

## Emergence of extended-spectrum beta-lactamase-producing *Escherichia coli* in community-acquired urinary infections in Casablanca, Morocco

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### Abstract

**Introduction:** Extended-spectrum beta-lactamase- (ESBL)-producing *Escherichia coli* are an increasingly significant cause of community-acquired infection worldwide. The aim of this study was to assess the prevalence of ESBL-producing *E. coli* in a community, to analyze the relationship between strains studied, and to characterize the ESBL genes involved in this resistance.

**Methodology:** ESBL production was detected by the double disk synergy test. Genes encoding ESBLs (*bla*<sub>TEM</sub>, *bla*<sub>CTM</sub>, *bla*<sub>SHV</sub>) were identified by PCR and DNA sequencing. Conjugation experiments were performed to check the transferability of antibiotic resistance genes. Strain inter-relationships were studied by pulsed field gel electrophoresis.

**Results:** Seven ESBL-producing *E. coli* were identified among the 535 *E. coli* isolates. Most of them expressed a CTX-M enzyme (6/7) with a predominance of CTX-M-15 (6/6). Two strains possessed TEM in combination with CTX-M-15 or SHV-5. Plasmid content and gene transfer analysis showed that resistance genes were carried by high molecular weight conjugative plasmids. PFGE analysis showed that the strains were not clonal.

**Conclusions:** ESBL-producing *E. coli* from urinary tract infections in Casablanca belong to different clones and carry mobile beta-lactamase genes. It is therefore essential to monitor the epidemiology of ESBLs in *E. coli* and related organisms locally to effectively combat resistance.

**Key words:** urinary tract infection; *Escherichia coli*; community; ESBL; PFGE; Morocco

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### Introduction

Beta-lactam agents such as penicillins, cephalosporins, monobactams and carbapenems are molecules of choice to treat a variety of infections. Their introduction into therapy was rapidly followed by the reports of resistance. Microorganisms producing extended-spectrum beta-lactamases (ESBL) were identified in early the 1980s, shortly after the introduction of oxyimino-beta-lactam.

ESBLs are enzymes that compromise the efficacy of all beta-lactams, except cephamycine and carbapenems, by hydrolysis of the beta-lactam ring. They are encoded by plasmids generally derived from TEM or SHV, but the predominance of CTX-M has increased considerably since 1995 in most parts of the world. The CTX-M genes appear to have been captured by transferable plasmids of *enterobacteriaceae* from the chromosome of an

environmental *Kluyvera spp.* More than 80 CTX-M types have been identified and they are divided into five groups based on their amino acid identities [1]. CTX-M types have now replaced TEM and SHV as the most common type of ESBL.

ESBLs are often encoded by genes located on large plasmids, which also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulfonamide, tetracycline, and the chloramphenicol. Furthermore, recent studies have demonstrated the co-transfer of *qnr*, encoding reduced susceptibility to the quinolones, with ESBLs on a plasmid [2]. The acquisition and accumulation of resistance determinants have given rise to multidrug resistant ESBL producers, further limiting therapeutic options and subsequent dissemination of these populations because of their co-selection by various antimicrobials [3].

ESBL-producing *Escherichia coli* were initially described in the hospital setting, but these organisms have begun to disseminate into the community and have become widely prevalent in community settings of many parts of the world [4-8]. Furthermore, these bacteria seem to have been imported from the community into hospital settings.

The aim of our study was to determine the prevalence of ESBL-producing *E. coli* from urinary tract infections in a Moroccan community setting, to analyze the relationship between the clones isolated, and to identify genes involved in resistance and their genetic support.

## Methodology

### *Bacterial isolates*

A total of 535 *E. coli* isolates were collected from urine specimens at the medical biology centre at the Pasteur Institute of Morocco during the period of July 2004 to July 2007. All species were identified both by conventional techniques and the API 20E system (BioMérieux, Marcy l'Etoile, France)

### *Antimicrobial susceptibility testing*

Susceptibility to antimicrobials was determined by disc diffusion on Mueller–Hinton agar-containing plates, following the Clinical and Laboratory Standards Institute recommendations [9]. Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam or cefepime. Minimum inhibitory concentration (MICs) were determined using the E-test method, according to the manufacturer's recommendations (AB Biodisk, Solna, Sweden).

### *Detection of beta-lactamase genes and sequencing*

Total DNA extraction was performed for all samples using the heat-shock technique [10]. The presence of betalactamase genes was detected by PCR using the following primers: *bla*<sub>SHV</sub>, ( SHV-F: CGCCGGGTTATTCTTATTTGTCGC, SHV-R : CGCCGGGTTATTCTTATTTGTCGC ), *bla*<sub>CTX-M</sub> (CTX-M-F :GGTAAAAAATCACTGCGTC; CTX-M-R: TTGGTGACGATTTAGCCGC) and *bla*<sub>TEM</sub> (TEM-F: ATAAAATTCTTGAAGACGAAA; TEM-R :GACAGTTACCAATGCTTAATCA) as previously described [11].

