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Article *in* Canadian Journal of Microbiology · August 2012

DOI: 10.1139/w2012-089 · Source: PubMed

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Occurrence of clinical isolates of *Klebsiella pneumoniae* harboring chromosomally mediated and plasmid-mediated CTX-M-15 β-lactamase in a Tunisian hospital

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Abstract: The spread of multidrug-resistant strains of *Klebsiella pneumoniae* in hospitals is of concern to clinical microbiologists, health care professionals, and physicians because of the impact infections caused by these bacteria have in causing morbidity and mortality. Clinical isolates of *K. pneumoniae* have been found to show resistance to third-generation cephalosporins as a result of acquiring extended-spectrum β-lactamase-producing genes, such as $bla_{\text{CTX-M}}$. Since little is known about the mechanisms of antibiotic resistance observed in Kasserine hospital, Tunisia, this study was undertaken to investigate the mechanisms by which clinical isolates of *K. pneumoniae* resist β-lactam antibiotics. Twelve strains of *K. pneumoniae* were collected from patients admitted to Kasserine hospital; these isolates showed multiresistance phenotypes. Molecular genetics investigations using polymerase chain reaction, S1 digestion, and pulsed-field gel electrophoresisshowed that $bla_{\text{CTX-M-15}}$ in association with IS*Ecp1* is responsible for the resistance of these strains to third-generation cephalosporins. It has been determined that $bla_{\text{CTX-M-15}}$ is chromosomally mediated and plasmid mediated, which alarming need for infection control to prevent the outbreak of such a resistance mechanism.

Key words: Klebsiella pneumoniae, extended-spectrum β-lactamase, ESBL, $bla_{\text{CTX-M-15}}$, IS*Ecp1*, Tunisia.

Résumé : La propagation dans les hôpitaux de souches de *Klebsiella pneumoniae* multi-résistantes aux médicaments est inquiétante pour les microbiologistes cliniques, les professionnels de la santé et les médecins à cause de l'impact des infections causées par ces bactéries en termes de morbidité et de mortalité. Des isolats cliniques de *K. pneumoniae* se sont avérés porteurs de résistance à la troisième génération de céphalosporines résultant de l'acquisition de gènes produisant des β-lactamases à spectre étendu comme $bla_{\text{CTX-M}}$. Parce que l'on a peu d'information sur les mécanismes de résistance aux antibiotiques à l'hôpital de Kasserine, cette étude a été entreprise afin d'examiner les mécanismes de résistance par lesquels les isolats cliniques de *K. pneumoniae* résistent aux antibiotiques de la famille des β-lactames. Douze souches de *K. pneumoniae* ont été récoltées de patients admis à l'hôpital de Kasserine ; ces isolats présentaient des phénotypes de résistance multiple. Des études en génétique moléculaire par PCR, digestion par la S1 et électrophorèse sur gel en champ pulsé ont montré que $bla_{\text{CTX-M-15}}$ associé à IS*Ecp1* sont responsables de la résistance des ces souches à la troisième génération de céphalosporines. La localisation de $bla_{\text{CTX-M-15}}$ étant chromosomique et plasmidique, ceci met en évidence l'urgence de contrôler l'infection afin de prévenir l'éclosion de la résistance.

Mots-clés : Klebsiella pneumoniae, β-lactamases à spectre étendu, BLSE, $bla_{\text{CTX-M-15}}$, IS*Ecp1*, Tunisie.

[Traduit par la Rédaction]

Introduction

The emergence and spread of antimicrobial resistance in pathogenic bacteria exists in many forms but always represents a process of evolution in response to selective antimicrobial pressure (Paterson 2000). This selective pressure is most commonly generated during human colonization and antimicrobial agents use (Tenover et al. 1995). The clinical

Received 3 April 2012. Revision received 26 June 2012. Accepted 29 June 2012. Published at www.nrcresearchpress.com/cjm on 20 August 2012.

