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ORIGINAL ARTICLE

Prevalence and genotypic analysis of plasmid-mediated β-lactamases among urinary *Klebsiella pneumoniae* isolates in Moroccan community

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The aim of this study is to assess the prevalence and molecular characterization of the extended spectrum β-lactamases (ESBL)-producing *Klebsiella pneumoniae* isolated from community acquired urinary tract infections and collected in five Moroccan cities during a 2010 survey. In all, 34 (7.5%) of the 453 *K. pneumoniae* isolates studied were positive for an ESBL phenotype and 91.1% of these isolates were multidrug resistant. The $bla_{CTX\text{-}M\text{-}15}$ ($n=31$) was the most frequent ESBL genes detected, followed equally by $bla_{SHV\text{-}28}$ and $bla_{SHV\text{-}12}$ ($n=3$), then $bla_{TEM\text{-}3}$, $bla_{SHV\text{-}36}$, $bla_{SHV\text{-}110}$ and $bla_{CTX\text{-}M\text{-}1}$ with one isolate for each ($n=1$). Eight isolates co-expressed more than one ESBL with $bla_{CTX\text{-}M\text{-}15}$. The non-ESBL genes detected were $bla_{SHV\text{-}1}$, $bla_{SHV\text{-}11}$, $bla_{SHV\text{-}32}$, $bla_{SHV\text{-}26}$, $bla_{SHV\text{-}76}$, $bla_{TEM\text{-}1}$, $bla_{TEM\text{-}1b}$ and $bla_{OXA\text{-}1}$. Plasmid-mediated AmpC β-lactamase genes, $bla_{ACT\text{-}2}$, $bla_{DHA\text{-}1}$ and a new β-lacatamase named $bla_{EBC\text{-}1464}$, were detected in 11.7% of isolates. Fourteen (41.1%) isolates harbored *qnr* genes; *qnrA6* ($n=1$), *qnrB1* ($n=8$), *qnrB2* ($n=1$) and *qnrS1* ($n=4$) types were detected. Twenty-six isolates (76.4%) were positive for *aac(6′)-Ib-cr* gene. Results of conjugation experiments indicated that $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1b}$, $bla_{OXA\text{-}1}$, *aac(6′)-Ib-cr* and *qnrB1* genes were co-transferred and that these genes were carried by a conjugative plasmid of high molecular weight. With the exception of *qnrB1*, all the antibiotic resistance genes were clustered in a 12-kb region. The results of this work report the genetic diversity of ESBL genes, with the CTX-M-15 enzyme being most common among ESBL-producing *K. pneumoniae* in Moroccan community. Furthermore, a major finding is that $bla_{EBC\text{-}1464}$ detection is a first in Morocco.
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INTRODUCTION

Urinary tract infections (UTIs) remain the common infections diagnosed in outpatients, as well as in hospitalized patients.[1] *Escherichia coli* and *Klebsiella pneumoniae* have been reported as the most common organisms causing UTIs.[2] Furthermore, *K. pneumoniae* is an important pathogen both in the community and the hospital setting to cause severe infections.[3] During the last decade, extended spectrum β-lactamases (ESBLs) type have emerged in the community setting among *K. pneumoniae* isolates, on plasmids that frequently bear additional resistance determinants. The class A ESBLs, TEM, SHV and CTX-M types, are the most widespread and clinically relevant. TEM and SHV types are derived from penicillinases TEM-1, TEM-2 and SHV-1 and are characterized by several single amino-acid substitutions. These mutations allow them to hydrolyze extended spectrum cephalosporins. More than 200 TEM enzymes and more than 160 different mutants of SHV have been reported (www.lahey.org/Studies/). The plasmid-encoded CTX-M family members, which confer high levels of resistance to extended spectrum cephalosporins, have emerged in several continents around the world. More than 130 different variants of CTX-M enzymes are currently known (www.lahey.org/Studies/). The genes encoding these β-lactamases are often located on large plasmids that also encode genes for resistance to other antibiotics.[4] Furthermore, there is an increasing tendency for pathogens to produce multiple β-lactamases.[5]

ESBL producing *Enterobacteriaceae* strains, predominated by *E. coli* and *K. pneumoniae,* have been isolated from different hospitals in Morocco.[6,7] The β-lactamase genes detected in Moroccan hospitals isolates were bla_{TEM}, bla_{SHV}, bla_{DHA} and bla_{OXA} types.[6,8] Recently, $bla_{OXA\text{-}48}$ carbapenemase gene has been detected in one *K. pneumoniae* isolate in Rabat city.[4] Here, we report the prevalence of ESBL producing *K. pneumoniae* isolated from UTIs in Moroccan community and the characterization of their β-lactamases enzymes. Current knowledge on antimicrobial susceptibility pattern of this uropathogen is mandatory for appropriate therapy, so the accumulation of other resistance genes by isolates was also investigated.

