Dissemination and genetic support of broad-spectrum beta-lactam-resistant *Escherichia coli* strain isolated from two Tunisian hospitals during 2004-2012.

Khaoula Ayari[1], Amel Bourouis[1], Hela Chihi[1], Sihem Mahrouki[1], Thierry Naas[2], Omrane Belhadj[1]

1. Laboratory of Biochemistry and technobiology, Faculty of Sciences of Tunis, University Tunis El Manar, 2092 El Manar II, Tunisia.
2. Bacteriology-Virology service. CHU Bicetre 78 rue du Général Leclerc, 94275 Le Kremlin-Bicetre.

Abstract

Background: The dissemination of extended-spectrum β-lactamase (ESBL)-producing bacteria presented a great concern worldwide. Gram-negative organisms such as *Escherichia coli* and *Klebsiella pneumoniae* are the most frequently isolated pathogens responsible for nosocomial infections.

Objectives: The aim of this study was to investigate and to follow the emergence of resistance and the characterization of Extended-Spectrum Beta-Lactamases (ESBL) among broad-spectrum beta-lactam-*Escherichia coli* clinical isolates recovered from the military hospital and Habib Thameur hospital in Tunisia.

Methods: A total of 113 *E.coli* isolates obtained during the period 2004 through 2012 showed a significant degree of multi-resistance. Among these strains, the double-disk synergy test confirmed the ESBL phenotype in 46 isolates. These included 32(70%) strains from Hospital A and 14(30%) from Hospital B.

Results: The ESBL was identified as CTX-M-15. The ESBL resistance was transferred by a 60 kb plasmid CTXM-15-producing isolates were unrelated according to the PFGE analysis and characterization of the regions surrounding the blaCTX-M-15 showed the ISEcp1 elements located in the upstream region of the bla gene and 20 of them truncated by IS26.

Conclusion: ESBL producing *E. coli* strains are a serious threat in the community in Tunisia and we should take into consideration any possible spread of such epidemiological resistance.

Keywords: CTX-M-15, diverse clones, ESBLs, *Escherichia coli*.

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Hospital A: Military hospital of Tunis; Hospital B: Habib Thameur hospital

Introduction

The production of extended-spectrum β-lactamases continues to be the important cause of resistance among gram negative bacteria.

The TEM- and SHV- are the first ESBLs type, mutant derivatives of established plasmid-mediated β-lactamases , however , a novel type of plasmid mediated ESBLs, the CTX-M enzymes, cefotaximases, emerged worldwide in the last decade, and preferentially hydrolyze cefotaxime over ceftazidime and are inhibited by clavulanic acid, sulbactam, and tazobactam[1] were reported in the second half of the 1980s classified in Ambler class A and in group 2be of the Bush, Jacoby and Medeiros classification[2].

More than 65 CTX-M β-lactamases were revealed worldwide. The phylogenic study clustered them in five major groups: CTXM 1,-2, -8, -9, and -25 groups[3-5] .

The number of reports studing the CTX-M variants continue to increase,CTX-M-28 enzyme was recently reported in Tunisa[6,7].

CTX-M-9, first described in 1994 in Spain[8], Guyana and the United Kingdom[9] also in Tunisa[10].

Also, in 2006, Boyd et al mention the first report of

Corresponding author:
Belhadj Omrane,
Laboratory of Biochemistry and technobiology,
Faculty of Sciences of Tunis,
University Tunis El Manar,
2092 El Manar II, Tunisia,
Tel/fax: +216 70 860 336.
Email: omrane.belhadj@fst.rnu.tn

CTX-M-16 type-producing *Enterobacteriaceae* in Tunisia and in Africa[11].
Another variant of CTX-M type, CTX-M-8 was detected in cefotaxime-resistant *Proteus mirabilis* strain in association with a plasmid mediated AmpC lactamase[12].
CTX-M-15 is the most prevalent ß-lactamase detected amongst the ESBL-positive *K. pneumoniae* and *E. coli* strains derived from CTX-M-3 by a substitution of Asp-240-Gly which increases its catalytic efficiency against ceftazidime[13,14] first described in 2001[15,16]
Many reports have documented the emergence of CTX-M gene[9], and the first report of the CTX-M-15 in Tunisia was cited in the Charles Nicolle Hospital in 1984 and it was described in various studies in Tunisia including that of coque et al, the gene has been found in E. coli strains in a Tunisian Hospital[17], France[18], and Central African Republic[19-25] .91% of the ESBL-producing isolates carried blaCTX-M-15 genes[21].
The production of CTX-M enzymes is an emerging phenomenon that has been called 'the CTX-M pandemic'[16].

