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ORIGINAL ARTICLE

First detection of a transferable *bla*$_{\text{CTX-M-14b}}$ gene in a *Klebsiella pneumoniae* clinical isolate from Tunisia and analysis of its genetic context

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Abstract *Klebsiella pneumoniae* ML2508 was isolated from a patient at the surgery unit of the Military Hospital (Hôpital Militaire de Tunis), Tunisia. It was identified as a producer of extended-spectrum β-lactamases (ESBLs) by the double-disk synergy test. The β-lactamases produced by the strain were characterized by isoelectric focusing, determination of the specific activities against penicillins and cephalosporins, determination of the inhibitory concentration required to inhibit 50% of enzyme activity (IC_{50}), and the inhibition effect of EDTA on putative metallo-β-lactamases. The crude extract of *K. pneumoniae* ML2508 contains five different β-lactamases with pI 5.5, 7.3, 7.6, 8.1, and 8.6. Only the β-lactamase with pI 8.1 was transferred by transformation and conjugation experiments. Molecular characterization of these genes was performed by PCR and sequencing. The four chromosomal β-lactamases are TEM (pI 5.5), 2 SHV (pI 7.3 and 7.6), and CTX-M-28 (pI 8.6). The β-lactamase with pI 8.1 was encoded by *bla*$_{\text{CTX-M-14b}}$ gene located on a 50-kb highly conjugative plasmid. The study of the genetic context of *bla*$_{\text{CTX-M-14b}}$ was realized by PCR-mapping and DNA sequencing. A novel variant of *tnp*$_{\text{IS}Ecp1}$ designated IS*Ecp1C* was detected upstream of the gene.

Keywords β-lactamase · *Klebsiella pneumoniae* · PCR-mapping · DNA sequencing

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Introduction

CTX-M-type extended-spectrum β-lactamases constitute a growing group of enzymes encoded by transferable plasmids that vary in size from 7 to 160 kb (Bonnet 2004; Eckert et al. 2004; Pieboji-Gangoue et al. 2005). A phylogenetic study of the CTX-M family of β-lactamases showed five major types: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups (Bradford 2001; Bonnet 2004; Shah et al. 2004; Canton and Coque 2006). The majority of CTX-M enzymes hydrolyse cefotaxime preferentially over ceftazidime, and the new enzymes were able to confer higher levels of resistance to ceftazidime than to cefotaxime (Sturenburg et al. 2004). The CTX-M enzymes have been found mainly in strains of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Shah et al. 2004), but have also been commonly found in *Klebsiella pneumoniae*, nontyphoid *Salmonella*, *Shigella*, *Citrobacter freundii*, *Enterobacter* spp., *Morganella morganii*, *Serratia marcescens*, and *Vibrio cholerae*, amongst other species (Radice et al. 2001; Di conza et al. 2002; Petroni et al. 2002).

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Some of the CTX-M enzymes have demonstrated an endemic occurrence in particular countries, such as CTX-M-9 and CTX-M-14 in Spain and CTX-M-1 in Italy, whereas other CTX-M enzymes, such as CTX-M-2 and CTX-M-15, have shown a widespread international distribution (Canton and Coque 2006).