PCR products were analyzed by agarose gel electrophoresis and purified amplicons were sequenced using an ABI 310 sequencer.

### *Genetic transfer*

Conjugation experiments were performed by the liquid mating out assay as previously described [12] using sodium azide-resistant *E. coli* K<sub>12</sub>J<sub>5</sub> as a recipient. Transconjugants were selected on brain heart infusion (BHI) agar supplemented with 200 mg/L sodium azide, and 2 mg/L ceftazidime. When not successful at the first attempt, mating experiments were repeated up to three times.

PCR was used to test for *bla* genes in transconjugants as described above for clinical isolates.

### *Plasmid analysis*

Plasmid DNA extraction was performed by a rapid alkaline lysis procedure [13]. The plasmid separation was done onto a 0.8% agarose gel. The size of the separated plasmid was estimated by using plasmids with known molecular weight.

### *Genetic relationships*

Genetic relationships of the ESBL-producing *E. coli* were assessed using pulsed field gel electrophoresis (PFGE) after digestion with XbaI. The PFGE analysis of XbaI-digested genomic DNA was performed using a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA) according to the instruction manual. Bacterial DNA and XbaI digestion was prepared as previously described [14]. Fragment separation was done in 1% agarose gel with the following conditions: 6 V/cm, for 24 hours with switch times of 5-60 seconds in TBE buffer pre-chilled to 12°C. PFGE patterns were interpreted according to criteria described previously by Tenover *et al.* [15] and then according to the Dice similarity coefficient and demonstrated graphically. The patterns were considered to belong to the same type if there was a difference of no more than three bands.

## Results

A total of 535 *E. coli* isolates were collected at the Center of Medical Biology at the Pasteur Institute of Morocco, Casablanca, between July 2004 and July 2007. Of these strains, 7 single ESBL-producing *E. coli* isolates (1.3%) collected from seven patients were selected in accordance with CLSI criteria.

**Table 1.** Antimicrobial susceptibility profiles and *bla* genes detected by PCR in seven ESBL-producing *E. coli*:

Strains	Amx	Amc	Cf	Tic	Fox	Ctx	Caz	Fep	imp	Fos	Gm	Tm	Ak	Sxt	Nal	Cip	<i>bla</i> types
E1	R	R	R	R	S	R	R	R	S	S	R	R	S	R	R	R	CTX-M-15
E2	R	R	R	R	S	R	R	R	S	S	R	R	S	R	S	S	CTX-M-15
E3	R	R	R	R	S	R	R	R	S	S	R	R	S	R	R	R	CTX-M-15
E4	R	R	R	R	S	R	R	R	S	S	R	R	S	R	R	R	TEM-1/CTX-M-15
E5	R	R	R	R	S	R	R	R	S	S	S	R	S	R	R	R	CTX-M-15
E6	R	R	R	R	S	R	R	R	S	S	R	R	S	R	S	S	CTX-M-15
E7	R	R	R	R	S	R	R	R	S	S	R	R	R	R	R	R	SHV-5/TEM-1

Amx: Amoxicillin; Amc: amoxicillin/clavulanate; Cf: Cefalotine; Tic: Ticarcilin; Fox: Cefoxitin; Ctx: Cefotaxime; Caz: ceftazidime; Fep: Cefepime; Fos: Fosfomycine; Imp: Imepinem; Gm: Gentamycine; Tm: Tobramycine; Ak : Amikacine ; Sxt: Sulfamethoxazol; Cip: Ciprofloxacin; Nal: Nalidixic Acid.

#### Antimicrobial susceptibility of clinical isolates

All ESBL-producing *E. coli* were resistant to amoxicillin, amoxicillin + clavulanate, cefalotin, ticarcillin, cefotaxime, ceftazidime and trimethoprim-sulfamethoxazole. In contrast, these isolates were susceptible to fosfomycin, cefoxitine and imipenem.

All but one of the ESBL-producers were resistant to gentamicin and tobramycin. Only one of the seven ESBL-producing *E. coli* isolates showed resistance to amikacin. Regarding quinolone resistance, five isolates were resistant to nalidixic acid and ciprofloxacin (Table 1).

#### Characterization of beta lactamases

Multiple beta-lactamase genes were identified in the ESBL-producing isolates. The CTX-M-1 group was detected in all strains except the E7 isolate. It was also observed in the combination of *bla* genes in two isolates. Sequencing with group-specific primers showed that the *bla* types identified were CTX-M-15, TEM-1 and SHV-5 (Table 1).

