAGCATTC	3–24	X92507
	CTXM2R	GATACCTCGCTCCATTTATTG	884–904	X92507
$bla_{\text{CTX-M-8}}$ group	CTXM8F	TGAATACTTCAGCCACACG	162–180	AY750914
	CTXM8R	TAGAATTAATAACCGTCGGT	1065–1084	AY750914
$bla_{\text{CTX-M-9}}$ group	CTXM9F	ATGGTGACAAAGAGAGTGCAAC	1–22	AF325134
	CTXM9R	CAGCCCTTCGGCGATGATTC	854–873	AF325134
$bla_{\text{CTX-M-26}}$ group	CTXM26F	ATGATGAGAAAAAGCGTAAGG	1–21	AY157676
	CTXM26R	ACCGTCGGTGACAATTCTGG	851–870	AY157676

fication Kit (G.E. Healthcare) and sequenced on both strands using a 3730 DNA analyzer (Applied Biosystem). The nucleotide sequences were analyzed with the software available at the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov/Blast.cgi).

Results

Forty-seven *E. coli* isolates were typed by ERIC-PCR. Genotypes presented by single isolates were called unique patterns. ERIC-PCR genotyping showed extensive diversity and identified 32 different types of 3 to 11 fragments, ranging in size from approximately 5 kb to less than 0.250 kb. Twenty-four of the 32 genotypes were unique (75%), whereas 8 genotypes (A, B, D, E, G, I, K, and L) included more than one isolate (Table 2). Based on the ERIC-PCR results, 32 of 47 isolates were further studied: 24 with unique banding patterns and 1 isolate each for the eight genotypes (A, B, D, E, G, I, K, and L).

Of the 32 *E. coli* isolates, 11 (34%) were recovered from urine, 10 (31%) from fecal swab, and 11 (34%) from various other specimens (Table 2). All 32 isolates, except 1, were resistant to ceftaxime (MIC >16 μg/ml); strain 22063 showed reduced susceptibility (MIC of 1 μg/ml). However, they displayed various degrees of resistance to ceftazidime: 8 isolates were resistant (MIC >8 μg/ml), 8 isolates showed intermediate resistance (MIC of 6 or 8 μg/ml), whereas 16 isolates were susceptible (MICs ranging from 0.5 to 4 μg/ml). The phenotypic screening method used in this study determined that all 32 isolates produced an ESBL. For all but one isolate, resistance to cefotaxime was reduced by clavulanic acid to a susceptible range (MICs, 0.016 to 0.125 μg/ml). Moreover, clavulanic acid enhanced the activity of ceftazidime in all cases. In particular, an 84- to >500-fold reduction in the MIC of ceftazidime, when combined with the inhibitor, was observed for ceftazidime-resistant isolates (Table 2).

PCR experiments performed on plasmid DNA with primers specific for 10 different *bla* genes yielded bla_{TEM}-positive

Table 2. Characteristics of CTX-M-Producing *Escherichia coli* Isolates from Children

