Characterization of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from the community in Morocco

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Of 803 community *Escherichia coli* (n=767) and *Klebsiella pneumoniae* (n=36) isolates collected from patients with urinary tract infections in three Moroccan cities, 10 *E. coli* (1.3%) and 2 *K. pneumoniae* (5.6 %) isolates were shown to produce extended-spectrum β-lactamases (ESBLs). PFGE revealed that the *E. coli* isolates comprised seven distinct genotypes. The presence of plasmids in the 12 isolates was revealed by conjugation experiments of plasmids from these *Enterobacteriaceae* strains with *E. coli* $K_{12}J_5$, with further isolation of the plasmids in the transconjugants. Subsequent nucleotide sequencing indicated that the plasmids encoded the bla_{CTX-M}, bla_{OXA}, bla_{TEM} and bla_{SHV} genes, including genes for CTX-M-15 (n=11), OXA-1 (n=11), TEM-1b (n=4), SHV-5 (n=1) and SHV-1 (n=2). Identification of plasmid-mediated quinolone-resistance genes was performed by PCR. The *aac(6′)Ib-cr* variant was detected in all strains, and two strains co-expressed *qnrS1*, $bla_{CTX-M-15}$ and bla_{OXA-1} genes. The presence of ESBLs in the *Enterobacteriaceae* strains studied was probably due to the dissemination of resistance plasmids with the predominant genotype of $bla_{CTX-M-15}$.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are enzymes that compromise the efficacy of all β-lactams, except cephamycins and carbapenems, by hydrolysis of the β-lactam ring, and are inhibited by β-lactamase inhibitors (Coque *et al.*, 2008). There are more than 400 ESBLs described so far, most derived from the groups TEM, SHV and CTX-M, with 183, 134 and 103 variants, respectively (http://www.lahey.org/Studies/).

Since the early 2000s, CTX-M-type ESBLs have been increasingly reported, and these enzymes have now replaced TEM and SHV as the most common type of ESBL. The genes encoding ESBLs are usually located on plasmids that are highly mobile and can harbour resistance genes to several other unrelated classes of antimicrobials (Baudry *et al.*, 2009), such as the plasmid-mediated quinolone-resistance (PMQR) genes and aminoglycoside-resistance genes (Crémet *et al.*, 2011; Rodríguez-Baño *et al.*, 2009).

CTX-M enzymes, particularly CTX-M-15, have been involved in various epidemiological situations and have disseminated throughout all continents as a result of epidemic plasmids and/or particular epidemic strains (Ruppé, 2010). CTX-M-producing *Escherichia coli* and *Klebsiella pneumoniae* are becoming increasingly involved in urinary tract infections, especially among outpatients. Furthermore, these bacteria seem to have been imported from the community into the hospital setting (Arpin *et al.*, 2009).

Some studies have shown a high prevalence of ESBL-producing *Enterobacteriaceae* in hospitals in North African countries (Iabadene *et al.*, 2008; Sekhsokh *et al.*, 2008), including Morocco (Lahlou Amine *et al.*, 2009; Sekhsokh *et al.*, 2008). However, little information is available regarding their prevalence within the community. In 2004, a survey conducted on antibiotic resistance in community *E. coli* strains isolated from various specimens collected in Casablanca (Morocco) revealed that ESBL

Abbreviations: DDST, double-disc synergy test; ESBL, extended-spectrum β-lactamase; PFP, pulsed-field profiles; PMQR, plasmid-mediated quinolone resistance.

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producers were detected at a rate of 1.3% (Bourjilat *et al.*, 2009). Until recently, no data were available regarding the types and frequency of ESBLs in Moroccan communities. In this study, we investigated the dissemination of these enzymes among community *E. coli* and *K. pneumoniae* strains responsible for urinary tract infections. The antimicrobial-resistance profiles, the presence and transferability of resistance genes, and the genetic diversity of these isolates were also investigated.

METHODS

Bacterial isolates. In this study, 767 *E. coli* and 36 *K. pneumoniae* non-repeated strains isolated from patients suffering from community-acquired urinary tract infections were studied. The strains were collected from the medical analysis laboratories of three Moroccan cities: Casablanca, situated in the centre of the Atlantic coast of Morocco; El Jadida, located 100 km south west of Casablanca; and Settat, situated 72 km south of Casablanca. The isolates were collected from September 2004 to November 2009.