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impact of antimicrobial resistance may be great or insignificant, depending on the level of resistance, the site of infection, and the availability of effective, nontoxic therapeutic alternatives (Bell et al. 2002).

Extended-spectrum β-lactamases (ESBLs) that hydrolyze extended-spectrum cephalosporins and are inhibited by clavulanic acid have been detected spreading among *Enterobacteriaceae*. The CTX-M enzymes are replacing SHV and TEM enzymes as the prevalent type of ESBLs, principally in community-acquired infections caused by *Escherichia coli* (Bonnet 2004). Associated infectious syndromes include urinary tract, bloodstream, and intra-abdominal infections, and may be serious enough to warrant hospitalization. Affected patients commonly have various underlying risk factors. This is also observed in hospital-acquired infections (Girlich et al. 2001). The rates of ESBL expression among nosocomial *Enterobacteriaceae* isolates, particularly *Klebsiella pneumoniae*, have risen substantially in several countries (Paterson et al. 2003).

In hospitalized patients, ESBL producers may cause nosocomial pneumonia and meningitis (Paterson 2000). It has been shown that mortality rates varying from 42% to 100% were reported in patients infected with ESBL-producing bacteria (Colodner 2005). ESBL-producing bacteria often show cross-resistance with other groups of antibiotics, such as fluoroquinolones, and the early detection of ESBLs is therefore extremely important because the prevalence of ESBLs is severely underestimated (Colodner 2005).

Most ESBL-producing bacteria can be divided into 3 groups: TEM, SHV, and CTX-M types, and Gram-negative β-lactamases are often mediated by bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ genes (Tzouvelekis et al. 2000). The SHV enzymes are named after the sulfhydryl variable active site and are commonly associated with *K. pneumoniae*. Initially, these bacteria contained a single ESBL gene, but now multiple ESBL genes are commonly present in a single strain, further complicating the process of detection (Samaha-Kfoury and Araj 2003). The original TEM was first discovered in *E. coli* in Greece, but it has spread rapidly to other genera of the *Enterobacteriaceae*, such as *Citrobacter freundii* and *Acinetobacter* spp. (Colodner 2005). More recently, *Enterobacteriaceae* producing novel ESBLs such as CTX-M enzymes have emerged within hospitals and communities worldwide (Pitout et al. 2007). The name CTX is an abbreviation for cefotaximase and refers to the potent hydrolytic activity of this enzyme against cefotaxime (Tzouvelekis et al. 2000).

The CTX-M β-lactamases exceed 450 different types based on their amino acid identities and can be divided into 5 groups: CTX-M group 1 (including CTX-M-1, -3, -10, -12, -15, -28, and -30, and FEC-1); CTX-M group 2 (including CTX-M-2, -4, -5, -6, -7, and -20, and Toho-1); CTX-M group 8 (including CTX-M-8); CTX-M group 9 (including CTX-M-9, -13, -14, -16, -17, -19, -21, -24, and -27, and Toho-2); and CTX-M group 25 (including CTX-M-25 and -26) (Perez et al. 2007).

Despite their widespread distribution, the prevalence of ESBL-producing organisms remains underestimated because a large number of laboratories, mainly within cities of the same countries, do not perform routine tests that specifically detect ESBLs. *Klebsiella pneumoniae* is a well-described health-care-associated pathogen and a cause of sepsis, urinary tract infections, pneumonia, and soft tissue infections in patients in the neonatal intensive care unit (Lebessi et al. 2002). The emergence of ESBL production in *K. pneumoniae* was first reported in 1983 (Knothe et al. 1983), but outbreaks associated with increased morbidity and mortality have been noted with increasing frequency in intensive care units, including neonatal intensive care units (Lebessi et al. 2002). In this study, we report the occurrence of chromosomally encoded and plasmid-encoded CTX-M-15 producing *K. pneumoniae* from the neonatal intensive care unit in Kasserine hospital in Tunisia.

