REPORT FOR POST-DOCTORAL RESEARCH
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INTRODUCTION

Gram-negative bacteria of the *Enterobacteriaceae* family as *Escherichia coli*, *Enterobacter* ssp, *Citrobacter* ssp and *Serratia* ssp are nosocomial pathogen responsible of various types of infections, such respiratory tract infections, urinary tract infections, bacteremia and meningitis, particularly in intensive care, neonatal and surgical units. β-lactam antibiotics are the most frequently prescribed in clinical medicine. However, problems associated with the development and spread of β-lactam resistance among bacteria have been increasing and are viewed as a major threat to clinical practice. Production of extended-spectrum β-lactamase (ESBLs) is the major mechanism of resistance to expanded-spectrum β-lactam antibiotics (as cefotaxime, ceftriaxone, cefpirome and ceftazidime) in Gram-negative rods.

The aim of our study was to investigate the involvement of ESBLs in resistance to expanded-spectrum β-lactams in strains of *Enterobacteriaceae* isolated at the Children's Hospital of Tunis, Tunisia in 2006.

MATERIAL AND METHODS

Bacterial strains

Clinical isolates, including *Escherichia coli* (n=60), *Enterobacter cloacae* (n=18), *Enterobacter aerogenes* (n=1), *Citrobacter koseri* (n=1), *Citrobacter freundi* (n=1), *Serratia marcescens* (n=2) and *Serratia liquefaciens* (n=1), were collected in 2006 from patients hospitalized at the Children's Hospital of Tunis. The isolates were obtained from different specimens.

Susceptibility testing

Routine antibiograms were determined by the disk diffusion method on Muller-Hinton agar using cefotaxime, ceftazidime, cefepime, aztreonam and ciprofloxacin disks. Phenotype of the strain (resistant or susceptible) was determined by measuring the inhibition zone of bacteria growth around the disk. Results were interpreted as recommended by the French Society for Microbiology Guidelines. The presence of ESBLs was inferred by a synergy image, using the double-disk synergy test that was performed with a expanded-spectrum of cephalosporin and amoxicillin-clavulanic acid disks.

Nucleic acid extractions

Whole-cell DNAs and plasmid DNAs were extracted from strains using Illustra bacteria genomic prep mini spinKit and miniprep kit fermentas, repectively.

Genomic fingerprinting by repetitive extragenomic palindromic (REP) and enterobacterial repetitive intergeneric consensus (ERIC)-PCR

Strain relationships were established by ERIC- and REP-PCR. The PCR mixture contained 0.25 mM each dNTP, 0.4 μM primer, 100 ng of genomic DNA, 1.5 U of GoTaq

polymerase in a total volume of 50 µl. The amplification protocol consisted of the following steps: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation 95°C for 30 s, annealing at 50°C for 1min, and a final extension step at 72°C for 10 min. Amplified PCR products were separated using 1.5% agarose gels and visualized by UV transillumination. DNA fingerprints were compared by visual inspection.

PCR of ESBLs genes and sequencing

The detection of ESBLs genes encoding to TEM-, SHV-, CTX-M (groups 1, 2 8, 9 and 25), PER-1 and PER-2 enzymes were performed on plasmid preparations using appropriate primers leading to the amplification of the total gene. The nucleotide sequence of the upstream region of the *bla*$_{CTX-M}$ genes were determined using primers derived from the nucleotide sequence of the *tnpA* gene of IS *Ecp1* insertion sequence.

The PCR mixture contained 0.25 mM each dNTP, 0.4 µM primer, 100 ng of genomic DNA, 1.5 U of GoTaq polymerase in a total volume of 50 µl. The amplification protocol consisted of the following steps: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation 95°C for 1min, annealing at 52°C for 1min, and a final extension step at 72°C for 10 min. Amplified PCR products were separated using 1% agarose gels and visualized by UV transillumination. PCR products were sequenced with the 3730 sequencer (applied biosystem). The nucleotide and deduced amino acid sequence were analyzed and compared to sequences available over the internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.gov).

Crude extract

ESBL-producing strains were grown in 100 ml of brain heart infusion broth. The bacteria collected by centrifugation were suspended with 25 mM of phosphate buffer pH 7 and disrupted by ultrasonic treatment. Crude extracts were obtained after centrifugation of the sonicate at 13000 rpm. Protein concentration was determined using the BCA Kit.

Isoelectric focusing and spectrophotometry

Isoelectric focusing was performed using 40 µg of crude extract, of strains expressing different ESBLs, with polyacrylamide gel containing ampholines with pH ranging from 3.5-9.5 at 1500 volts/1h30min (Multiphor II, Pharmacia). Visualization of β-lactamase activity was carried out with a filter paper overlay containing 1 mM of nitrocefin.

Spectrophotometric ESBL activities were performed against the following substrates (0.08 mM) at the corresponding wavelengh: 255 nm for the ceftriaxone ($\Delta\varepsilon$ -7700$M^{-1}.cm^{-1}$), 260 nm for ceftazidime $\Delta\varepsilon$ (-9000 $M^{-1}.cm^{-1}$) and cefpirome $\Delta\varepsilon$ (-4500$M^{-1}.cm^{-1}$).

Transferability of *bla*$_{CTX-M}$ gene

Transferability of *bla*$_{CTX-M}$ gene was tested by broth mating assay using *E. coli* DH5α strain (resistant to nalidixic acid, free of plasmids) as a recipient at a 1:10 donor:recipient ratio. Transconjugants were selected on brain-heart (or LB) agar plates containing ceftriaxone at 2 µg/ml and nalidixic acid at 75 µg/ml and incubated for 24 h.