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MATERIALS AND METHODS

Bacterial isolates

A total of 453 non-duplicate community acquired uropathogenic *K. pneumoniae* isolated from urine specimens (corresponding to an overall incidence in UTIs of 17%) were examined in this study. They were collected from January to December 2010 from medical analysis laboratories of five Moroccan cities: Casablanca situated in the center of Atlantic Coast of Morocco; El Jadida located 100 km south west of Casablanca; Settat situated 72 km South of Casablanca; Rabat located 95 km north east of Casablanca and Meknes located 230 km north east of Casablanca.

The *K. pneumoniae* isolated from hospitalized or previous hospitalized patients during the study year were excluded. Strains were named (Kpp (*K. pneumoniae*)) and numbered independently from isolation date.

Antimicrobial drug susceptibility testing

Antimicrobial drug susceptibility was determined by the disk-diffusion method on Mueller-Hinton (MH) agar plates (Bio-Rad, Marnes-la-Coquette, France) as recommended by the French Society for Microbiology (FSM; 2009) (http://www.sfm-microbiologie.org). The following antimicrobial agents (Bio-Rad) 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), kanamycin (30 μg), amikacin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), trimethoprim (5 μg) and tetracycline (30 μg). Multidrug resistance (MDR) was defined as those resistant to at least two of the antibiotic classes in addition to the β-lactams. *E. coli* ATCC 25922 was used as quality control strain.

ESBL production was detected by the double-disc synergy test (DDST) using clavulanic acid-amoxicillin (20/10 μg) and ceftazidime (30 μg), cefotaxime (30 μg), aztreonam (30 μg) and cefepime (30 μg) on MH agar as recommended by the FSM (2009) (http://www.sfm-microbiologie.org). Isolates showing a DDST-negative and resistant to third generation cephalosporins were screened for ESBL phenotype; ESBL + AmpC screening identification kit (Rosco Diagnostica, Taastrup, Denmark) that included antibiotic impregnated tablets: cefotaxime 30 μg, cefotaxime 30 μg + clavulanate, cefotaxime 30 μg + cloxacillin, cefotaxime 30 μg + cloxacillin + clavulanate were used. By measuring differences in zone of inhibition, they were categorized into strains that produced ESBL or AmpC or both according to the manufacturer's instructions.

Preparation of DNA template for PCR

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

Identification of phylogenetic groups

The identification of phylogenetic group affiliation of strains was conducted by gyrA PCR–restriction fragment length polymorphism using restriction enzymes *Taq*I and *Hae*III as previously described.[3]

Detection of β-lactamase-encoding genes

K. pneumoniae isolates 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}, bla_{VEB} and bla_{AmpC} as described previously.[9,10]

Detection of plasmid-mediated quinolone resistance genes

All ESBL-producing strains were screened by PCR for *qnr* genes (*qnrA*, *qnrB* and *qnrS*) and *aac(6′)-Ib-cr* gene as described previously.[9]

All primers used in this work are shown in Table 1.

Sequencing of resistance genes

The primers used for resistance gene amplification targeting a region allow the sequencing of the whole coding sequence. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130 × 1 sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. The nucleotide and deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information website (see www.ncbi.nlm.nih.gov).

Conjugation experiments and plasmid analysis

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

The putative transconjugants were tested for susceptibility to all antibiotics, as indicated previously, to identify transferable antibiotic resistance determinants. Minimal inhibitory concentrations of β-lactams (amoxicillin, cefotaxime, ceftazidime, cefoxitin and cefepime), quinolones (nalidixic acid, ciprofloxacin), aminoglycosides (gentamicin, kanamycin, amikacin and streptomycin) and tetracycline were determined by agar dilution method according to guidelines of the FSM (2009) (http://www.sfm-microbiologie.org) for ESBL-carrying strains and their transconjugants.