The insertion sequence ISEcp1 was found to be involved in the mobility of blaCTX-M,was located upstream the bla CTX-M-27 gene e in a neonatal ward of the maternity department of Farhat Hached Hospital, Sousse[26] .It has been found also upstream the CTX-M-14 producing *E. coli* isolated from hospitalized patients in a university Hospital of Tunisia[27]., and upstream the CTX-M-15 gene in *Proteus mirabilis* and *Morganella morganii* isolated at the Military Hospital of Tunis[24].
ISEcp1 was located upstream of the blaCTX-M gene on *E. coli* isolates from food samples[28].
CTX-M genes may spread through clonal dissemination or horizontal gene transfer[19].

Methods

Bacterial strain

These clinical strains were isolated from samples collected in different wards, including the emergency (25, 86 %), reanimation (16.07 %), hemodialysis (4.56 %), neonatal (4.24 %), pediatrics (4.39 %), gastroenterology (13.32 %), external (12.56 %) and urology (19 %).

68% of strains were from urine, 17.8% from blood culture and 14.2% from Pus.

All the isolates were identified by the Vitek automated system (bioMérieux, Vitek 32) and API 20E system (bioMérieux, Marcy l'Etoile, France).

E. coli DH5a (recA1, F_, end A1, gyrA96, thi-1, hsdR17, rK_, mK+, supE44, relA1, DlacU69, F80lazDM15) and *E. coli* HB101 (F_, D(gpt-proA) 62, leuB6, supE44, ara-14, galK2, lac Y1, D(mcrc-mrr), rps, L26, Xyl-rmtl 1, thi-1, IncFI, rec AB, strr), were used respectively for the transformation and conjugation experiments.

Antimicrobial susceptibility and synergy testing

Routine antibiograms were determined by the disk diffusion method on Mueller–Hinton agar (MH, Diagnostics Pasteur) using susceptibility breakpoints as recommended by the Clinical and Laboratory Standards Institute (CLSI)[29].

The double-disk synergy test was used to detect the ESBL production as previously described[30,24] by using amoxicillin–clavulanate against cefotaxime, ceftriaxone, ceftazidime and aztreonam.
Minimum inhibitory concentrations (MICs) of selected anti-microbial agents were determined by using the dilution method on Mueller–Hinton agar according to CLSI guidelines[29].

Table 1 shows MICs (μg/mL) of various antimicrobial agents obtained for the clinical isolate *E. coli*, transconjugant and transformant, and the *E. coli* recipients.
CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; ATM, aztreonam; TIC, ticarcillin; STR, streptomycin; IMP, imipenem; ERT, ertapenem; CHL, chloramphenicol; TET, tetracycline; OFX, ofloxacin. NM, not measured.

Table 1: Primers used for detection of resistance genes.

PCR Target	Primer name	Primer sequence	Amplicon sizes (pb))	Annealing temperatures (°C)	References
CTX-M	CTX-M-A	5' TTT GCG ATG TGC AGT ACC AGT AA3'	544	57°C	(Edelstein et al., 2003)
	CTX-M-B	5' CGA TAT CGT TGG TGG TGC ATA3'			
TEM	TEM-F	5'ATGAGTATTCAACATTTCCGTG3'	844	55°C	(Yagi et al., 2000)
	TEM-R	5'TTACCAATGCTTAATCAGTGAG3'			
SHV	SHV-F	5' ATTTGTCGCTTCTTTACTCGC3'	861	55°C	(Essack et al., 2001)
	SHV-R	5' TTTATGGCGTTACCTTTGACC'			
ISEcp1	ISEcp1-A	5'GCAGGTCTTTTTCTGCTCC3'	490	57 °C	Lartigue et al., 2004)
	ISEcp1-B	5'ATTTCCGCAGCACCGTTTGC3'			

β-Lactamase extraction and isoelectric focusing

IEF was performed as described previously[17,31], using a culture grown overnight at 37°C in Trypto-Caseine Soy broth (TCS). These exponentially growing bacteria were harvested at 17,400 _ g (Rotor F 0650, Bekman) and bacterial suspensions were prepared by sonication in UP 400 S (dv. Hielscher, Germany) five times for 45 s each time.

Crude extract was centrifuged at 17,400 _ g (Universel 32 R, Hettich) for 15 min at 4°C.