RESULTS

Resistance patterns

As determined by disk-diffusion antibiotic susceptibility testing, the majority of isolates are resistant to cefotaxim. Table 1 showed the number of strains having resistant phenotype of the studied strains, including those with intermediate phenotype. All isolates, with intermediate or resistant phenotype demonstrated synergy between clavulanic acid and cefotaxim, or cefepime, indicating ESBL production.

Table 1. Number of strains with resistant phenotypes

Strains	Antibiotics			
	cefotaxime	Ceftazidime	Aztreonam	Ciprofloxacin
E.coli	57	50	53	14
E. cloacae	17	18	17	1
E.aerogenes	1	1	1	0
C. freundi	1	1	1	0
C.Koseri	1	0	0	0
S.marcescens	2	2	2	0
S.liquefaciens	1	1	1	0

Differentiation of *E. coli* and *E. cloacae* strains

ERIC-PCR and REP-PCR amplifications of genomic DNA of the *E. coli* and *E. cloacae* strains generated 38 and 13 different patterns, respectively. These strains, and the remaining enterobacteria of our collection, were investigated for the identification of ESBLs.

ESBLs patterns

DNA sequencing of PCR products, using plasmid preparations and appropriate primers, revealed that the predominant ESBL type is the CTX-M type, in particular those of group1. Thus, CTX-M-15 ESBL was found in: *E. coli* (37 strains), *E. cloacae* (6), *C. koseri* (1), *C. freundi* (1), *S. marcescens* (2) and *S. liquefaciens* (1). CTX-M-3, a group 1 CTX-M type ESBL, was found in only one case, a strain of *E. coli*. The SHV-type ESBL: SHV-12 and SHV-2a were also detected. SHV-2a was found only in *E. cloacae* (5 strains), SHV-12 was found in *E. coli* (2 strains), *E. cloacae* (1), *E. aerogenes* (1), *C. koseri* (1), *C. freundi* (1), *S. marsescens* (2).

PCR amplifications were negative for the genes encoding the following ESBLs: CTX-M- (of groups 2, 8, 9 and 25), TEM, PER-1 and PER-2. Partiel *tnpA* gene sequence of the IS *Ecp1* insertion sequence was located upstream the $bla_{CTX\text{-}M\text{-}3}$ and the $bla_{CTX\text{-}M\text{-}15}$ genes (9 *E. coli*, 6 *E. cloacae* and the 3 *Serratia ssp*)

ESBL activities and isoelectric points

Crude extracts of four strains expressing the four different ESBLs, were tested spectrophotometrically for their activities against ceftriaxone, cefpirome and ceftazidime (table 2).

Table 2. Specific activities against expanded-spectrum cephalosporins

Strains expressing ESBL	A.S μmoles/min/mg of proteins	
	ceftriaxone	cefpirome
E. coli (CTX-M-3)	0.47	0.24
E. coli (CTX-M-15)	0.96	0.37
E. coli (SHV-12)	0.018	0.04
E. cloacae (CTX-M-15)	1.11	0.55
E. cloacae (SHV2a)	Not detected	Not detected

Activities against ceftazidime were not detected in all cases.

Analytical IEF of crude extracts, of the four strains expressing different ESBLs, revealed the nature of CTX-M-type enzymes, detected by molecular analysis, consistent with the presence of ESBLs with pI of 8.6 for CTX-M-3 and CTX-M-15. However, SHV-12 and SHV-2a activities were not detected by IEF in conditions applied in the present study.

Transferability of the $bla_{CTX\text{-}M\text{-}15}$ gene

According to our results, CTXM-15 is the most frequent ESBL. Transferability of the corresponding determinant was assayed in mating experiments with strains representative of clones or species. Transfert of plasmid was successful in 7 cases (4 *E. coli*, 2 *E. cloacae* and 1 *C. koseri*). In the 7 case, transconjugates were positive by PCR for $bla_{CTX\text{-}M\text{-}15}$ gene.

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ABSTRACT OF THE WHOLE WORK
(POST-DOCTORAL RESEARCH
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ESBLs were investigated in a collection of *Enterobacteriacea* of non repetitive strains of 38 *E. coli* and 13 *E. cloacae*, *Enterobacter aerogenes* (n=1), *Citrobacter koseri* (n=1), *Citrobacter freundi* (n=1), *Serratia marcescens* (n=2) and *Serratia liquefaciens* (n=1). These strains, and the remaining enterobacteria of our collection, were investigated for the identification of ESBLs. DNA amplifications by PCR and sequencing revealed the presence of four types of genes encoding CTX-M-3, CTX-M-15, SHV-2a and SHV-12 ESBLs. CTX-M-15 is the most frequently encountered (49 strains), whereas CTX-M-3 was found in one strains, SHV-2a in five strains and SHV-12 in eight strains. ESBLs of TEM and PER-types were not detected. Specphotometric activities against expanded-spectrum β-lactam antibiotics (ceftriaxone, cefpirome and ceftazidime) were investigated in crude extracts of strains expressing ESBLs. Specific activities (SA) against ceftriaxone were higher in strains of *E. coli* and *E. cloacae* (1 U/mg proteins) producing CTXM-15 and CTX-M-3 (1 U/mg).Whereas, ceftriaxone was less efficiently hydrolyzed in the case of strains producing SHV-ESBLs. Cefpirome, was also more efficiently hydrolyzed in the cases of strains producing CTXM-3 and CTX-M-15 than those expressing SHV-ESBLs. $bla_{CTX\text{-}M\text{-}15}$ gene, the most frequently detected in the present study, was transferable by mating experiments tested. The $bla_{CTX\text{-}M\text{-}15}$ gene was located downstream the *tnpA* gene of the IS *Ecp1* insertion sequence.