#### PFGE analysis

PFGE fingerprinting patterns of most of the seven ESBL-positive isolates were apparently different from each other indicating that these strains were not clonally related (Figure 1). The Dice coefficient analysis revealed that strains E4 and E5, presenting a similarity of more than 85%, could be classified together.

#### Transfer of antibiotic resistance

We tested whether *bla* genes in studied isolates were transferable by conjugation. Transconjugants were obtained from only three of the isolates.

Generally, the transconjugants had antibiotic beta-lactam resistance profiles similar to those of the donor clinical isolates (Table 2).

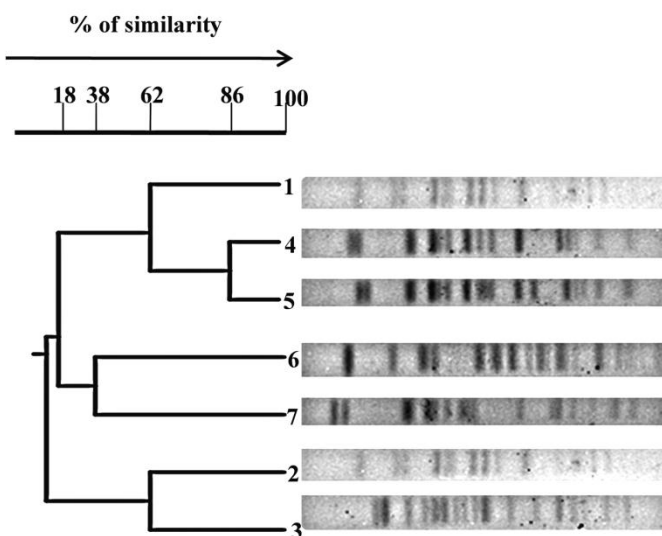
All transconjugants (Except Tc3, which is tobramycin susceptible) were resistant as donor strains to gentamicin and tobramycin indicating a co-transfer of *bla* genes and aminoglycoside resistance genes.

The clinical strain E3 was resistant to nalidixic acid and ciprofloxacin, but the transconjugant made from this strain was quinolone sensitive.

Resistance profiles and PCR confirmation of the transconjugants indicated co-transfer of *bla*<sub>CTX-M-15</sub> and *aac* (6')-Ib-cr.

Analysis of plasmid content of strains hosting the ESBL genes and their transconjugants, depicted in

**Figure 1.** Estimates of genetic relationship according to Dice similarity coefficient of *Xba*I patterns obtained for ESBL-producing *E. coli* strains



**Table 2.** The MICs of clinical isolates, *E. coli* transconjugants and conjugation recipient to seven antibacterial agents

Codes	CAZ		CTX		FEP		AN		K		GM		TM		NAL		CIP	
	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC	MIC	CC
E2	16	R	>256	R	>256	R	8	S	1	S	48	R	32	R	8	S	0.5	S
Tc2	24	R	24	R	96	R	3	S	0.064	S	8	R	16	R	2	S	0.02	S
E3	8	I	8	R	64	R	6	S	64	R	16	R	12	R	512	R	32	R
Tc3	48	R	>256	R	>256	R	3	S	0.094	S	12	R	2	S	2	S	0.25	S
E6	12	R	>256	R	128	R	6	S	2	S	64	R	16	R	4	S	0.02	S
Tc6	48	R	>256	R	256	R	2	S	2	S	8	R	8	R	2	S	0.5	S
K <sub>12J5</sub>	0.5	S	0.5	S	3	S	0.75	S	0.75	S	0.5	S	0.5	S	2	S	0.02	S

Figure 2, showed that these resistance genes were carried by conjugative plasmids of high molecular weight.

**Discussion**

Over the past 20 years there has been increased resistance to beta-lactam antibiotics by production of ESBL mediated by plasmids. This type of resistance is now observed in all species of *Enterobacteriaceae* and currently disseminated worldwide.

ESBL-producing *E. coli* have been described in hospitals as causing infectious outbreaks, but the presence of this microorganism has also been reported in community [16,17,18]. During our survey, seven ESBL-producing *E. coli* strains were isolated from the urinary tracts of seven non-hospitalized patients in Casablanca city, Morocco. This study showed an overall prevalence rate of 1.3%. This value is similar to those recorded in the community of some countries such as France (1.1%); Spain (1.4% to 1.7%); and Brazil (1.48%) [17, 19,20]. In the United States and Tunisia, this prevalence is around 3% [21,22].

Data on the sensitivity of ESBL-producing strains showed that these strains are not only resistant to beta-lactams but also to other classes of antibacterials including as gentamicin, trimethoprim-sulfamethoxazole and quinolones.