Antimicrobial drug susceptibility testing and ESBL detection. Antimicrobial drug susceptibility was determined by a disc-diffusion method on Mueller–Hinton (MH) agar plates (Bio-Rad), according to the recommendations of the Antibiogram Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org). The following antimicrobial agents were tested: amoxicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), aztreonam (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tobramycin (10 µg), kanamicin (30 µg), amikacin (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and tetracycline (30 µg). Quality control was conducted using the reference strain *E. coli* ATCC 25922.

ESBL production was screened using a double-disc synergy test (DDST) as a standard disc-diffusion assay on MH agar. Discs containing aztreonam (30 µg), ceftazidime (30 µg), cefepime (30 µg) and cefotaxime (30 µg) were placed at a distance of 30 mm (centre to centre) around a disc containing amoxicillin/clavulanic acid (20/10 µg), as recommended by the Antibiogram Committee of the French Society for Microbiology (http://www.sfm-microbiologie.org). Isolates that were DDST negative and resistant to third-generation cephalosporins were screened for an ESBL phenotype using an ESBL+AmpC screening identification kit (Rosco Diagnostic) according to the manufacturer's instructions.

Preparation of DNA template for PCR. DNA templates for PCR were generated by suspending five colonies of an overnight growth of *Enterobacteriaceae* isolates on Luria–Bertani agar (Bio-Rad) in 500 µl DNase- and RNase-free water (Invitrogen). The suspension was boiled at 100°C for 10 min in a thermal block (Polystat 5; Bioblock Scientific), then centrifuged at 19 000 ***g*** for 5 min. An aliquot of 1 µl of the supernatant was used as DNA template for PCR.

Detection of β-lactamase-encoding genes. *Enterobacteriaceae* isolates included in the study were screened by PCR for the following β-lactamase-encoding genes: $bla_{\text{CTX-M}}$ phylogenetic lineage groups 1, 2 and 9, bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-1}}$, bla_{PER} and bla_{VEB}, as described by Guessennd *et al.* (2008).

For all β-lactamase detection methods, the known β-lactamase-producing strains *E. coli* U2A1790 (CTX-M-1), *E. coli* U2A1799 (CTX-M-9), *Salmonella* sp. U2A2145 (CTX-M-2), *Salmonella* sp. U2A1446 (TEM-1 and SHV-12), *Pseudomonas aeruginosa* U2A1125 (PER), *Acinetobacter baumannii* U2A2026 (VEB) and *E. coli* U2A2446 (OXA-1) were used as positive controls. *E. coli* $K_{12}J_5$ strain was used as a negative control.

Detection of PMQR genes associated with ESBL genes. All ESBL-producing strains were screened by multiplex PCR for *qnr* genes (*qnrA*, *qnrB* and *qnrS*) as described by Guessennd *et al.* (2008), and for *aac(6′)-Ib* and *qepA* genes as described by Périchon *et al.* (2007). The PMQR-producing *E. coli* strains U2A1528 [*aac(6′)-Ib*], U2A2118 (*qnrA1*), U2A2119 (*qnrB1*) and U2A2120 (*qnrS1*), and *E. coli* TOP10/pAT791 (*qepA*) were used as positive controls. The primers used in this work are shown in Table 1.

Sequencing of ESBL and PMQR genes. All amplified products were sequenced to validate their identities. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130x1 sequencer (Applied Biosystems), using the same primers as used for PCR amplification. Nucleotide and deduced protein sequences were analysed with software available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Conjugation experiments and plasmid analysis. Conjugation assays were performed using a broth mating method with an azide-resistant mutant of *E. coli* $K_{12}J_5$ as the recipient strain. Transconjugants were selected on MH agar containing azide (200 mg l^{-1}) and ceftazidime (2 mg l^{-1}) (Bio-Rad), and incubated for 18–24 h at 37°C. If not successful at the first attempt, mating experiments were repeated up to three times.

The putative transconjugants were tested for their susceptibility to all 16 antibiotics to identify transferable antibiotic-resistance determinants. MICs of β-lactams (cefotaxime, ceftazidime, and cefepime), quinolones (nalidixic acid, ciprofloxacin and levofloxacin) and aminoglycosides (gentamicin, tobramycin, kanamicin and amikacin) were determined using the Etest method (AB Biodisk) for ESBL-carrying strains and their transconjugants.

Plasmid DNA extraction from donors and transconjugants was performed using a plasmid midi prep kit (Qiagen) according to manufacturer's instructions. The sizes of plasmids were estimated by electrophoresis on 0.7% agarose gels using plasmids from *E. coli* V517 as the standard markers (Macrina *et al.*, 1978).

PFGE. PFGE was performed to determine the genetic relatedness among the *E. coli* isolates, following a standardized protocol developed by Durmaz *et al.* (2009) using *XbaI*. The Dice similarity coefficient was calculated between pairs of lanes, and the strains were grouped using UPGMA, using the dendrogram construction utility DendroUPGMA (Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php).