Plasmid DNA extraction from both donor and transconjugants was performed using a Plasmid Midi Prep Kit (Qiagen Ltd, West Sussex, UK) according to manufacturer's instructions. The sizes of plasmids were estimated by electrophoresis on 0.7% agarose using the plasmids from *E.coli* V517 as the standard markers.[11]

Genetic environment of $bla_{\text{CTX-M}}$

The genetic organization of $bla_{\text{CTX-M}}$ genes and the sequencing of the regions surrounding these genes were investigated by PCR. The internal IS*26* and IS*Ecp1* forward primers and the CTX-M reverse consensus primer (MA1 reverse) were used to investigate the promoter regions of the $bla_{\text{CTX-M}}$ genes.[12] Primer orf477-IRR matches the alternative IS*Ecp1* IRR.[13]

The plasmid DNA obtained from the transconjugants (*K. pneumoniae* Kpp.319 and Kpp.20) was used to investigate the flanking regions of the $bla_{\text{CTX-M}}$ genes by long PCR technique, partial sequencing of the regions surrounding these genes (Table 1) and PCR analysis as described previously.[12–14]

RESULTS

Prevalence of extended spectrum β-lactamases producing *K. pneumoniae* isolates

Among the 453 community *K. pneumoniae* strains responsible for UTIs studied, 34 were ESBL producers, so the overall prevalence was 7.5% (3.7–9.0% depending on the cities) (Table 2).

Antimicrobial drug susceptibility

Disk-diffusion susceptibility testing indicated high prevalence of resistance to various antimicrobial agents: 91.1% of these isolates were MDR. All strains were resistant to amoxicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, cefepime and aztreonam. Eight (23.5%) strains were resistant to cefoxitin. Five (14.7%) strains show intermediate level of resistance to imipenem. We observed a substantial level of resistance to quinolones; 28 strains (77.7%) had an intermediate level of resistance or were fully resistant to nalidixic acid, and ciprofloxacin. Of the 34 ESBL-carrying strains, only one was susceptible to nalidixic acid and had intermediate resistance level to ciprofloxacin. Cotrimoxazole and tetracycline resistance was observed in 27 (79.4%) and 31 (91.1%) strains, respectively. Twenty-nine strains (85.3%) were resistant to at least one of the four