Material and methods

Bacterial isolates and minimum inhibitory concentration determination

Twelve isolates of ESBL-producing *K. pneumoniae* were included in the study. All samples were obtained from Kasserine hospital, Tunisia. The samples were collected between January and March 2010. Bacterial species identity and minimum inhibitory concentrations (MICs) were confirmed by the Phoenix automated phenotypic identification criteria (Becton Dickinson, Oxford, UK).

Polymerase chain reaction amplification of $bla_{CTX\text{-}M\text{-}15}$ resistance gene and sequence analysis

The genetic incidence of the $bla_{CTX\text{-}M\text{-}15}$ genes and their genetic environment were performed by polymerase chain reaction (PCR) and sequencing. Detection of the upstream IS*Ecp1* insertion sequence was carried out using the following primers: CTX-M-F — TCTTCCAGAATAAGGAATCCC and CTX-M-R — CCGTTTCCGCTATTACAAAC; IS-F — GTGCCCAAGGGGAGTGTATG and IS-R — ACATTACTGGTGCTGCACAT. Bacterial DNA of the 12 *K. pneumoniae* isolates was prepared by suspending 1 fresh colony in 500 μL of sterile distilled water and heated at 95 °C for 10 min. PCR amplification of the $bla_{CTX\text{-}M\text{-}15}$ genes was carried out under the following conditions: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min.

The amplicons were purified and sequencing was performed using corresponding primers specific for $bla_{CTX\text{-}M\text{-}15}$ genes according to the method of Sanger et al. (1977), using an automated sequencer (377, ABI Prism, PerkinElmer, Connecticut, USA).

Pulsed-field gel electrophoresis and S1 digestion

To separate chromosomal and plasmid DNA, total DNA of *K. pneumoniae* isolates was treated with S1 followed by pulsed-field gel electrophoresis (PFGE) as described previously (Barton et al. 1995). One colony of *K. pneumoniae* isolate was grown at 37 °C for 8–16 h in brain heart infusion broth (Oxoid, Hampshire, UK) and was then resuspended in 100 μL of phosphate-buffered saline. Fifty microlitres of each sample solution was used for plug preparation. After the serial extraction procedures were completed, the plugs were serially treated with proteinase K, followed by treatment with phenylmethylsulfonyl fluoride and washing buffer (50 mmol/L Tris–HCl (pH 7.5)).

Each plug was rinsed with 200 μL of 1× nuclease S1 buffer (50 mmol/L NaCl, 30 mmol/L sodium acetate (pH 4.5),

Table 1. Antibiotic susceptibilities of 12 clinical isolates of *Klebsiella pneumoniae*, as measured by minimum inhibitory concentration (MIC).

	MIC (μg/mL)										
Isolate	AMP	AMC	CFZ	CTX	CAZ	CFP	ATM	CIP	GM	KAN	IMP
1	>64	>16/4	>64	>64	32	>64	>64	0.125	0.25	0.25	0.125
2	>64	>16/4	32	>64	16	>64	32	0.25	0.125	0.125	0.125
3	>64	>16/4	32	>64	>64	>64	>64	0.125	0.125	0.25	0.125
4	>64	>16/4	>64	>64	32	>64	>64	0.125	0.125	0.25	0.25
5	>64	>16/4	16	>64	>64	>64	>64	0.25	0.25	0.125	0.125
6	>64	>16/4	>64	>64	>64	>64	>64	0.25	0.25	0.25	0.125
7	>64	>16/4	32	>64	>64	>64	32	0.125	0.125	0.25	0.125
8	>64	>16/4	>64	>64	>64	>64	>64	0.25	0.125	0.25	0.25
9	>64	>16/4	32	>64	>64	>64	>64	0.125	0.125	0.25	0.125
10	>64	>16/4	16	>64	32	>64	>64	0.125	0.25	0.125	0.125
11	>64	>16/4	32	>64	>64	>64	>64	0.25	0.25	0.25	0.125
12	>64	>16/4	>64	>64	>64	>64	16	0.125	0.25	0.125	0.25

Note: AMP, ampicillin; AMC, amoxicillin–clavulanate; CFZ, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; CFP, cefepime; ATM, aztreonam; CIP, ciprofloxacin; GM, gentamicin; KAN, kanamycin; IMP, imipenem.