RESULTS

Among the *E. coli* (n=767) and *K. pneumoniae* (n=36) isolates studied, 37 and 8, respectively, were resistant to third-generation cephalosporins. Screening using the DDST and ESBL+AmpC identification kit revealed that only ten *E. coli* (1.3%) and two *K. pneumoniae* (5.6%) were ESBL producers.

The antimicrobial-resistance patterns of the 12 ESBL-producing isolates are shown in Table 2. All isolates were multidrug resistant but were susceptible to imipenem and cefoxitin. We observed a substantial level of resistance to

Table 1. Primers used for PCR amplification and sequencing

Gene	Primer	Primer sequence (5′→3′)	Amplicon (bp)	Reference
$bla_{\text{CTX-M group1}}$	CTX-M1(+)	GGTTAAAAAATCACTGCGTC	863	Guessennd *et al.* (2008)
	CTX-M1(−)	TTGGTGACGATTTTAGCCGC		
$bla_{\text{CTX-M group2}}$	CTX-M2(+)	ATGATGACTCAGAGCATTCG	865	Guessennd *et al.* (2008)
	CTX-M2(−)	TGGGTTACGATTTTCGCCGC		
$bla_{\text{CTX-M group9}}$	CTX-M9(+)	ATGGTGACAAAGAGAGTGCA	869	Guessennd *et al.* (2008)
	CTX-M9(−)	CCCTTCGGCGATGATTCTC		
bla_{TEM}	a-216	ATAAAATTCTTGAAGACGAAA	1079	Guessennd *et al.* (2008)
	a-217	GACAGTTACCAATGCTTAATCA		
bla_{SHV}	Os-5	CGCCGGGTTATTCTTATTTGTCGC	795	Guessennd *et al.* (2008)
	Os-6	CGCCGGGTTATTCTTATTTGTCGC		
$bla_{\text{OXA-1}}$	Oxa-1	CCAAAGACGTGG	440	Guessennd *et al.* (2008)
	Oxa-2	GTTAAATTCGACCCCAAGTT		
bla_{PER}	per(+)	CCTGACGATCTGGAACCTTT	716	Guessennd *et al.* (2008)
	per(−)	GCAACCTGCGCAAT(GA)ATAGC		
bla_{VEB}	veb (+)	ATTTCCCGATGCAAAGCGT	542	Guessennd *et al.* (2008)
	veb(−)	TTATTCCGGAAGTCCCTGT		
qnrA	qnrA(+)	TTCTCACGCCAGGATTTGAG	571	Guessennd *et al.* (2008)
	qnrA(−)	TGCCAGGCACAGATCTTGAC		
qnrB	qnrB(+)	TGGCGAAAAAAATT(GA)ACAGAA	594	Guessennd *et al.* (2008)
	qnrB(−)	GAGCAACGA(TC)GCCTGGTAG		
qnrS	qnrS (+)	GACGTGCTAACTTGCGTGAT	388	Guessennd *et al.* (2008)
	qnrS (−)	AACACCTCGACTTAAGTCTGA		
qepA	qepA(+)	TGGTCACGCCATGGACCTCA	1137	Périchon *et al.* (2007)
	qepA(−)	TGAATTCGGACACCGTCTCCG		
aac(6′)-Ib	aac(6′)-Ib(+)	ATGACTGAGCATGACCTTG	476	Périchon *et al.* (2007)
	aac(6′)-Ib(−)	AACCATGTACACGGCTGG		

quinolones: the majority of the isolates (91.6%) were resistant to nalidixic acid, except for E.c4. Cotrimoxazole resistance was observed in all strains except one *K. pneumoniae* (K.p30). All strains were resistant to at least one of the four aminoglycosides tested. The most frequently observed phenotypic profile included resistance to gentamicin and tobramycin (11/12 isolates). Three strains (E.c1, E.c9 and E.c64) were resistant to kanamycin and none was resistant to amikacin. Only four strains were resistant to tetracycline.