Table 1 Primers sets used for PCR amplification and sequencing

Gene or location	*Primer*[a]	*Primer sequence (5′→3′)*	*Reference*
$bla_{\text{CTX-M group1}}$	CTX-M1(+)	GGTTAAAAAATCACTGCGTC	Barguigua *et al.*[9]
	CTX-M1(−)	TTGGTGACGATTTTAGCCGC	
$bla_{\text{CTX-M group2}}$	CTX-M2(+)	ATGATGACTCAGAGCATTCG	Barguigua *et al.*[9]
	CTX-M2(−)	TGGGTTACGATTTTCGCCGC	
$bla_{\text{CTX-M group9}}$	CTX-M9(+)	ATGGTGACAAAGAGAGTGCA	Barguigua *et al.*[9]
	CTX-M9(−)	CCCTTCGGCGATGATTCTC	
bla_{TEM}	a-216	ATAAAATTCTTGAAGACGAAA	Barguigua *et al.*[9]
	a-217	GACAGTTACCAATGCTTAATCA	
bla_{SHV}	Os-5	CGCCGGGTTATTCTTATTTGTCGC	Barguigua *et al.*[9]
	Os-6	CGCCGGGTTATTCTTATTTGTCGC	
bla_{OXA}	Oxa-1	CCAAAGACGTGG	Barguigua *et al.*[9]
	Oxa-2	GTTAAATTCGACCCCAAGTT	
bla_{PER}	per (+)	CCTGACGATCTGGAACCTTT	Barguigua *et al.*[9]
	per(−)	GCAACCTGCGCAAT(GA)ATAGC	
bla_{VEB}	veb (+)	ATTTCCCGATGCAAAGCGT	Barguigua *et al.*[9]
	veb(−)	TTATTCCGGAAGTCCCTGT	
qnrA	qnrA(+)	TTCTCACGCCAGGATTTGAG	Barguigua *et al.*[9]
	qnrA(−)	TGCCAGGCACAGATCTTGAC	
qnrB	qnrB(+)	TGGCGAAAAAAATT(GA)ACAGAA	Barguigua *et al.*[9]
	qnrB(−)	GAGCAACGA(TC)GCCTGGTAG	
qnrS	qnrS (+)	GACGTGCTAACTTGCGTGAT	Barguigua *et al.*[9]
	qnrS (−)	AACACCTCGACTTAAGTCTGA	
aac(6′)-Ib	aac(6′)-Ib(+)	ATGACTGAGCATGACCTTG	Barguigua *et al.*[9]
	aac(6′)-Ib(−)	AACCATGTACACGGCTGG	
bla_{FOX}	foxM(+)	AACATGGGGTATCAGGGAGATG	Pérez-Pérez and Hanson[10]
	foxM(−)	CAAAGCGCGTAACCGGATTGG	
bla_{ACC}	accM(+)	AACAGCCTCAGCAGCCGGTTA	Pérez-Pérez and Hanson[10]
	accM(−)	TTCGCCGCAATCATCCCTAGC	
bla_{EBC}	ebcM(+)	TCGGTAAAGCCGATGTTGCGG	Pérez-Pérez and Hanson[10]
	ebcM(−)	CTTCCACTGCGGCTGCCAGTT	
bla_{MOX}	moxM(+)	GCTGCTCAAGGAGCACAGGAT	Pérez-Pérez and Hanson[10]
	moxM(−)	CACATTGACATAGGTGTGGTGC	
bla_{CIT}	citM(+)	TGGCCAGAACTGACAGGCAAA	Pérez-Pérez and Hanson[10]
	citM(−)	TTTCTCCTGAACGTGGCTGGC	
bla_{DHA}	dhaM(+)	AACTTTCACAGGTGTGCTGGGT	Pérez-Pérez and Hanson[10]
	dhaM(−)	CCGTACGCATACTGGCTTTGC	
IS26-$bla_{\text{CTX-M}}$	IS26	GTTTATCACCACCGAC	Eckert *et al.*[12]
	CTX-MA3 (−)	ACYTTACTGGTRCTGCACAT	
IS*Ecp1*-$bla_{\text{CTX-M}}$	IS*Ecp1*	AAAAATGATTGAAAGGTGGT	Eckert *et al.*[12]
	CTX-MA3 (−)	ACYTTACTGGTRCTGCACAT	
bla_{TEM}-IS*Ecp1*	ISEcp (−)	TTCAATAAAATCAAAAATCCCA	Lavollay *et al.*[14]
	Tem 1 (+)	ATACCGCACCACATAGCAGA	
$bla_{\text{CTX-M}}$ – *Orf477*	P1A (+)	GGCGATCCGCGTGATACCAC	Literacka *et al.*[13]
	orf477 (−)	CCTGGGACCTACGTG	

[a]+, Primer forward; −, Primer reverse.

Table 2 Distribution and prevalence of ESBL-producing *K. pneumoniae* isolates in Moroccan cities

City	*Total* K. pneumoniae	*ESBL-producing K. pneumoniae*	*Frequency (%)*
Rabat	136	10	7.3
Casablanca	161	14	8.7
El Jadida	107	7	6.5
Meknès	22	2	9.0
Settat	27	1	3.7
Total	453	34	7.5

aminoglycosides tested. The most frequently observed phenotype profile included resistance to gentamicin, tobramycin and kanamycin ($n=23$; 79.3% of the strains) followed by resistance to gentamicin and tobramycin ($n=4$; 13.7% of the strains) (Table 3).

Phylogenetic types of extended spectrum β-lactamase producing *K. pneumoniae*

In total, 29 (82.3%) isolates were identified as *K. pneumoniae* phylogenetic group I (KpI), 4 (11.7%) as *K. pneumoniae* phylogenetic group II (KpII) and 2 (5.8%) as *K. pneumoniae* phylogenetic group III (KpIII).

Table 3 Characteristics of the ESBL-producing *K. pneumoniae* isolates collected in Moroccan community