Different genetic elements might be involved in the mobilization of $bla_{\text{CTX-M}}$ genes. IS*Ecp1* and IS*Ecp1*-like insertion sequences have repeatedly been observed upstream of genes encoding the CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-15, CTX-M-17, CTX-M-19, CTX-M-20, and CTX-M-21 enzymes (Bonnet 2004; Canton and Coque 2006). CTX-M-9- and CTX-M-2-encoding genes have also been observed in unusual class 1 integrons, designated InS21, In35, In116, and In60 (Arduino et al. 2002; Di Conza et al. 2002; Partridge and Hall 2003; Power et al. 2005). For integrons InS21, In116, and In35, there is a common region (CR) containing a 2,185-bp sequence that harbors the $bla_{\text{CTX-M-2}}$ gene and an Orf3, which probably originated from *Kluyvera ascorbata* (Arduino et al. 2002; Di Conza et al. 2002; Partridge and Hall 2003; Power et al. 2005). For integron In60, this CR contains the $bla_{\text{CTX-M-9}}$ gene, an Orf-3-like element, and a putative insertion sequence designated IS*3000* (Bonnet 2004). Other resistance genes have been observed in the CR, such as the *catA2* gene (chloramphenicol resistance, formerly *catII*), the *dfrA10* gene (trimethoprim resistance, formerly *dhfrX*), and the $bla_{\text{DHA-1}}$ gene associated with its *ampR* regulator gene, in In6, In7 and the integron of plasmid pSAL-1 from *Salmonella enterica* serovar Enteritidis, respectively (Partridge and Hall 2003; Bonnet 2004). In addition, it has been suggested that orf513, the so-called IS*CR* element, might encode a putative site-specific DNA recombinase, which may have been able to capture genes from the chromosome of environmental microorganisms, e.g., $bla_{\text{CTX-M}}$ genes associated with loci from *Kluyvera* (Toleman et al. 2006). Another hypothesis suggests complex mobilization processes involving the Orf513 putative recombinase and the IS*Ecp1* insertion sequence (Partridge and Hall 2003; Canton and Coque 2006). In addition, the CR element, the associated variable region, and the second 3'CS element could be transferred by homologous recombination between 3'CS elements (Partridge and Hall 2003; Bonnet 2004; Canton and Coque 2006; Toleman et al. 2006). Other elements may be involved in the mobilization of $bla_{\text{CTX-M}}$ genes, such as IS*10* and IS*26*, partial sequences of which have been observed upstream of $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-1}}$, respectively, and an IS*903*-like element observed downstream of the $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-17}}$ genes (Bonnet 2004). In addition, Oliver et al. (2001) suggest that the transfer of $bla_{\text{CTX-M-10}}$ from the chromosome of *Kluyvera* spp. to a transferable plasmid may have been mediated by transduction by a bacteriophage, highlighting the potential role of phages in the dissemination of resistance determinants. In this work, we describe the first isolation of $bla_{\text{CTX-M-14b}}$ in Tunisia and the analysis of its genetic context.

Materials and methods

Bacterial strains and antimicrobial susceptibility testing

Klebsiella pneumoniae ML2508 was isolated in October 2004 from a surgery unit at the Military Hospital (Hôpital Militaire de Tunis) in Tunisia. The isolate was identified using an API 20 E system (bio-Mérieux, Marcy l'Etoile, France). *E. coli* DH5α (*recA1*, F^-, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, rK^-, mK+, *supE44*, *relA1*, *ΔlacU69*, *Φ80lazΔM15*), and *E. coli* HB101 (F^-, *Δ*(*gpt-proA*) 62, *leuB6*, *supE44*, *ara-14*, *galK2*, *lacY1*, *Δ*(*mcrc-mrr*), *rps*, L26, Xyl-rmtl 1, *thi-1*, IncFI, *recAB*, str^r) were used for the transformation and conjugation experiments, respectively. Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar (Bio Rad, France), microdilution, and E-test (Biodisk, Solna, Sweden), according to the Clinical and Laboratory Standards Institute recommendations (table 2 in Clinical Laboratory Standards 2009). Production of ESBLs was phenotypically confirmed using synergism between amoxicillin-clavulanate and cefotaxime, ceftriaxone, ceftazidime, and aztreonam.

Biochemical characterization of β-lactamases

β-Lactamase activity was measured by spectrophotometric monitoring of antibiotic hydrolysis (spectrophotometer Varian UV-Visible Cary 50 Bio) at 25°C in 25 mM sodium phosphate buffer (pH 7.0). The concentrations and wavelengths used for each solution of β-lactam were: 0.4 mM at 235 nm for benzyl-penicillin and ampicillin; 0.1 mM at 235 nm for ticarcillin; 0.05 mM at 230 nm for cloxacillin; 0.05 mM at 262 nm for cephalothin; 0.025 mM at 255 nm for ceftriaxone; 0.025 mM at 260 nm for cefotaxime and ceftazidime; 0.05 mM at 265 nm for cefoxitin; 0.025 mM at 258 nm for cefpirome; 0.3 mM at 318 nm for aztreonam; and 0.05 mM at 297 nm for imipenem. In inhibition assays, crude extracts were incubated with different concentrations of clavulanic acid or sulbactam at room temperature in the same buffer, and remaining activity was then determined by spectrophotometry at 262 nm with cephalothin as substrate (0.05 mM). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of substrate in 1 min at 25°C. The inhibitory concentration required to inhibit 50% of enzyme activity (IC_{50}) was determined. The effect of EDTA inhibition of metallo-β-lactamase was also tested.