The results of ESBL-encoding gene detection by PCR revealed that the *E. coli* and *K. pneumoniae* strains harboured a diversity of β-lactamases, namely SHV, CTX-M, OXA and TEM enzymes, and that bla_{PER} and bla_{VEB} were not detected in any of the isolates (Table 2). Further analysis of the bla_{TEM} sequences indicated that these *Enterobacteriaceae* harboured the TEM-1b subgroup, whereas for bla_{SHV}, the subgroup found in the isolates was SHV-5 and SHV-1. In the case of the $bla_{\text{CTX-M}}$ gene, DNA sequence analysis indicated that 11 isolates were carriers of the subgroup CTX-M-15 and no amplicons were obtained for the other two tested CTX-M subgroups. For bla_{OXA}, the subgroup found in the isolates was OXA-1 (Table 2). All isolates except E.c1 harboured the CTX-M-15 gene associated with the $bla_{\text{OXA-1}}$ gene. The $bla_{\text{SHV-5}}$ and $bla_{\text{SHV-1}}$ genes were detected in one *E. coli* strain (E.c1) and two *K. pneumoniae* strains, respectively. The bla_{TEM} gene was harboured by one *K. pneumoniae* and three *E. coli* isolates. All combinations of *bla* genes detected are shown in Table 2. Of the 11 $bla_{\text{CTX-M}}$ isolates, three also harboured bla_{TEM} and two had bla_{SHV}. It is interesting to note that four β-lactamases genes were co-expressed in *K. pneumoniae* isolate K.p18: bla_{SHV}, bla_{OXA}, bla_{TEM} and $bla_{\text{CTX-M}}$. None of the 12 isolates carried the *qepA*, *qnrA* or *qnrB* genes, whereas the *aac(6′)-Ib-cr* variant gene was detected in all isolates, two of which (E.c64 and K.p18) also harboured the *qnrS1* gene (Table 2).

Conjugation experiments were carried out for all ESBL producers, but transfer of this phenotype to the recipient sodium azide-resistant *E. coli* $K_{12}J_5$ was successful in only seven isolates (Table 3). All transconjugants were resistant to β-lactam and gentamicin. Kanamycin resistance was co-transferred in the Tc1, Tc9 and Tc64 transconjugants, whereas for the Tc64 transconjugant, trimethoprim, sulfamethaxazole and tetracycline resistances were also transferred (Table 3).

Plasmids of variable sizes were detected (Table 2): a plasmid of ~125 kb was detected in 75% (9/12) of the isolates tested, and was always found with the CTX-M-15 ESBL type and OXA-1. All transconjugants had plasmids of 125 kb (Table 3).

The E.c1 isolate successfully transferred the $bla_{\text{SHV-5}}$ and $bla_{\text{TEM-1b}}$ genes to the *E. coli* $K_{12}J_5$ recipient strain. These

Table 2. Characteristics of the ESBL-producing enterobacteria strains studied

Code	Isolate	Date	City	Sex/age (years)	Resistance to antibiotics other than β-lactams*	ESBL gene†	PMQR genes	Plasmid size (kb)	PFGE patterns
E.c1	*E. coli*	10/2004	Casablanca	F/55	NA, CIP, GM, TM, AN, SXT, K	SHV-5; TEM-1b	*aac(6′)-Ib-cr*	<125	CE7
E.c2	*E. coli*	09/2006	Casablanca	F/71	NA, CIP, GM, TM, SXT	CTX-M-15, TEM-1b, OXA-1	*aac(6′)-Ib-cr*	125	CE2
E.c3	*E. coli*	07/2007	Casablanca	M/64	NA, CIP, GM, TM, SXT	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	125, 15.2	CE1
E.c4	*E. coli*	02/2007	Casablanca	F/1	GM, TM, SXT	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	125	CE5
E.c5	*E. coli*	06/2005	Casablanca	F/5	NA, CIP, GM, TM, SXT	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	<125	CE3
E.c6	*E. coli*	12/2005	Casablanca	F/66	NA, CIP, GM, SXT	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	125	CE2
E.c7	*E. coli*	02/2006	Casablanca	F/49	NA, CIP, GM, TM, SXT	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	125, 7.6, 2.4	CE4
E.c95	*E. coli*	03/2008	El Jadida	F/20	NA, CIP, GM, TM, SXT, TET	CTX-M-15, TEM-1b, OXA-1	*aac(6′)-Ib-cr*	125	CE2
E.c64	*E. coli*	12/2008	El Jadida	F/25	NA, CIP, GM, TM, SXT, TET, K	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*, *qnrS1*	125, 54	CE4
E.c9	*E. coli*	07/2008	Settat	F/20	NA, CIP, GM, TM, SXT, TET, K	CTX-M-15, OXA-1	*aac(6′)-Ib-cr*	<125, 125, 3, 2.5	CE6
K.p30	*K. pneumoniae*	08/2008	Settat	M/28	NA, GM, TM, TET	CTX-M-15, SHV-1, OXA-1	*aac(6′)-Ib-cr*	125	ND
K.p18	*K. pneumoniae*	07/2008	Settat	F/40	NA, CIP, GM, TM, SXT	CTX-M-15, TEM-1b, SHV-1, OXA-1	*aac(6′)-Ib-cr*, *qnrS1*	<125	ND