Code	Date (day month year)	City	Phylogenetic group	β-Lactamases genes	qnr	aac(6′)-Ib (variant)	Resistance to antibiotics pattern[a]
Kpp.334	04 March 2010	Meknes	KpI	CTX-M-1; TEM-1; SHV-11; OXA-1	*qnrS1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT
Kpp.396	22 January 2010	EL Jadida	KpI	CTX-M-15	–	+ (cr)	NA; CIP; GM; TET; TM; SXT; TMP
Kpp.390	04 October 2010	Rabat	KpI	CTX-M-15; SHV-1	–	–	–
Kpp.329	24 April 2010	EL Jadida	KpI	CTX-M-15; SHV-1; OXA-1	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.373	22 September 2010	Rabat	KpI	CTX-M-15; SHV-36; OXA-1	–	+ (cr)	NA[b]; CIP[b]; GM; K; TM; TET
Kpp.359	07 September 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-1; OXA-1	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.391	09 November 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-1; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.392	11 November 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-12	*qnrS1*	–	GM; TMP
Kpp.370	05 October 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-26	–	+ (cr)	CIP[b]; GM; K; TM; TET[b]; SXT; TMP
Kpp.377	15 September 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-26; OXA-1	–	+ (cr)	GM; K; TM; TET; SXT; TMP
Kpp.394	12 November 2010	Casablanca	KpI	CTX-M-15; TEM-1; SHV-28; OXA-1	–	+ (cr)	NA; CIP; K; TM; TET; SXT; TMP; IMP[b]
Kpp.328	16 June 2010	Casablanca	KpI	CTX-M-15; TEM-1; SHV-32	–	–	TET[b]; SXT; TMP
Kpp.374	20 September 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-76; OXA-1	*qnrB2*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.318	11 May 2010	Casablanca	KpI	CTX-M-15; TEM-1b; EBC-1464; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K[b]; TM; TET; SXT; TMP
Kpp.20	01 June 2010	EL Jadida	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	–	+ (cr)	NA; CIP; GM; K[b]; TM; TET; SXT; TMP
Kpp.323	12 June 2010	EL Jadida	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP; IMP[b]
Kpp.313	01 April 2010	Casablanca	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K; TM; AN[b]; TET; TMP; IMP[b]
Kpp.319	13 May 2010	Casablanca	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.317	10 May 2010	Casablanca	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.350	23 July 2010	Casablanca	KpI	CTX-M-15; TEM-1b; SHV-1; OXA-1	*qnrS1*	+ (Ib)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.366	01 October 2010	Casablanca	KpI	CTX-M-15; TEM-1b; SHV-12; OXA-1	*qnrS1; qnrA6*	+ (cr)	NA; CIP; GM[b]; K; TM; TET; SXT; TMP
Kpp.343	17 July 2010	Casablanca	KpI	CTX-M-15; TEM-3; EBC-1464	–	–	NA; CIP; TET[b]; IMP[b]
Kpp.307	01 March 2010	EL Jadida	KpI	CTX-M-15; TEM-1; SHV-1; OXA-1	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP; IMP[b]
Kpp.386	26 October 2010	Rabat	KpI	CTX-M-15; TEM-1; SHV-11; OXA-1	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT[b]; TMP
Kpp.342	17 July 2010	Casablanca	KpI	CTX-M-15; TEM-1; SHV-11; OXA-1	*qnrB1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.332	08 June 2010	EL Jadida	KpI	CTX-M-15; TEM-1; SHV-11; OXA-1	*qnrS1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.380	15 September 2010	Casablanca	KpI	CTX-M-15; TEM-1; SHV-28; OXA-1	–	+ (cr)	NA; CIP; GM; K; TET
Kpp.347	26 July 2010	Casablanca	KpI	SHV-12; ACT-2	–	–	–
Kpp.395	07 August 2010	Casablanca	KpII	CTX-M-15; TEM-1; SHV-28	–	+ (cr)	NA; CIP; GM; TM; TET; SXT; TMP
Kpp.399	23 August 2010	Settat	KpII	CTX-M-15; TEM-1; SHV-110	–	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.335	10 May 2010	Meknes	KpII	CTX-M-15; TEM-1; SHV-76; OXA-1	*qnrB1*	+ (cr)	NA[b]; CIP; GM; K; TM; TET; SXT; TMP
Kpp.303	01 March 2010	EL Jadida	KpII	CTX-M-15; TEM-1b; SHV-1; OXA-1	*qnrS1, qnrB1*	+ (cr)	NA; CIP; GM; K; TM; TET; SXT; TMP
Kpp.338	05 July 2010	Casablanca	KpIII	CTX-M-15; TEM-1; DHA-1	–	–	NA; CIP; GM; TET; SXT; TMP
Kpp.364	04 September 2010	Rabat	KpIII	CTX-M-15; TEM-1b	–	–	NA; CIP; GM; K[b]; TM; TET; SXT; TMP

Abbreviations: AN, amikacin; CIP, ciprofloxacin; GM, gentamicin; IMP, imipenem; K, kanamycin; NA, nalidixic acid; SXT, Trimethoprim/sulfamethoxazole; TET, tetracycline; TM, tobramycin; TMP, trimethoprim; +, presence; –, negative.
[a]All strains were resistant to the ampicillin, cefotaxime, ceftazidime and amoxicillin/clavulanic acid.
[b]Intermediate resistance level.