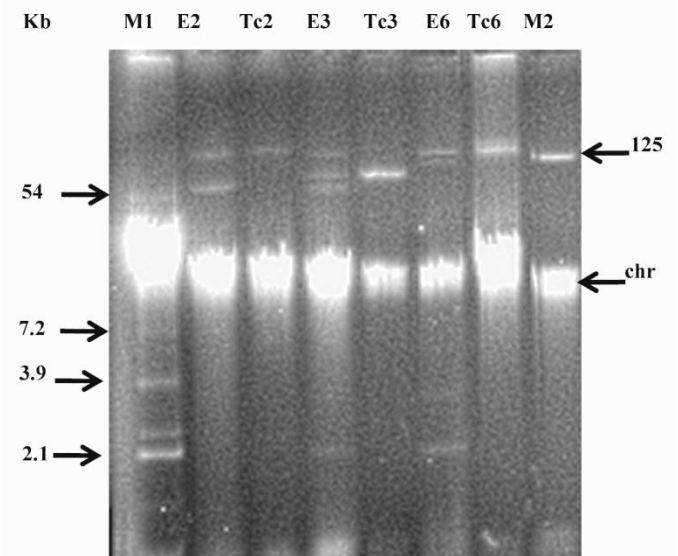
Until now, no study has shown the frequency of certain ESBL types in Moroccan communities. In this study, a variety of enzymes were detected among the ESBL-producing *E. coli* isolates, similar to results previously reported in other countries [5,19,23].

The most frequently encountered mechanism of resistance to beta-lactams found in this study was the production of CTX-M-type beta-lactamase. These results are consistent with those reported by previous studies performed elsewhere in which the majority

(over two thirds) of the ESBL producing *enterobacteriaceae* reported was *E. coli* and most of them ( $\geq 85\%$ ) expressed CTX-M enzymes [24-29]. This is the reason why some authors suggest that an increase in ESBL-producers could be ascribed to clonal expansion of CTX-M producing *E. coli*.

In this study, pulsed-field gel electrophoresis of *Xba*I macro-restricted genomic DNA yielded 7 distinct PFGE profiles for the studied isolates. Computer-assisted cluster analysis of the band profiles using the unweighted-pair group method and Dice similarity coefficient showed a minimum of 85% similarity among the *E. coli* isolates gathered into 6 pulsotypes. This result showed the genomic heterogeneity of the studied isolates and also demonstrated that the

**Figure 2.** Plasmid Profile of clinical strains and their transconjugants M1 and M2 are the molecular weight markers: M1: *E. coli* V517; M2: plasmid pIP 173



majority of the isolates were not derived by clonal expansion from a single source. Despite being genetically heterogeneous, all six *E. coli* pulsotypes showed almost identical phenotypes.

Gene transfer experiments revealed that a single conjugative plasmid carried both resistance to beta-lactams and aminoglycosides. The PCR reactions followed by sequencing confirmed the presence of *bla*<sub>CTX-M-15</sub> and *aac*(6')-Ib genes in the same plasmid. This result agrees with those reporting that genes encoding ESBLs are usually located in transferable plasmids that may also carry other resistance determinants, such as those for resistance to aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulphamides, and quinolones [30, 31, 32, 33].

The high level fluoroquinolone MIC observed in the transconjugants could be attributed to the association of *bla*<sub>CTX-M-15</sub> and a gene encoding the ciprofloxacin and aminoglycoside-modifying enzyme AAC(6')-Ib-cr in the same transmissible plasmid. Indeed, PCR with primers targeting these determinants gave positive results with both clinical strains and their transconjugants.

An increase was also noted in the MICs of ceftazidim and cefotaxim in transconjugants compared to their parental strains. These results are reminiscent of a previous study [34] indicating that some determinants may be expressed at variable levels, possibly depending on host genotypes.

In addition, the resistance of strains producing the most ESBL to the other classes of antibiotics, in particular fluoroquinolones and /or trimethoprim-sulphamethoxazole, would complicate the treatment of urinary tract infections especially as these antibacterials are commonly prescribed by general practitioners in such infections.

## Conclusion

ESBL-producing *E. coli* is a growing risk for infection in the community in Morocco. To control the emergence and spread of the new multidrug-resistant *E. coli*, it is essential for the public to practice good hygiene habits and to comply with recommendations on the proper use of antibiotics. The worrisome development of antibiotic resistance to ESBL-producing *E. coli* in communities also requires enhanced capabilities for the detection of resistance mechanisms by private as well as public laboratories to implement appropriate infection control practices, and to prescribe appropriate chemotherapeutic agents.

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