5 mmol/L $ZnSO_4$) at room temperature for 20 min before being incubated with 1 U of S1 nuclease (Sigma, St. Louis, Missouri, USA) in 200 μL of 1× nuclease S1 buffer at 37 °C for 45 min. The reaction was stopped by adding 10 mL of 0.5 mol/L EDTA (pH 8.0). Electrophoresis was performed on CHEF-DR III apparatus (Bio-Rad Laboratories, Bath, UK). The initial switch time was 5 s, the final switch time was 45 s, and the run time was 20 h at 6 V/cm and 120° angle at 14 °C. After gel electrophoresis, the gels were stained with ethidium bromide and were photographed under UV light at 302 nm.

Localization of $bla_{CTX\text{-}M\text{-}15}$ by hybridization

The S1-digested gel was dried overnight on a Whatman filter paper (15 cm × 15 cm) blotting paper, the gel was then rehydrated, denatured using a denaturing buffer (0.5 mol/L NaOH, 1.5 mol/L NaCl) for 30 min at room temperature, neutralized using a neutralizing solution (0.5 mol/L Tris–HCl, pH 7.5; 1.5 mol/L NaCl) for 30 min at room temperature. The gel was then transferred to hybridization tube contains prehybridization solution (6× SSC (sodium citrate), 0.1% (*m*/*v*) polyvinylpyrrolidine, 1 mL of 0.5% (*m*/*v*) SDS, 400 μL of 0.1% (*m*/*v*) ficoll, 400 μL of milk, and 300 μL of 150 μg/mL denatured spermatozoid DNA), incubated overnight at 65 °C.

Labelling of $bla_{CTX\text{-}M\text{-}15}$ gene probe

A 15 μL volume of a purified $bla_{CTX\text{-}M\text{-}15}$ template PCR product was mixed with 8 μL of DNA-free water and 10 μL of random 9-mer primers (Agilent Technologies, Stratagene, USA Products) and was added into a screw-cap Eppendorf tube. First, the mixture was boiled in a water bath for 5 min. Then, a 10 μL volume of 5× dCTP buffer (Agilent), 2.5 μL of the radioactive phosphorus ^{32}P (PerkinElmer, Boston, Massachusetts, USA), and 1 μL of Exo(–) Klenow (Agilent) were added to the mixture, transferred to a lead jar, and incubated at 37 °C for 15 min to allow the production of radiolabelled $bla_{CTX\text{-}M\text{-}15}$ template DNA.

The radiolabelled product was pipetted into a silica gel column (Nick columns Sephadex, G-50 DNA Grade, illustra, GE Healthcare, Life Science, UK). The column was then washed twice, once with 320 μL of washing buffer (0.1 mol/L Tris–HCl buffer, pH 7.5 and once with 430 μL of the same washing buffer to elute the radiolabelled $bla_{CTX\text{-}M\text{-}15}$ gene to purification. The radiolabelled PCR product was then boiled in a water bath for 6 min to denature the double-stranded template DNA; the probe was then added to the bottom of the incubated gel in the hybridization tube and incubated overnight at 65 °C.

Hybridization of S1-digested PFGE gel

The hybridized gel was probed with a radiolabelled $bla_{CTX\text{-}M\text{-}15}$ gene template DNA and incubated overnight. After probing, the gel was washed twice, once with 2× SSC (sodium citrate 0.1% (*m*/*v*)) plus SDS and once with 0.1× SSC plus 0.1% (*m*/*v*) SDS. The gel was subsequently warped in a cling film, and a Hyperfilm (Amersham, GE Healthcare, Life Sciences) was firmly pressed on the gel in a cassette and frozen at –80 °C for 18 h. Developer and fixer solutions were used to detect the appearance of any radiolabelled spot on the Hyperfilm.