Analytical isoelectric focusing (IEF)

Isoelectric focusing on crude extract from *K. pneumoniae* ML2508 and its transformants and transconjugants was performed (Ben-Mahrez et al. 1999), in non-denaturing polyacrylamide gels containing ampholines (pH range of 3 to 10) at 4°C in a 111Mini IEF Cell, (Bio-Rad). β-Lactamase activities were detected with benzyl-penicillin (0.5 mM) and cefotaxime (3 mM) in 25 mM sodium phosphate buffer (pH 7). The pI standards included TEM-1 (pI 5.4), TEM-2 (pI 5.6), SHV-1 (pI 7.6), SHV-12 (pI 8.2), and CTX-M-15 (pI 8.6).

β-Lactam resistance transfer assays

Plasmid DNA was extracted with the GFX Micro Plasmid Prep kit (Amersham Biosciences, UK), according to the manufacturer's instructions, and transformed into *E. coli* DH5α competent cells. Transformants were selected on Luria Bertani agar plates supplemented with ampicillin (100 μg/ml). Conjugation was carried out on Luria Bertani broth, with *E. coli* HB101 as the recipient. Transconjugants were selected on Luria Bertani agar containing ampicillin (100 μg/ml) and streptomycin (256 μg/ml).

PCR assays for molecular characterization

The presence of TEM, SHV and CTX-M-type β-lactamases encoding genes and $tnpA_{ISEcp1}$ was investigated by PCR. Primers used to amplify β-lactamase genes, annealing temperatures and predicted amplicon sizes are shown in Table 1.

The occurrence of $tnpA_{ISEcp1}$ upstream blaCTX-M was investigated by a mapping PCR using the CTX-M-9R and ISEcp1F primers, with a predicted amplicon size of 2.3 kb (Table 1).

Cloning and sequencing of PCR products

PCR products were purified with the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences), ligated in the pTZ57R/T vector (Fermentas, USA), and transformed into *E. coli* DH5α cells. Positive clones were selected on Luria Bertani Agar supplemented with ampicillin (100 μg/ml). Plasmid DNA was extracted with GeneJet Plasmid Miniprep kit (Fermentas). The insert size was confirmed by *Xba*I and *Bam*HI (Promega, USA) digestion. The inserts were sequenced at the GIGA Genomics Facility in Belgium, and the nucleotide and deduced amino acid sequence analyses were performed by the NCBI (www.ncbi.nlm.nih.gov/) and EBI (www.ebi.ac.uk/) analysis tools, and by comparison with data available in GenBank and at www.lahey.org/studies/webt.html.

Results

Antimicrobial susceptibility testing

Klebsiella pneumoniae ML2508 was resistant to all the antibiotics tested except for imipenem (Table 2). This resistance was transferred to the transformants and transconjugants strains except for resistance to cefoxitin, aminoglycosides, quinolones, and phenicols. The disk diffusion method showed synergy between ceftazidime, cefotaxime, aztreonam, ceftriaxone, and amoxycillin-clavulanic acid against the strain and its transformants and transconjugants, suggesting the presence of a class A extended-spectrum β-lactamase.

Biochemical characterization of the β-lactamases

The β-lactamase-containing crude extract from *K. pneumoniae* ML2508 was active against benzyl-penicillin, ampicillin, ticarcillin, cephalothin, cefotaxime, ceftazidime, ceftriaxone, cefpirome, and cloxacillin. The specific activity varied from 0.23 to 5.87 U/mg proteins. Cephalothin was more efficiently hydrolyzed compared to the rest of tested antibiotics. In fact, cephalothin was hydrolyzed at 8-, 17-, and 4-fold higher rates than cefotaxime, ceftazidime, and ceftriaxone, respectively. No hydrolytic activity was detected against cefoxitin, aztreonam, and imipenem.

The production of a class A extended-spectrum β-lactamase (ESBL) and the absence of B-metallo-β-lactamases were confirmed by inhibition studies with clavunic acid and EDTA, respectively. The β-lactamases present in the crude extract of *K. pneumoniae* ML2508 were strongly inhibited by clavulanic acid, with an IC_{50}=11.3 μM. A three-fold higher concentration of sulbactam was needed to obtain an inhibition at 50% (IC_{50}=33 μM). β-Lactamases activities were not inhibited by 1 mM EDTA.