The supernatant of the sonicate was subjected to isoelectric focusing on ampholine polyacrylamide gel with a pH range of 3-10 at a voltage range of 100-300, at 4°C in a 111Mini IEF Cell (Bio-Rad). TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-3 (pI 6.3), SHV-1 (pI 7.6) and SHV-12 (pI 8.2) were used as pI markers.

β-lactamase activities were revealed by iodemetric method using benzylpenicillin to 1 mM and cefotaxime (3 mM) as substrates in phosphate buffer (25 mM; pH 7).

β-Lactamase assay

Hydrolytic activities of crude extracts for β-lactam antibiotics were determined by the spectrophotometric method at the wave length of maximal absorbance for the

β-lactam ring of each antibiotic.[32]

The decrease in absorbance of the antibiotics at an appropriate concentration was measured in a temperature controlled spectrophotometer (Varian R CARY 50 Bio UV-visible) at 37°C.

Specific activity is calculated on depending of Ross and O'Callaghan equation in 1975[33]

Effect of inhibitors (IC50 determination)

Crude enzyme extract was incubated with clavulanic acid and sulbactam increasing concentrations. EDTA was used at a fixed concentration of 1 mM. Residuals β-lactamases activities were determined by the spectrophotometric method using cephalothin 1 mM as substrate. The inhibitor concentration required to inhibit 50% of enzyme activity was determined graphically (IC50)[34].

Analysis of plasmids and transfer of resistance

Plasmid DNA was extracted with the alkaline lysis method, as described by Sambrook et al[35].

Conjugation experiments were carried out with *E. coli* HB101, as previously described[7,24]. (31; 9;3;4).

The transconjugants were selected on LB agar supplemented with streptomycin (100 µg/ml) and ampicillin (100 µg/ml).

Transformation experiments were carried out by using *E. coli* DH5α as the recipient as previously described[31,36].

Transformants were selected on Luria-Bertani medium agar plates supplemented with ampicillin (100 mg/ml).

Transformants were subjected to DDST to confirm the presence of ESBL genes and were examined for co-transfer of other antibiotic resistance determinants present in the donor clinical isolates by disk diffusion.

Characterization of the resistance genes using PCR method and sequencing

Primers used for amplification of resistance genes, annealing temperatures and predicted amplicon sizes are shown in Table 1. They were used to detect blaCTX-M, blaSHV, blaTEM and the sequences surrounding the bla CTX-M gene, ISEcp1and IS26[37,38] also the aac(6')-ib-cr gene.

The DNA amplification programs consisted of initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing temperatures differed according to the primer pair used and were for 45 s at 57°C for the blaCTX-M and blaISEcp1; 55°C for blaTEM and blaSHV. Finally, the polymerization for 5 min at 72°C.

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, USA) and sequenced using the Big-Dye Terminator v.3.1 Cycle Sequence Kit and ABI Prism 310 automatic sequencer (Applied Biosystems, USA).
The nucleotide sequences were compared with those included in the GenBank database using BLAST (http:/www.ncbi.nlm.nih.gov/blast/).

Detection of ISEcp1
PCR for ISEcp1 was used to examine the region upstream of the the blaCTX-M gene with the primers shown in Table 1. An annealing temperature,57°C was used followed by sequencing of the PCR product.

Typing of isolates by MLST
Molecular typing was performed for one representative *E. coli* isolate by MLST by PCR amplification of the seven housekeeping loci (adk, fumC, gyrB,icd, mdh, purA and recA).
The amplicon was sequenced and compared with the MLST database.(http://mlst.ucc.ie/mlst/dbs/Ecoli)[39].

Molecular typing using Pulsed-field gel electrophoresis (PFGE)
The epidemiological relationship of CTX-M-15 positive strains was studied by PFGE using XbaI as restriction enzymes (Bio-Rad Laboratories, France).
Restriction fragments of DNA were separated by electrophoresis which was performed in a 1.2% agarose gel on a CHEF DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA) with 6V/cm for 19 h at 14ºC with an initial switch time of 2.2 to 52.0 sec.
XbaI-digested DNA were compared visually based on differences in the number and mobility of bands[40].