Extended spectrum β-lactamase genes detected

The CTX-M ESBL type was detected in 32 isolates (94.1%), all belonged to CTX-M-1 group. Twenty-nine (85.3%) belonged to the SHV family and twenty-nine (85.2%) belonged to the TEM family. Twenty-three isolates (67.6%) harbored bla_{OXA-1} gene. Eight isolates produced more than one ESBL. The two *bla* genes encoding VEB and PER β-lactamases were not detected in any isolate (Table 3).

Plasmid-mediated AmpC β-lactamse genes detected

Among eight cefoxitin-resistant isolates, four harbored plasmid-mediated AmpC β-lactamase (PABLs) genes as revealed by PCR multiplex; one isolates carried *bla* genes of the DHA group and three isolates carried genes belonging to the EBC group. Three types of PABLs were detected by PCR with specific primers and confirmed by sequencing analysis as DHA-1 (Kpp.338 isolate), ACT-2 (Kp.374 isolate) and a new PABLs named EBC-1464 (GenBank: FJ237368.1) (Kpp.318 and Kpp.343 isolates).

Plasmid-mediated quinolone resistance genes

Fourteen (41.1%) ESBL-producing *K. pneumoniae* isolates were positive for *qnr* genes; *qnrA*-, *qnrB*- and *qnrS*-type alleles were detected in 1 (7.1%), 8 (57.1%) and 4 (28.5%) *K. pneumoniae* isolates, respectively. These were found to be *qnrA6*, *qnrB1*, *qnrB2* and *qnrS1* alleles by sequencing of PCR products (Table 3). Interestingly, two isolates carried simultaneously two types of *qnr* genes: Kpp.303 harbored both *qnrB1* + *qnrS1* and Kpp.366 carried both *qnrA6* + *qnrS1*.

Twenty-seven isolates (79.4%) were positive for *aac(6′)-Ib* gene, of which 26 (96% of all) isolates carried the –cr variant. The *aac(6′)-Ib-cr* gene was detected in 85.7% (12/14 isolates) of *qnr*-positive isolates,

Table 4 Transconjugants compared with their parental ESBL-producing *K. pneumoniae*

	MIC ($\mu g\,ml^{-1}$)												Transfer			
Code	*AMX*	*CTX*	*CAZ*	*FEP*	*AN*	*K*	*GM*	*Str*	*TET*	*NA*	*CIP*	*FOX*	*Others resistance*	*Plasmid (kb)*	*β-lactamases genes*	*PMQR genes*
Kpp.20	>256	>256	32	32	8	256	128	>256	>256	512	256	2	TM,TMP	125	CTX-M-15; TEM-1b; OXA-1	*aac(6′)-Ib-cr*
Tc20	>256	>256	32	16	2	64	64	64	128	0.50	0.12	0.50				
Kpp.319	>256	>256	96	>256	8	256	128	>256	256	512	256	4	TM, TMP, SXT	125	CTX-M-15; TEM-1b; OXA-1	*qnrB1*; *aac(6′)-Ib-cr*
Tc319	>256	>256	48	>256	4	128	8	>256	128	32	4	0.50				
Kpp.343	>256	>256	>256	>256	0.50	8	1	4	4	8	0.25	>256	—	150	EBC-1464	—
Tc343	>256	256	256	0.12	0.12	2	0.50	2	2	0.50	0.02	128				
$K_{12}J_5$	4	0.50	0.50	0.12	0.12	0.75	0.50	2	1	0.50	0.02	0.50	—	—	—	

Abbreviations: AMX, amoxicillin; AN, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; GM, gentamicin; K, kanamycin; NA, calidixic acid; Str, streptomycin; SXT, trimethoprim/sulfamethoxazole; Tc, transconjugant; TET, tetracycline; TM, tobramycin; TMP, trimethoprim.

whereas only 51.8% (14/27 isolates) of *qnr*-negative isolates harbored this gene.