Isolate	*Source*	*ERIC-PCR type (number of isolates)*	*MIC (μg/ml)* CTX	CTX/CLA	CAZ	CAZ/CLA	*β-lactamase(s)*	*Result for IS*Ecp1-bla$_{CTX-M}$ *(GenBank accession number)*
2941	Fecal swab	UP	>16	0.023	3	≤0.064	CTX-M-15; TEM-1B	
3373	Urine	Genotype A (4)	>16	0.064	16	0.125	CTX-M-15; TEM-1B	
4268	Intra-abdominal peritonitis	UP	>16	0.125	>32	0.094	CTX-M-15; IRT-6	
5455	Fecal swab	Genotype B (2)	>16	0.064	4	≤0.064	CTX-M-15; IRT-6	
7023	Pus	UP	>16	0.023	2	≤0.064	CTX-M-15; TEM-1B	
7336	Fecal swab	UP	>16	0.032	6	≤0.064	CTX-M-15; IRT-6	
7891	Fecal swab	UP	>16	≤0.016	2	≤0.064	CTX-M-15; TEM-1B	
9517	Fecal swab	UP	>16	0.023	3	≤0.064	CTX-M-15; TEM-1B	
10080	Fecal swab	UP	>16	0.064	8	0.125	CTX-M-15; TEM-1B	
13235	Fecal swab	Genotype D (2)	>16	0.032	3	≤0.064	CTX-M-15; TEM-1B	
13464	Urine	UP	>16	0.064	0.5	≤0.064	CTX-M-15; TEM-1B	+[a] (FR718870)
13888	Intra-abdominal peritonitis	UP	>16	≤0.016	3	≤0.064	CTX-M-15	
14236	Fecal swab	Genotype E (2)	>16	0.032	4	≤0.064	CTX-M-15; TEM-1B	
14237	Fecal swab	UP	>16	0.032	8	0.094	CTX-M-15; TEM-1B	+ (FR717863)
14700	Urine	UP	>16	0.094	>32	0.38	CTX-M-15; TEM-1B	
14970	Urine	UP	>16	0.023	6	≤0.064	CTX-M-15; TEM-1B	
15062	Urine	UP	>16	0.047	6	≤0.064	CTX-M-15; TEM-1B	+ (FR717864)
15149	Urine	UP	>16	0.125	24	0.125	CTX-M-15; TEM-1B	+ (FR718869)
16594	Blood	UP	>16	0.047	16	≤0.064	CTX-M-15; TEM-1B	+ (FR717862)
17685	Fecal swab	Genotype G (6)	>16	≤0.016	4	≤0.064	CTX-M-15; TEM-1B	
18402	Blood	UP	>16	0.023	6	0.094	CTX-M-15; TEM-1B	+ (FR717865)
18378	Intra-abdominal peritonitis	UP	>16	0.023	4	0.094	CTX-M-15; TEM-1B	
19038	Trachea	Genotype I (2)	>16	0.064	32	≤0.064	CTX-M-15; TEM-1B	+ (FR717866)
19596	Urine	UP	>16	0.047	8	0.094	CTX-M-15; TEM-1B	+ (FR717892)
19961	Urine	UP	>16	0.094	32	0.125	CTX-M-15; TEM-1B	+ (FR717893)
20283	Wound	UP	>16	>1	4	≤0.064	CTX-M-15; TEM-1B	
21104	Trachea	UP	>16	0.023	3	≤0.064	CTX-M-15; TEM-1B	+ (FR717894)
21977	Urine	UP	>16	0.023	3	≤0.064	CTX-M-15; TEM-1B	
22063	Urine	UP	1	≤0.016	3	≤0.064	SHV-12	
23093	Lung	Genotype K (2)	>16	0.047	32	0.125	CTX-M-15; TEM-1B	+ (FR718868)
23321	Pus	UP	>16	0.032	6	≤0.064	CTX-M-15; SHV-12	
23369	Urine	Genotype L (3)	>16	0.023	4	≤0.064	CTX-M-15; TEM-1B	

[a]+: presence of IS*Ecp1* upstream from $bla_{CTX-M-15}$ gene.

CAZ, ceftazidime; CAZ/CLA, ceftazidime/clavulanate; CTX, cefotaxime; CTX/CLA, cefotaxime/clavulanate; UP, unique pattern; ERIC-PCR, enterobacterial repetitive intergenic consensus-polymerase chain reaction; MIC, minimum inhibitory concentration.

results for 29 out of 32 *E. coli* isolates, whereas bla_{SHV} genes were found in 2 isolates. bla_{CTX-M} group1 genes were identified in 31 isolates resistant to cefotaxime. PCR-based screening for bla_{PER-1}, bla_{PER-2}, and the remaining groups of bla_{CTX-M} was negative in all isolates. Further, sequencing of the PCR products confirmed the identity of the genes and revealed the presence of bla_{TEM-1B} (26 isolates, 81%), $bla_{TEM-34(IRT-6)}$ (3 isolates, 9%), bla_{SHV-12} (2 isolates, 6%), and $bla_{CTX-M-15}$ (31 isolates, 97%). Our findings revealed that most strains had the same *bla* gene profiles. The *bla* genes were found alone or in various combinations in a single isolate. Thus, bla_{TEM-1} and $bla_{CTX-M-15}$ genes were detected in 26 out of the 32 isolates. Three isolates harbored both $bla_{TEM-34(IRT-6)}$ and $bla_{CTX-M-15}$. bla_{SHV-12} was identified either alone or with $bla_{CTX-M-15}$ in a single isolate (Table 2).

Sequence analysis of the region upstream from $bla_{CTX-M-15}$ gene indicated the presence of the IS*Ecp1* transposable element that was inserted in different locations. Thus, it was located 48 base pairs (bp) (10 isolates) and 128 bp (1 isolate) upstream from the $bla_{CTX-M-15}$ gene.

Discussion

Several studies that have examined ESBL types have illustrated an alarming increase in the prevalence of ESBLs, with CTX-M-type being the most dominant non-TEM-, non-SHV-type ESBLs among Enterobateriaceae.[7] Reports on ESBL types in Enterobacteriaceae in Tunisia found that more than 90% of the ESBL-positive *E. coli* isolates produced CTX-M-type ESBLs,[15,26] with similar findings in studies conducted in other countries such as Sweden,[17] Thailand,[22] Switzerland,[24] Lebanon,[28] Algeria,[35] and recently, CTX-M-type ESBLs were reported in 76% of *E. coli* isolates from Saudi Arabia.[4] The majority of the CTX-M-type ESBLs belonged to CTX-M group 1, mostly CTX-M-15, which has been found in *E. coli* isolates over a wide geographical area as reported in Tunisia,[15,25] Canada,[11] France,[18] Cameroon,[19] Turkey,[20] Thailand,[22] Egypt,[21] Portugal,[27] Lebanon,[28] and Algeria.[37]