Results
Studied strains show multidrug resistance phenotype with various antibiotics. They were highly resistant to antibiotics such as penicillins, cephalosporins, aminoglycosides, quinolones and tetracycline, (MIC 512- 64µg/mL) whereas remained susceptible to imipinem (MIC <2µg/ml) and meropenem (MIC <0,5µg/ml) (Table 2). Similar results were observed with the transformants and transconjugants except with quinolones, phenicol and aminoglycosides, suggesting that this resistance could be in chromosome.
46 *E. coli* strains were phenotypically confirmed as ESBL with the double disc synergy showing a marked synergy between ceftazidime, céfotaxime, aztreonam, ceftriaxoneand amoxicillin–clavulanic acid on MH agar plates as well as the transformants and transconjugants suggested the presence of a class A ESBL.

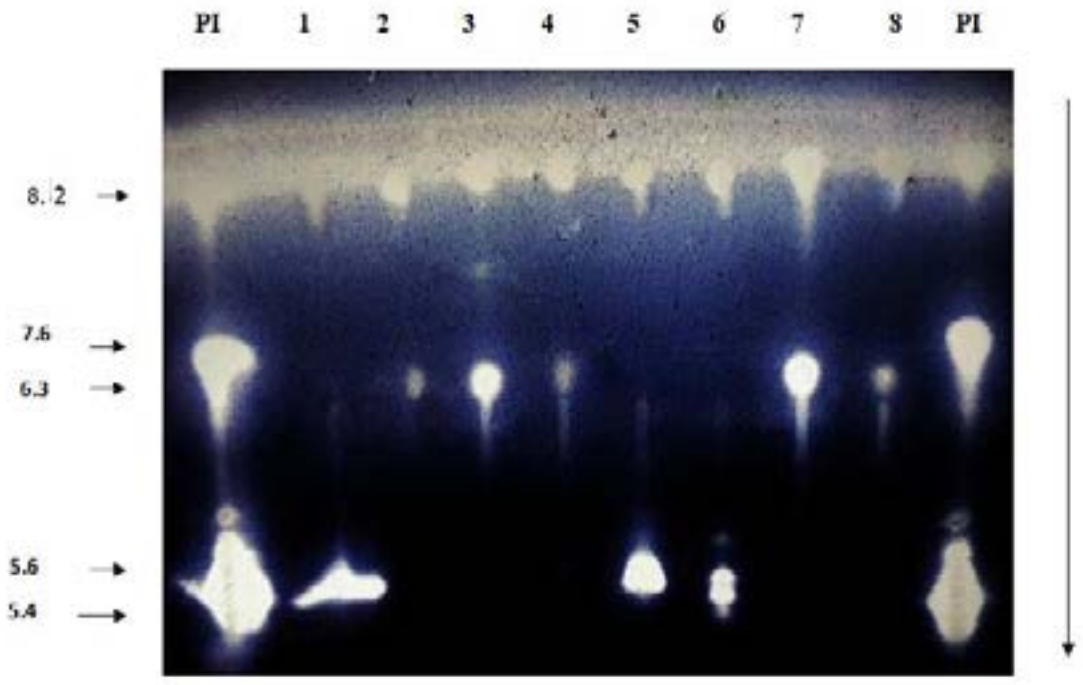
Table 2: MICs (µg/mL) of various antimicrobial agents obtained for the clinical isolate *E. coli*, transconjugant and transformant, and the *E. coli* recipients.

Isolates	Year of isolation	Ward	Specimen	MIC (mg/l)										
				Cefotaxime	Ceftriaxon	Ceftazidime	Aztreonam	Ticarcillin	Streptomycin	Imipenem	ERT	CHL	Tetracyclin	OFX
E.coli 4836	2011	urology	urine	>512	>512	>512	>512	>512	128	<2	0 .5	128	64	64
E. coli HB101 X E. coli 4836				512	512	512	128	512	256	<2	0.5	32	64	128
E. coli HB101				<2	<2	<2	<2	8	2	<2	<2	<2	<2	<2
E. coli DH5a/ E. coli 4836				512	512	16	512	>512	256	≤0 .006	<0.5	8	4	<2
E. coli DH5a				<2	<2	<2	<2	<2	<2	≤0 .006	<0.5	2	<2	<2

The resistance to expanded-spectrum cephalosporins was successfully transferred to a recipient strains, *E. coli* HB101 with the frequency of transfer equivalent to 5×10^{-5} transconjugant/recipient and this indicated that the gene was plasmidic transferred with an estimated molecular size of 60 kb of the transferable plasmid.

An extended-spectrum β-lactamase band with a pI 8.6 with cefotaxime as substrate was detected in the 46 ESBL strains (Figure 1), transformants and transconjugants.