AN, Amikacin; CIP, ciprofloxacin; F, female; GM, gentamicin; M, male; NA, nalidixic acid; ND, not done; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TM, tobramycin.
*All strains were susceptible to the imipenem and cefoxitin, and all were resistant to ampicillin, cefotaxime, ceftazidime and amoxicillin/clavulanic acid.
†TEM-1b, OXA-1 and SHV-1 are not ESBLs.

genes were carried by one conjugative plasmid of high molecular mass (~125 kb). The E.c64 isolate contained two plasmids of ~125 and ~54 kb. In conjugation experiments, the E.c64 isolate transferred successfully β-lactam and fluoroquinolone resistance to the *E. coli* $K_{12}J_5$ recipient strain. PCR and sequencing confirmed that the transconjugant carried the *qnrS1*, *aac(6′)-Ib-cr*, bla_{OXA-1} and $bla_{CTX-M-15}$ genes on the large plasmid (~125 kb).

Among the *E. coli* isolates, seven different pulsed-field profiles (PFPs) were identified and classified as PFP types CE1–CE7 (Fig. 1). The patterns CE1, CE3, CE5, CE6 and CE7 were represented by only one isolate. PFP type CE2 was prevalent, containing three *E. coli* isolates, and two subtypes were further determined within this PFP type, CE2a and CE2b, showing 88% similarity. The CE2b subtype was composed of two *E. coli* strains (E.c6 and E.c95) with high-level similarity (95%); these strains were collected in two different community settings (Casablanca and El Jadida). Pattern CE4 comprised two isolates (E.c7 and E.c64) showing 100% similarity, isolated from different outpatients clinics and were obtained in different years (2006 and 2008, respectively).

DISCUSSION

Enterobacteriaceae have become one of the most important causes of nosocomial and community-acquired infections. β-Lactams (mainly extended-spectrum cephalosporins and carbapenems) and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these microorganisms. However, resistance to these compounds has been reported increasingly frequently from different parts of the world in recent years (Cantón *et al.*, 2008; Reinert *et al.*, 2007). The last European Antibiotic Resistance Surveillance System (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net) report from 2006, covering over 800 laboratories from 31 countries, showed a continuous increase since 2000 in invasive *E. coli* and *K. pneumoniae* isolates resistant to third-generation cephalosporins, with a prevalence of >10% for half of the enrolled countries.

In our study, we found 37/767 (4.8%) *E. coli* and 8/36 (22.2%) *K. pneumoniae* strains tested to be resistant to third-generation cephalosporins, but only 10 (1.3%) *E. coli*

Table 3. Characteristics of clinical strains and their transconjugants

Code	Species	MICs (μg ml^{-1})										Transfer		Genes	
		LEV	NA	CIP	GM	TM	AN	K	CTX	CAZ	FEP	Resistance profile other than *β*-lactams	Plasmid (kb)	ESBL*	PMQR
E.c4	*E. coli*	0.032	8	1	48	32	8	1	>256	16	>256	GM-TM	125	CTX-M15, OXA-1	*aac(6′)-Ib-cr*
Tc4	*E. coli*	0.02	2	0.02	8	16	3	0.064	24	24	96			CTX-M15, OXA-1	*aac(6′)-Ib-cr*
E.c5	*E. coli*	6	>256	32	16	12	6	64	>256	8	64	GM	125	CTX-M15, OXA-1	*aac(6′)-Ib-cr*
Tc5	*E. coli*	0.012	2	0.25	12	2	3	0.094	8	48	>256			CTX-M15, OXA-1	*aac(6′)-Ib-cr*
E.c7	*E. coli*	0.032	4	0.5	64	16	6	2	>256	48	128	TM	125	CTX-M15, OXA-1	*aac(6′)-Ib-cr*
Tc7	*E. coli*	0.02	2	0.02	8	8	2	2	>256	12	256			CTX-M15, OXA-1	*aac(6′)-Ib-cr*
E.c9	*E. coli*	4	512	32	16	16	3	24	>256	128	>256	GM-K-SXT-TET	125	CTX-M15, OXA-1	*aac(6′)-Ib-cr*
Tc9	*E. coli*	0.006	2	0.02	6	1.5	2	12	>256	32	>256			CTX-M15, OXA-1	*aac(6′)-Ib-cr*
E.c64	*E. coli*	6	512	128	12	6	1.5	12	>256	96	>256	GM-K-TM-SXT-TET	125	CTX-M15, OXA-1	*aac(6′)-Ib-cr\qnrS1*
Tc64	*E. coli*	0.094	8	0.5	6	4	1.5	12	>256	48	>256			CTX-M15, OXA-1	*aac(6′)-Ib-cr\qnrS1*
K.p30	*K. pneumoniae*	0.012	2	0.02	8	8	1	4	>256	64	>256	GM-TET	125	CTX-M15, SHV-1, OXA-1	*aac(6′)-Ib-cr*
Tc30	*K. pneumoniae*	0.004	2	0.02	8	1	1	3	>256	64	>256			CTX-M15, OXA-1	*aac(6′)-Ib-cr*
E.c1	*E. coli*	>32	512	128	12	32	8	12	>256	128	>256		125	SHV-5, TEM-1b	*aac(6′)-Ib-cr*
Tc1	*E. coli*	0.02	2	0.02	6	16	3	12	>256	32	128	GM-TM-K		SHV-5, TEM-1b	*aac(6′)-Ib-cr*
$K_{12}J_5$	*E. coli*	0.006	2	0.02	0.5	0.5	0.75	0.75	0.5	0.5	0.5	–	–	–	–