Transfer of extended spectrum β-lactamase genes by conjugation experiment

Transfer of β-lactamase genes was observed for three (Kpp20, Kpp319 and Kpp343) out of six isolates examined. Transfer frequencies ranged from 1.10^6 to 2.10^4 per donor cell. Susceptibility results of the clinical strains and their transconjugants are shown in Table 4. All transconjugants were resistant to amoxicillin, amoxicillin/clavulanic acid, cefotaxime and ceftazidime. Cefoxitin resistance was transferred in the Tc.343 transconjugant, whereas trimethoprim, tobramycin, gentamicin, kanamycin, streptomycin and tetracycline resistances were cotransferred in the Tc.20 and Tc.319 transconjugants (Table 4).

Kpp.343 isolate transferred successfully $bla_{\text{EBC-1464}}$ gene in *E. coli* $K_{12}J_5$ recipient strain; this gene is carried by one conjugative plasmid of high molecular weight (150 kb). Analysis of gene of the isolates tested was also located on a plasmid of high molecular weight (125 kb).

The plasmid pK319 of Tc.319 transconjugant strain additionally harbored the following four antibiotic resistance genes: $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1b}}$, *aac(6′)-Ib-cr* and *qnrB1* genes. The plasmid pK20 of Tc.20 transconjugant strain carried $bla_{\text{TEM-1b}}$, $bla_{\text{OXA-1}}$ and *aac(6′)-Ib-cr*.

Exploration of the regions surrounding $bla_{\text{CTX-M}}$ genes

PCR identified the insertion sequence IS*Ecp1* upstream of the $bla_{\text{CTX-M}}$ gene in 32 strains. The sizes of the PCR products were about 0.4 kb in all isolates except Kpp.443 strain. For this strain, the PCR fragment was about 1.1 kb, suggesting the insertion of an additional DNA fragment. PCR with the IS*26*-specific primer produced no amplicon.

The genetic organization of the $bla_{\text{CTX-M-15}}$ genes has been elucidated in the two host plasmids obtained by conjugation experiments (Tc20 and Tc319), in which $bla_{\text{CTX-M-15}}$ gene transfer was successful. In addition, these transconjugants were characterized by their resistance genes diversity. In the mapping of the 3′ends, only the PCR with primers P1A and orf477-IRR worked in all these cases, indicating that both modules terminated at the IS*Ecp1* IRR within *orf477*. The $bla_{\text{TEM-1b}}$ gene was found to be located in a Tn3-like transposon. The *tnpA* gene of the Tn3 transposon is disrupted by IS*Ecp1*-$bla_{\text{CTX-M-15}}$. Long-PCR analysis confirmed that $bla_{\text{CTXM-15}}$ was located just upstream from *aac*(6′)-*Ib-cr*. With the exception of *qnrB* in pK.319, all the antibiotic resistance genes were clustered in a 12-kb region.

DISCUSSION

The ESBL producing *K. pneumoniae* isolates are increasingly causing UTIs both in hospitalized and outpatients.[1,15–16] The prevalence of ESBL producing isolates of *K. pneumoniae* varies in different countries. In European hospitals, it ranges from as low as 5% in Iceland and Estonia to as high as 59.3 to 100% in Ireland, Spain, Germany, Bulgaria and Romania.[16–17] However, there is limited data regarding the prevalence of ESBLs producing *K. pneumonia* in community. In the present study, 34 of the 453 (7.5%) *K. pneumoniae* isolates were ESBL producers. Higher rates of ESBL production among community-onset bacteremic UTIs caused by *E. coli* and *K. pneumoniae* have been reported from Taiwan (17.9%) and Scotland (12.5%).[3,5]

KpI is the most prevalent phylogenetic group in *K. pneumoniae* isolates from Moroccan community as reported in previous studies.[3,15] These preliminary results should be expanded by pulsed field gel electrophoresis or multilocus sequence typing methods to determine epidemiologic relationships among isolates.

ESBL-producing *K. pneumoniae* are commonly resistant to different antibiotic families, which contributes to the selection and persistence of MDR–ESBL strains and plasmids in community settings, resulting in the limitation of therapeutic options.[18–20] In this study, the rate of MDR was alarmingly high (91.1%), imipenem being the most active among the antibiotics tested. All these observations indicate the real threat of this problem, particularly when we know that the increasing use of carbapenems that have been widely used to treat serious infections with MDR *K. pneumoniae*, has led to the emergence of carbapenem-resistant isolates via acquired genes encoding carbapenem-hydrolyzing enzymes.[20]