Figure1: Isoelectric focusing (7%) with cefotaxime for 1mM as substrate of the E. coli strains 1-8: E. coli strains; pI: pI markers TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-3 (pI 6.3), SHV-1 (pI 7.6) and SHV-12 (pI 8.2)



Kinetics resuts showed that *E. coli* strains hydrolyze benzylpenicillin, ticarcillin with a high level of hydrolytic activity, specific activities ranged between (8.77-6.67U/mg of protein) and hydrolyze cephalosporins with a higher hydrolytic activity to cefotaxime (U/mg of protein) (Table 3).

Table 3: Specific activities β -lactamases of *E. coli* strain 4836 and its transconjugant (µmol of substrate hydrolyzed/min/mg of protein).

Antibiotics	*E. coli* 4836	*E. coli* HB101 X *E. coli* 4836
Benzylpenicilli	8.77	2.63
Ticarcillin	6.67	2.43
Imipenem	ND	ND
Cefoxitine	0.654	0.755
Cefotaxime	5.16	2.55
Ceftriaxone	0.239	0.192
Ceftazidime	0 .16	0.116
Aztreonam	0. 455	0.430
Cefpirome	0.31	0.02

ND: non detected.

These enzymes are not inhibited by EDTA and are not defined as metallo-enzymes. The analysis of the IC50 showed that the clavulanic acid with IC50 of 3.5 µM was the powerful inhibitor and categorized this enzyme to class A: serine active β-lactamases (Table 4).

PCR analyses confirmed the presence of blaCTX-M-3-related genes in parental strain *E. coli* 4836, and transformant *E. coli* DH5α/ E.coli 4836 and transconjugant *E. coli* HB101 X, *E.coli* 4836 indicating that this gene is located on conjugative plasmid.Sequencing of the deduced amino acid, followed by BLAST searches, and confirmed blaCTX-M as blaCTX-M-15.

This enzyme preferentially hydrolyzed cefotaxime over ceftazidime.

Table 4. Inhibitors effect of activities β-lactamases of *E. coli* 4836 strain (IC_{50})

E. coli 4836	IC_{50} (µM)		
	Clavulanic acid	Sulbactam	EDTA
	3.5	14.7	-

(–): without effect

15 strains produced the quinolone resistance determinants aac(6´)-Ib-cr whereas ISEcp1 sequence was found in CTX-M-15- producing isolates.

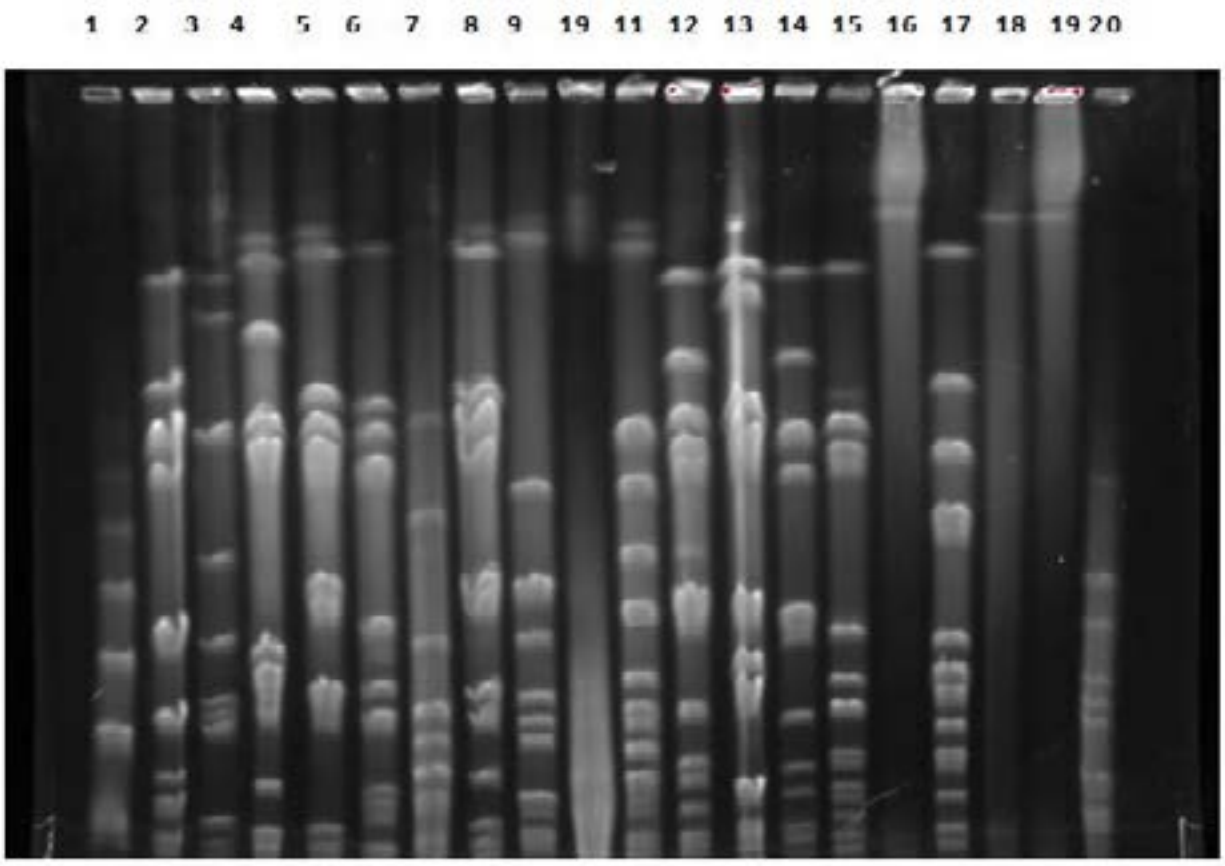
When using the specialized primers reversibly of IS sequences (ISEcp1 and the IS26) with blaCTX-M-15 primers, We came to the result that the ISEcp1 sequence was found upstream of the blaCTX-M-15 gene.