AN, Amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; GM, gentamicin; K, kanamycin; LEV, levofloxacin; NA, nalidixic acid; SXT, trimethoprim/sulfamethoxazole; Tc, transconjugant; TET, tetracycline; TM, tobramycin.

*TEM-1b, OXA-1 and SHV-1 are not ESBLs.

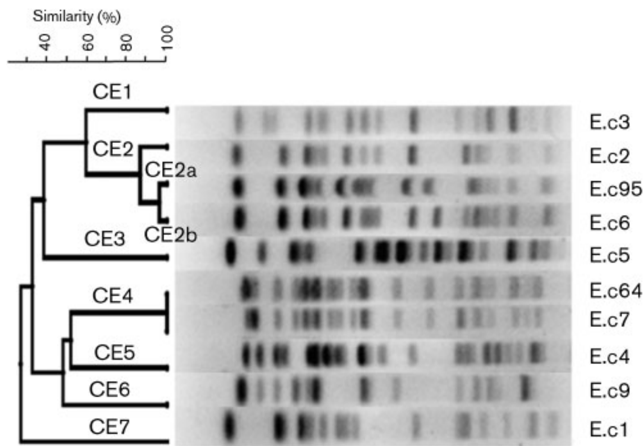

Fig. 1. Dendrogram produced by DendroUPGMA (a dendrogram-construction utility) using the Dice similarity coefficient and UPGMA on the basis of the PFGE profiles of *E. coli* strains.

and 2 (5.5%) *K. pneumoniae* gained their resistance by producing ESBLs. Third-generation cephalosporin resistance in non-ESBL producers (27 *E. coli* and 6 *K. pneumoniae* strains) could be due to some other resistance mechanism such as lack of permeation of porins and AmpC β-lactamase production (Mohamudha *et al.*, 2010).

The prevalence of 1.3% ESBL-producing *E. coli* in the community found in this study is similar to that recorded in Spanish and French communities (1.4%) (Arpin *et al.*, 2009; Rodríguez-Baño *et al.*, 2004) but lower than the 5.2% observed in a Spanish multicentre study covering 15 microbiology laboratories in 2006 (Andreu *et al.*, 2008). A study performed in Turkey showed a prevalence of 21% ESBL producers among *E. coli* causing community-acquired urinary tract infections during 2004 and 2005 (Yumuk *et al.*, 2008). The study showed a much higher prevalence of ESBL-producing isolates among *K. pneumoniae* than among *E. coli* isolates.

Overall, the resistance patterns of ESBL-producing bacteria studied here were similar to those commonly described in other studies, i.e. the ESBL producers were resistant to different antibiotic families including – besides β-lactams – fluoroquinolones, aminoglycosides and trimethoprim/sulfamethoxazole (Lahlou Amine *et al.*, 2009; Minarini *et al.*, 2007b; Mshana *et al.*, 2009; Pitout *et al.*, 2004; Sekhsokh *et al.*, 2008; Shi *et al.*, 2009), which contribute to the selection and persistence of multidrug-resistant ESBL strains and plasmids in both clinical and community settings (Cantón *et al.*, 2008). Nevertheless, all CTX-M-producing strains were susceptible to imipenem. However, the emergence of different types of carbapenemases could compromise the usefulness of this class of antibiotic (Walsh *et al.*, 2005; Walther-Rasmussen & Høiby, 2006).