ESBLs CTX-M types have been known for their rapid spread in African and European communities.[3,9,21–22] The first detection of $bla_{\text{CTX-M}}$ (CTX-M-15 and CTX-M-28) in Morocco was documented in 2010 at the teaching hospital Ibn Rochd in Casablanca.[6] In a previous study, we have investigated, for the first time, the dissemination of these enzymes among community ESBL producing *Enterobacteriaceae* isolates responsible for UTIs, including a restricted sample of *K. pneumoniae* strains.[9] To contribute to a better understanding of the epidemiology of

these enzymes at local and national level, other cities are included in the present work and therefore, the sample was larger. It was remarkable that, 91.1% of our ESBL-producing *K. pneumoniae* isolates carried $bla_{\text{CTX-M-15}}$; this proves that $bla_{\text{CTX-M-15}}$ gene is highly endemic in Moroccan community. Seventy-four percent of strains harboring the $bla_{\text{CTX-M-15}}$ gene also possessed a $bla_{\text{OXA-1}}$ gene (Table 3), presumably explaining the high percentage of non-susceptibility to amoxicillin-clavulanic acid. This genes combination has been found in the same strain in Portugal.[21] The $bla_{\text{CTX-M-15}}$ plus $bla_{\text{OXA-1}}$ plus $bla_{\text{TEM-1}}$ or $bla_{\text{TEM-1b}}$ was co-expressed by 23 strains. This genes association has been also reported in strains from Portugal.[21] However, the combination of $bla_{\text{CTX-M-15}}$ plus $bla_{\text{TEM-3}}$ reported here is, to our knowledge, first in Africa. Twenty-nine ESBL-producing *K. pneumoniae* isolates carried bla_{SHV}, of which, twenty-three encoded non-ESBL types. It is noteworthy that this study reported the detection of SHV-28, SHV-36 and -110-producing isolates for the first time in Morocco.

Plasmid analysis revealed that the transconjugants (Tc.20 and Tc.319) harbored large plasmids of about 125 kb and these plasmids (pK.319 and pK.20) carry only two known resistance genes, $bla_{\text{TEM-1b}}$ and $bla_{\text{CTX-M-15}}$. These findings suggest that the CTX-M-15 allele is carried on large conjugative plasmids that are well adapted and constantly exchanged by lateral gene transfer among the *K. pneumoniae* isolates. Previous studies have shown that bla_{TEM} genes are carried by three of the earliest described bacterial transposons, namely Tn1, Tn2 and Tn3. These transposons contain the transposase and resolvase genes, *tnpA* and *tnpR*, as well as a resolution site.[23] The $bla_{\text{TEM-1}}$ gene was found to be located in a Tn3-like. Interestingly, the *tnpA* gene of the Tn3 transposon is disrupted by IS*Ecp1*-$bla_{\text{CTX-M-15}}$ due to IS*Ecp1*-mediated transposition. The mechanism involves the left inverted repeat of IS*Ecp1* and a right inverted repeat (IRR1), which resembles the IRR of IS*Ecp1*.[24]

The resistance of *K. pneumoniae* to cephamycins have also arisen by acquisition of plasmids containing the chromosomally derived AmpC β-lactamase.[6] In Morocco, the PABLs types previously detected were DHA-1 and CMY-2.[6,25] In this study, we detected eight cefoxitin-resistant *K. pneumoniae*, of which four isolates harbored PABLs genes. They could become an important cause, in addition to ESBL-producing isolates, of increasing resistance to extended spectrum β-lactams in Moroccan community. The major finding of this study was the report of the occurrence of the ACT-2 β-lactamase for the first time in Morocco. Furthermore, we detected two strains that harbored $bla_{\text{EBC-1464}}$ gene. To the best of our knowledge, this is the second report of EBC-1464 β-lactamase in the world. The EBC-1464 β-lactamase was a novel PABL detected in a *K. pneumoniae* isolate from China (GenBank: FJ237368.1). Cefoxitin resistance in AmpC non-producers could be due to some other resistance mechanism, such as lack of permeation of porins.[26]

In conclusion, our results demonstrate that the resistance of ESBL-producing *K. pneumoniae* responsible for UTIs to a wide variety of common antimicrobials continue to become a serious global health concern that could complicate treatment strategies. We also report the first detection in Morocco of $bla_{\text{EBC-1464}}$ and the diversity of the β-lactamase genes of community *K. pneumoniae* isolates, with the CTX-M-15 being most common. To ensure appropriate therapy, current knowledge of antibiotic susceptibility of the organisms that cause UTIs is mandatory.[1] In this context, routine screening for ESBLs producers in the laboratory is of great importance for their early identification and management.

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