On the other hand, IS26 transposase region was detected upstream of the ISEcp1 sequence on 22 of our studied *E. coli* isolates.

According to the MLST analysis of one *E. coli* isolate, it showed that this belonged to ST 131, displayed specific O25 type (O 25b) and the *E. coli* isolate belonged to the B2 phylogenetic subgroup I.

PFGE (Figure 2) showed that the 46 strains presented different profiles, and our studied strains were unrelated suggesting that the dissemination of bla CTX-M-15gene among *E. coli* clinical strains due to horizontal transfer of multi- resistance or/and the genetic mobile element are responsible for the dissemination of these gene.

Figure 2. Pulsed-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA of the *E. coli* isolates producing CTX M-15 (Lane 1-20)



Discussion

Our study investigated the genetic environment of blaCTX-M genes in fourty six *Escherichia coli* extended spectrum β-lactamase (ESBL)-positive isolates. Of these 46 strains, 10 *E. coli* isolates (24 %) produced ß-lactamase activities with a varied isoelectric points (pIs) between 5.5 and 8,6 (lane 1,5 and 6, Figure 1), they carried the blaTEM gene (9 from hospital A and 1 from hospital B). Sequencing of PCR products of this latter showed that it corresponded to the TEM-1.

9 of the studied *E. coli* isolates (26 %) presented a basic pIs of 7.3 and 8.6 (lane 2,3,4,7 and 8, Figures 1). They carried the blaSHV gene (8 from hospital A and 1 from hospital B) and it corresponded to SHV-1 by sequencing.

The presence of the insertion sequence ISEcp1upstream the gene in all the CTX-M-15 strain producers is a real concern which plays an important role of the mobilization of this latter as has been reported in several studies[16,41].

According to the MLST analysis of one *E. coli* isolate, it showed that this belonged to ST 131, displays specific O25 type (O 25b) and the *E. coli* isolate belonged to the B2 phylogenetic subgroup I. This phylogroup B2 ST131 is previously described on *E.coli* CTX-M producers responsible for the clonal diffusing of the CTX-M-15 gene[41,42] and this isolate may belong to this clone.

The presence of the blaCTX-M-15 encoding gene amongst unrelated strains argued for genetic transit of mobile elements amongst unrelated strains. Our study confirms that the CTX-M-15 gene is the most prevalent ESBL found in Tunisian hospitals and the association of the bla CTX-M-15 with the insertion sequence ISEcp1 facilitates its dissemination.

In short with reference to our insightful study, we should control the emergence of ESBL producing *E. coli* strains from hospitals environment which in some cases lack both the efficient surveillance and cleaning services.

This finding is alarming for healthcare providers and reinforces other previous studies in Tunisia to prevent a further spread and in the light of the current and fabulous medical breakthroughs, that we are now witnessing on a daily basis of, has to be a wake-up call for us to further enhance our efforts and better our practical measures.

Conflict of interest

There is no conflict of interest.

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