We detected a variety of β-lactamases among the isolates of *E. coli* and *K. pneumoniae*, namely SHV-, CTX-M-, OXA- and TEM-type enzymes. The CTX-M type was the most common ESBL in our setting. The predominance of CTX-M-15 indicates that this allele is now common in Morocco, as in other countries as a result of worldwide dissemination (Arpin *et al.*, 2009; Minarini *et al.*, 2007a; Woodford *et al.*, 2007). These higher rates of CTX-M among total ESBL enzymes are most probably associated with high mobilization of the encoding genes. Barlow *et al.* (2008) found that $bla_{\text{CTX-M}}$ genes are mobilized to plasmids almost ten times more frequently than other class A β-lactamases.

The co-existence of different β-lactamases genes within the same isolate, as detected in this study, has also been reported in other countries (Arpin *et al.*, 2009; Woodford *et al.*, 2007). The combination of $bla_{\text{CTX-M-15}}+bla_{\text{OXA-1}}+bla_{\text{TEM-1b}}$ was reported in 30 strains from Portugal (Mendonça *et al.*, 2007), and an association between $bla_{\text{SHV-5}}$ and $bla_{\text{TEM-1b}}$ has been described in the Brazilian community (Minarini *et al.*, 2007a).

The association of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ in the same strain has also been reported in Portugal (Mendonça *et al.*, 2007) and the USA (Hanson *et al.*, 2008). Combined production of CTX-M and OXA enzymes by *E. coli and K. pneumoniae* improved resistance to β-lactamase inhibitors, presumably explaining their non-susceptibility to amoxicillin/clavulanate (Livermore & Hawkey, 2005).

Despite the importance of ESBLs as a serious clinical problem for treatment of patients carrying ESBL-expressing *K. pneumoniae*, the clinical importance of non-ESBL enzymes is yet to be revealed. Among these non-ESBL enzymes we identified SHV-1, an instrinsic gene in *K. pneumoniae*. Co-expression of CTX-M enzymes and SHV-1 penicillinases in *K. pneumoniae* strains is becoming very common (Kiratisin *et al.*, 2008; Romero *et al.*, 2005).

Among the 12 ESBL-producing isolates with reduced susceptibility to fluoroquinolones, *qnr* genes remained infrequent, with only two isolates carrying the *qnrS1* gene. Interestingly, as noted by other authors in hospitals and community settings (Crémet *et al.*, 2011; Jiang *et al.*, 2008; Pitout *et al.*, 2009), *qnr* genes were particularly recovered among *K. pneumoniae* isolates (1/2) compared with *E. coli* isolates (1/10), despite the low number of *K. pneumoniae* isolates in our collection. Although based on a limited number of *qnr*-positive isolates, our results underlined the absence of QnrA determinants in our community, which contrasts with studies reporting the presence of *qnrB1* and *qnrA* genes notably in Kenyan and Nigerian communities (Kariuki *et al.*, 2007; Soge *et al.*, 2006).

The *aac(6′)-Ib-cr* gene occurred in ESBL-producing enterobacterial strains with a higher prevalence than *qnr* genes. These results support previous findings suggesting a wider dissemination of AAC(6′)-Ib-cr than Qnr determinants, especially in multiple clones of *E. coli* carrying the $bla_{\text{CTX-M-15}}$ gene (Pitout *et al.*, 2009; Ruppé *et al.*, 2009; Soge *et al.*, 2006).

We noted the absence of the *qepA* gene among the studied strains. This new PMQR mechanism is still very rare, except

in China where recent studies underlined the predominance of the *qepA* gene in enterobacterial strains isolated from food-producing animals (Ma *et al.*, 2009). The now common combination of CTX-M-15, OXA-1, SHV-1 and TEM-1 β-lactamases and PMQR determinants *aac(6′)-Ib-cr* and *qnrS1* in a community *K. pneumoniae* strain (K.p18), as reported here, is, to our knowledge, a first.

Conjugation experiments were successful in only 7 of the 12 isolates. However, it should be noted that only broth matings were carried out in this study and not filter matings, and thus the transmissibility potential for the other five isolates could not be fully ascertained. In addition, the ceftazidime concentration used for transconjugant selection could be a limiting factor, although CTXM-15 ESBLs confer high-level resistance to ceftazidime (MIC range 64 to >128 μg ml^{-1}) (Younes *et al.*, 2011).