Results

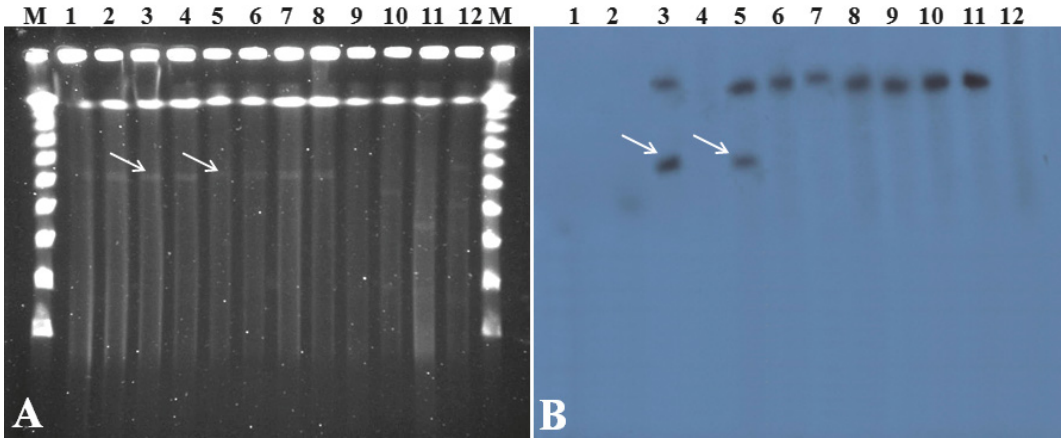
MICs of β-lactam antibiotics

The antibiotic susceptibility profiles of the 12 *K. pneumoniae* isolates showed resistance to third-generation cephalosporins, with MICs reduced by clavulanic acid. A high degree of resistance to ceftazidime, cefotaxime, aztreonam, and cefepime was also observed with values of 16 to >64 μg/mL. Among the 12 isolates; 8 had MICs of cefotaxime higher than that of ceftazidime, suggesting the expression of a CTX-M-type enzyme. The isolates were sensitive to ciprofloxacin, gentamicin, kanamycin, imipenem, and ertapenem, with values of 0.125–0.25 μg/mL (Table 1).

Amplification of $bla_{CTX\text{-}M\text{-}15}$ gene and its surrounding regions

PCR experiments showed the occurrence of $bla_{CTX\text{-}M\text{-}15}$ in addition to the insertion sequence IS*Ecp1* located upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene in 8 out of 12 *K. pneumoniae* isolates. IS*Ecp1* was detected by sequencing 48 nucleotides up-

Fig. 1. Localization of *bla*$_{CTX-M-15}$ with pulsed-field gel electrophoresis after S1 digestion. (A) Total DNA gel separation after S1 digestion. (B) Autorad of the gel A by *bla*$_{CTX-M-15}$ probe. The arrows show the plasmids harboring *bla*$_{CTX-M-15}$.



stream of *bla*$_{CTX-M-15}$. A 48 bp sequence was previously described for *bla*$_{CTX-M-15}$ from India and has formerly been named as the W sequence (Poirel et al. 2003; Karim et al. 2001; Eckert et al. 2006). The insertion sequence IS*Ecp1* has been repeatedly identified upstream of many *bla*$_{CTX-M}$ genes worldwide and plays an important role in the mobilization and expression of these genes.

S1 digestion and plasmid separation

S1 is an endonuclease able to cut plasmids in only 1 site, converting them to a linear form. After PFGE and S1 digestion of the 12 *K. pneumoniae* isolates whole DNA, profiles obtained show 3 types of plasmids with sizes of 150 kb in 8 isolates, 100 kb in 2 isolates, and 50 kb in 1 isolate (Fig. 1).