Reports describing the prevalence of ESBL types among *E. coli* isolates from children are scarce, especially in Tunisia. The results of our study provided insights into the genetic characteristics of ESBLs among *E. coli* isolates at the Children's Hospital in Tunis. The identification of ESBLs among ESBL-positive *E. coli* isolates revealed an overwhelming occurrence of CTX-M-type β-lactamases, owing exclusively to CTX-M group 1, particularly CTX-M-15, which is consistent with data reported in Tanzanian[6] and Swiss children.[24] It is worrying that the high CTX-M-15 prevalence found in our study in children was similar to results recently reported in adults on ESBL types in Enterobacteriaceae, including 31 *E. coli* isolates, in a Tunisian university hospital.[15] On the other hand, our findings differed from those reported by Mamlouk *et al*.[26] In fact, among eight ESBL-producing enterobacteria isolates from pediatric patients at Charles Nicolle Hospital (Tunisia) between 2000 and 2003 (four *E. coli* and four *K. pneumoniae*), only one isolate (*K. pneumoniae*) was a CTX-M-15 producer and the remaining seven isolates encoded CTX-M-16, an ESBL within the CTX-M-9 cluster. Moreover, considering isolates from children, the predominance of CTX-M-15 in our investigation is not always a consistent finding worldwide. According to a study conducted at a children's hospital in Taiwan, Wu *et al.* found that CTX-M-3 was predominant.[39] On the other hand, in a survey carried on healthy children in Bolivia and Peru,[31] an increased diversity of CTX-M determinants was observed among commensal *E. coli* isolates, which could play as a potential reservoir of these clinically relevant resistance determinants.

Eleven $bla_{CTX-M-15}$-carrying isolates in this study were positive for IS*Ecp1* upstream from $bla_{CTX-M-15}$ gene, as was previously described for the $bla_{CTX-M-15}$ gene in *Enterobacteriaceae* isolates.[20,21,30,33] The IS*Ecp1* element was found at variable distances from $bla_{CTX-M-15}$ gene, a finding in agreement with other studies.[1,19,30] It is worth mentioning that no IS*Ecp1* element was detected in 20 $bla_{CTX-M-15}$-carrying isolates. There may be two reasons for this: (i) probably the IS*Ecp1* element was located far upstream from $bla_{CTX-M-15}$ gene, beyond the region covered by the primer used in our study, or (ii) IS*Ecp1* was not associated with $bla_{CTX-M-15}$ gene.

In the present study, SHV-type ESBLs were scarce (6%) compared with CTX-M-type ESBLs (97%), whereas no TEM-type ESBLs were detected. Similar to our study, Wu et al.[39] found SHV-12 but no TEM-type ESBLs in *E. coli* isolates, whereas Blomberg *et al*.[6] identified TEM-type ESBLs but no SHV-type ESBLs among *E. coli* isolates. Compared with a finding reported by Dahmen *et al*.[15] in adults in Tunisia, no TEM-type ESBLs were detected for both studies and no SHV-type ESBLs were found in *E. coli* isolates from adults.

Further, we noted that TEM-1B β-lactamase was the most common TEM variant found, being recovered in 81% of the isolates, whereas three isolates expressed the inhibitor-resistant β-lactamase TEM-34 (IRT-6). In our investigation, all TEM-producing isolates expressed CTX-M-15 β-lactamase. To our best knowledge, this is the first report of the coexistence of CTX-M-15 and IRT-6. The combination TEM-1B/CTX-M-15 has already been described in *E. coli* isolates from neonatal and pediatric wards in Algeria.[35] Both TEM-1 and CTX-M-15 β-lactamases were detected in one *K. pneumoniae* isolate but not in *E. coli* isolates recovered in a pediatric ward of a Tunisian hospital,[26] whereas the combination TEM-1/CTX-M-15 has been frequently reported in the adult population in Tunisia.[15,26]

In conclusion, this study highlights the high prevalence of CTX-M-15 ESBL detected in *E. coli* isolates from children. On the whole, considering the β-lactamase contents, the results of our study are in accordance with our previous findings,[36] whereas ESBL prevalence is considerably different. The IS*Ecp1* transposable element could be implicated in the spread of the $bla_{CTX-M-15}$ gene. This article reports different associations of β-lactamases. It is worrying that bacteria harboring multiple β-lactamases are isolated from children and we described the occurrence of CTX-M-15 and SHV-12, TEM-1B, or IRT-6 produced by single *E. coli* isolates. Such bacteria should be a cause for grave concern and may have important implications for physicians threatening children as therapeutic options used in children are limited.

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Disclosure Statement

No conflicts of interest.

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