In this study, gentamicin and tetracycline resistance were transferable by conjugation in most of the isolates tested. Isolates harbouring CTX-M-15 were resistant to cefepime, with most of them exhibiting an MIC >64 μg ml^{-1}. It has been demonstrated that CTX-M ESBLs hydrolyse cefepime with higher efficiency compared with other ESBL types (Lavollay *et al.*, 2006; Mshana *et al.*, 2009). Plasmid analysis revealed that the majority of the transconjugants harboured large plasmids of ~125 kb. Most previous studies have found plasmids ranging from 7 to 200 kb in association with CTX-M-15 (Baudry *et al.*, 2009; Mshana *et al.*, 2009). These findings suggest that, in our setting, the CTX-M-15 allele was carried on large conjugative plasmids that were well adapted to, and constantly exchanged by, lateral gene transfer among the *E. coli* and *K. pneumoniae* isolates.

The *aac(6′)-Ib-cr* gene has been linked mainly to CTX-M-15 isolates in various surveys, whereas *qnr* genes are mostly associated with enzymes from the CTX-M-9 or CTX-M-1 groups, reflecting the fact that genes encoding resistance to β-lactams and quinolones are located on the same plasmid and thus passed on together among different enterobacterial species (Jones *et al.*, 2008; Nordmann & Poirel, 2005). The *qnr* family and *aac(6′)-Ib-cr* confer only low-level resistance, but their presence could potentially facilitate evolution of the bacterial host towards higher levels of resistance by mutational alterations in the target type II topoisomerases. In this study, five of the seven transconjugants harbouring *aac(6′)-Ib-cr* showed no elevation of ciprofloxacin MIC. Indeed, *aac(6′)-Ib-cr* alone confers a degree of resistance sufficiently low that the MIC might not distinguish organisms that carry it from those that do not (Robicsek *et al.*, 2006). Recently, Harajly *et al.* (2010) described transconjugants with no elevation of MIC after the transfer of *aac(6′)-Ib-cr*.

PFGE analysis was used to establish the genetic relatedness of the ESBL-producing *E. coli* strains. Using a similarity coefficient of 0.80, we were able to establish seven different clusters (CE1–CE7) among the *E. coli* isolates (Fig. 1) and to suggest some evidence of genetic relatedness among some strains producing CTX-M-15. Pattern CE4 contained two *E. coli* isolates (E.c7 and E.c64). These isolates were from two different outpatient clinics, collected in two different geographical regions (Casablanca and El Jadida) and were obtained over a 2 year period, and hence are likely to represent distinct pockets of community-acquired clonally related strain types. Pattern CE2 contained three *E. coli* isolates (E.c2, E.c95 and E.c6). Although E.c2 and E.c6 were isolated in the same city (Casablanca), they originated from two different patients who had contracted urinary tract infections in 2006 and 2005, respectively. These isolates showed 88 % similarity with E.c95 collected in El Jadida city in 2008 and hence are clonally related. Various factors may have contributed to this dissemination, including the proximity of the population areas and/or inadequate antibiotic use. In contrast, the other five isolates (E.c3, E.c5, E.c4, E.c9 and E.c1) produced different PFGE digest patterns, indicating that they were isolates from distinct and unrelated sources of infection in their respective communities. Studies from Spain and the UK, also using PFGE, have shown that most *E. coli* producing CTX-M enzymes from the community are not clonally related. However, the UK study did suggest some evidence of genetic relatedness among strains producing CTX-M-1 (Pitout *et al.*, 2005a, b).

The production of similar TEM and CTX-M-type enzymes in various genetically related strains and in isolates from distant regions suggests horizontal transfer of the corresponding genes. Isolates of profile type CE4 expressed CTX-M-15 and OXA-1 enzymes and were multidrug-resistant. Isolates of profile subtype CE2b were also multidrug resistant and expressed CTX-M, OXA and TEM enzymes.

In conclusion, this report offers an insight into the current prevalence and molecular types of ESBL-producing organisms in Moroccan community settings, contributing to a better understanding of the epidemiology of these enzymes at local and national levels. Our results indicate that a variety of ESBL producers are present in outpatient settings and indicate the spread of the CTX-M-15 type among *Enterobacteriaceae* isolates, and the occurrence of clonal and polyclonal dissemination of ESBL-producing strains in the community. In addition, we have reported for what is to the best of our knowledge the first time the association of $bla_{CTX-M-15}$, *qnrS1* and *aac(6′)-Ib-cr* in a community *K. pneumoniae* strain and in *E. coli* strains in a North African country. Our results emphasize the necessity for adequate screening of ESBL-producing strains in Moroccan hospitals and community environments, together with efforts to promote the judicious use of antibiotics and to prohibit their sale without prescription.

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