The examination of the crude extract of *K. pneumoniae* ML2508 by isoelectric focusing showed the presence of five β-lactamase bands with apparent pIs of 5.5, 7.3, 7.6, 8.1, and 8.6. Only the β-lactamase with pI 8.1 was transferred by transformation and conjugation experiments. This enzyme was revealed by isoelectric focusing with benzyl-penicillin and cefotaxime as substrates.

Plasmid profiles and transfer of resistance

Plasmid analysis revealed the presence of a large plasmid transferable by transformation experiments with an estimated molecular size of 50 kb. This plasmid was named pML2508. The frequency of conjugational transfer performed with *K. pneumoniae* ML2508 as donors and *E. coli* HB101 as the recipient was 10^{-3}/donor.

Table 1 Sequences of the primers used in PCR and TAI-PCR to study bla_{CTX-M} gene context

Primer	Sequence (5′-3′)	Expected size (bp)	Reference / acc. number
TEM-F	ATG AGT ATT CAA CAT TTC CG	858	J01749
TEM-R	CCA ATG CTT AAT CAG TGA GC		
SHV-F	ATG ATG AGC ACC TTT AAA GTA	621	M59181
SHV-R	ATT TCG CTC GGC CAT GCT CGC		
CTX-M-1F	ATG GTT AAA AAA TCA CTG C	864	X92506
CTX-M-1R	GGT GAC GAT TTT AGC CGC		
CTX-M-2F	TTA ATG ATG ACT CAG AGC ATT C	902	X92507
CTX-M-2R	GAT ACC TCG CTC CAT TTA TTG C		
CTX-M-8F	TGA ATA CTT CAG CCA CAC G	923	AF189721
CTX-M-8R	TAG AAT TAA TAA CCG TCG GT		
CTX-M-9F	ATG GTG ACA AAG AGA GTG C	876	AF174129
CTX-M-9R	TCA CAG CCC TTC GGC GAT G		
ISEcp1F	GCA GGT CTT TTT CTG CTC C	527	AJ242809
ISEcp1R	ATT TCC GCA GCA CCG TTT GC		

PCR assays for molecular characterization

PCR analyses showed the presence of bla_{SHV}, bla_{TEM}, and $bla_{CTX-M-1}$ group on the chromosomal DNA of *K. pneumoniae* ML2508. The $bla_{CTX-M-9}$ group was detected by PCR in pML2508 extracted from the *K. pneumoniae* ML2508 parental strain, and also in its derived transformant and transconjugant clones, finding that the encoded gene was harbored by the 50-kb conjugative plasmid. Sequence analysis of nucleotide sequence of bla_{CTX-M} genes showed the presence of $bla_{CTX-M-28}$ and $bla_{CTX-M-14b}$ in the chromosome and plasmid, respectively. The sequence of $bla_{CTX-M-14b}$ differs from the only $bla_{CTX-M-14b}$ entry present in GenBank (accession number DQ359215) by one nucleotide substitution in the codon stop TAA to TGA. The sequence has been deposited in GenBank and has been given GenBank accession number EU274579. Sequence analysis of the deduced amino acid sequence showed 100% identity with CTX-M-14.

An amplification product of 2.3 kb was obtained with CTX-M-9R (reverse) and ISEcp1F (forward) specific primers, suggesting that the insertion sequence was located

Table 2 MICs of various antimicrobial agents obtained for the clinical isolate *K. pneumoniae* ML2508, transformants, transconjugants, and the *E. coli* recipients

Antibiotics	MIC (μg/ml)				
	K. pneumoniae ML2508	*E. coli* [a]TF-DH5α/pML2508	*E. coli* DH5α	*E. coli* [b]TC-HB101/pML2508	*E. coli* HB101
Ampicillin	>512	>512	8	>512	4
Amoxicillin	>512	>512	8	>512	4
Ticarcillin	>512	>512	2	>512	2
Cephaloridine	>512	>512	0.13	>512	1
Cefoxitin	64	8	4	8	2
Cefotaxime	>512	>512	0.13	>512	1
Ceftazidime	128	64	0.13	64	1
Ceftriaxone	>512	>512	0.13	>512	1
Cefpirome	>512	>512	0.13	>512	2
Aztreonam	>512	256	0.13	256	1
Chloramphenicol	>256	2	2	2	2
Nalidixic acid	>512	1	0.06	1	1
Ciprofloxacin	256	1	0.06	1	1
Tetracyclin	>512	2	0.25	2	2
Streptomycin	64	0.25	0.25	>256	>256
Imipenem	0.75	0.38	0.06	0.38	0.25