Detection of chromosomally mediated and plasmid-mediated *bla*$_{CTX-M-15}$ genes

The autorad of the S1-digested PFGE gel confirmed that only 8 out of the 12 isolates were *bla*$_{CTX-M-15}$ positive. Among the 8 isolates, 2 harbored the *bla*$_{CTX-M-15}$ gene on both the chromosome and plasmid with a size of 150 kb. In contrast, 6 isolates harbored the *bla*$_{CTX-M-15}$ gene on only the chromosome (Fig. 1).

Discussion

Despite the high prevalence of CTX-M-15 β-lactamase in Tunisia, there have been few investigations on *Klebsiella* spp. with CTX-M enzymes (Mamlouk et al. 2006; Abbassi et al. 2008). This work confirms the emergence of hospital- and community-acquired CTX-M-15 enzymes in *K. pneumoniae* and their spread in a Tunisian hospital. Using PCR, several isolates were detected harboring chromosomally located and plasmid-located *bla*$_{CTX-M-15}$ gene and were associated with IS*Ecp1*. We identified 8 *K. pneumoniae* isolates conferring high-level resistance to third-generation cephalosporin with MICs of cefotaxime higher than that of ceftazidime, suggesting the expression of a CTX-M-type.

This report has also provided support for the seemingly frequent transposition events of the *bla*$_{CTX-M-15}$ gene linked to IS*Ecp1*. This element seems to have a preferred insertion site at the *tnpA* gene of a *bla*$_{CTX-M-15}$ carrying a Tn*3*-like transposon, the latter also being inserted by a transposition event (Eckert et al. 2006). Both transposition events prefer AT-rich target sequences, whereas the IS*Ecp1*–*bla*$_{CTX-M-15}$ elements prefer the same target sequence for all Tn*3* elements. The potential for transposition of *bla*$_{CTX-M-15}$ gene has public health implications, since extended-spectrum cephalosporins are extensively used in human and veterinary medicine. The mobile genetic element IS*Ecp1* is shown to play an important role in the plasticity of navigation of *bla* genes from chromosome to plasmid, plasmid to chromosome, and plasmid to plasmid (Boyd et al. 2004). IS*Ecp1* seems to facilitate the mobilization of not only chromosomal fragments but also fragments from other plasmids. Collectively, these data suggest that IS*Ecp1* plays a critical role in the evolution of diverse multiresistant plasmids found in clinical *Enterobacteriaceae* (Miriagou et al. 2005).

According to earlier experiments conducted in the same hospital and in the same period, clinical isolates of *E. coli* were found to harbor *bla*$_{CTX-M-15}$ gene carried by the IncA/C plasmid (Chouchani et al. 2012). In this study, analysis of the surrounding sequence of the *bla*$_{CTX-M-15}$ gene revealed a partially truncated fragment of IS*Ecp1 tnpA* transposase indicating the variety of genetic events that have enabled associations between IS*Ecp1* sequences and *bla*$_{CTX-M-15}$ genes in these clinical isolates. It has been reported that IS*Ecp1* is associated with a variety of *bla*$_{CTX-M}$ genes, and the genetic environment of these resistance genes may vary among different *bla*$_{CTX-M}$ genes (Lartigue et al. 2004). It has been shown that IS*Ecp1*B may recognize a variety of similar DNA sequences to the right inverted repeat during a mobilization process, and that the insertion site of IS*Ecp1*B-mediated transposition could be different (Poirel et al. 2005). We suggest that *bla*$_{CTX-M-15}$ genes and IS*Ecp1* sequences must have been in close contact somewhere to enable the arrangement of these hybrid genetic structures to provide a higher level of expression of the chromosomally located and (or) plasmid-located *bla*$_{CTX-M-15}$ genes.

Acknowledgments

This work was supported by the Islamic Development Bank offering a Post-Doc Scholarship to C. Chouchani in Department of Infection, Immunity & Biochemistry, School of Medicine, Cardiff University, United Kingdom. We would like to thank the Tunisian Ministry of Higher Education and

Scientific Research for their helpful assistance. Many thanks are extended to the Director of Kasserine Hospital for his helpful assistance in obtaining the clinical isolates.

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