[a] *TF* Transformant

[b] *TC* Transconjugant

upstream of the $bla_{\text{CTX-M-14b}}$ gene. Sequence analysis of common region of $tnpA_{\text{ISEcp1}}$ indicates an open reading frame of 532 bp, corresponding to 177 amino acid residues. Compared to $tnpA_{\text{ISEcp1}}$ (DDBJ/EMBL/GeneBank accession no. DQ821704), one amino acid substitution was found at position 191, resulting in a Met (ATG) to Ile (ATA) change; Met191Ile. This substitution has not been shown in other studied insertion sequences, therefore corresponding to a novel variant of $tnpA_{\text{ISEcp1}}$. The sequence has been deposited in GenBank and has been given GenBank accession no. EU285457. There is another IS*Ecp1C* (accession no. EU523120) described by Cattoir et al. (2008), which does not include the same mutation.

Discussion

Klebsiella pneumoniae ML2508 produced two different CTX-M type β-lactamases, CTX-M-28 (group 1) and CTX-M-14 (group 9). CTX-M-14 was encoded by $bla_{\text{CTX-M-14b}}$ (GenBank accession number: DQ359215), which interestingly demonstrated three synonymous nucleotide substitutions compared with $bla_{\text{CTX-M-14}}$ (GenBank accession number: AF252622), whereas it demonstrated only one non-synonymous nucleotide substitution compared with $bla_{\text{CTX-M-9}}$ (GenBank accession number: AF174129). Thus, $bla_{\text{CTX-M-14b}}$ probably represents a subsequent derivative of $bla_{\text{CTX-M-9}}$ (Navarro et al. 2007).The first $bla_{\text{CTX-M-14b}}$ was described in 2003 in an *E. coli* strain isolated from clinically relevant samples from Spain (Navarro et al. 2007).

Outbreaks of *Salmonella enterica* serovar Livingstone encoding $bla_{\text{CTX-M-27}}$, showing (Asp240Gly) nucleotide substitutions compared with $bla_{\text{CTX-M-14b}}$ have been previously reported in a neonatal ward in Sousse (Tunisia) in 2002 (Bouallegue-Godet et al. 2005), suggesting the occurrence of molecular evolutionary events in Tunisia.

During a period of 6 years and 5 months (January 1999 to May 2005) at Mongi Slim University Hospital Centre, 2 of 103 isolates of *K. pneumoniae* were found to carry $bla_{\text{CTX-M-27}}$, and two had $bla_{\text{CTX-M-14b}}$ (Elhani et al. 2010). CTX-M-14 is the most frequent CTX-M β-lactamase in eastern Europe, Latin America, Asia, and more recently in Spain, Kenya (Bonnet 2004; Canton and Coque 2006; Velasco et al. 2007; Yu et al. 2007). In Tunisia, the CTX-M-14 enzyme has been reported in *K. pneumoniae* and *E. coli* (Ben Slama et al. 2011; Elhani et al. 2010; Jouini et al. 2007).

A new insertion sequence IS*Ecp1C* (GenBank accession no. EU285457) detected on 250 bp upstream of $bla_{\text{CTX-M-14b}}$ was 532 bp long and possessed two 16-bp imperfect inverted repeats. A putative promoter consisting of the −10 (TACAAT) and −35 (TTGAAA) regions separated by 18 bp was observed within the 3' non-coding sequence of IS*Ecp1C*. Accordingly, the expression of $bla_{\text{CTX-M-14b}}$ was most likely associated to IS*Ecp1C*. In contrast, $bla_{\text{CTX-M-14b}}$ described in Spain had the orf513 upstream and the IS3000 downstream, in accordance with the In60 structure described for the $bla_{\text{CTX-M-9}}$ gene (Navarro et al. 2007).

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