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***Klebsiella pneumoniae* Producing CTX-M-15 Genes from Neonatal Intensive Care Unit in Saudi Arabia**

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Abstract: Reports on outbreak of extended-spectrum β -lactamases (ES β Ls) by Enterobacteriaceae and especially *Klebsiella pneumoniae*, are few in Saudi Arabia. This study was therefore devoted to describe the outbreak which occurred by ES β L-producing *K. pneumoniae*. Sixteen *K. pneumoniae* isolates were isolated from 16 neonatal patients hospitalized from September 2007 to December 2007 in the neonatal intensive care unit during the outbreak in Al-Qatif Hospital, Eastern Province, Saudi Arabia. These isolates were sent to microbiological laboratories, College of Pharmacy, King Saud University, for investigation. *Klebsiella pneumoniae* strains were found to produce antibiotic resistance and produce extended spectrum beta-lactamase. Genotypic characterization of extended spectrum beta-lactamase producing *K. pneumoniae* showed that all isolates carried TEM-1, SHV-1 and CTX-M-15 genes. Matting out assay revealed that all third generation cephalosporins were located on transferable plasmid. An outbreak which occurred in neonatal intensive care unit was due to CTX-M-15-producing *K. pneumoniae* isolates either single or in multiple clones. This is the first report of *bla*_{CTX-M-15} gene in Saudi Arabia from *K. pneumoniae* and the first outbreak in Saudi hospitals due to CTX-M-15 producing *K. pneumoniae*.

Key words: Antibiotic resistance, ES β L, CTX-M-15 genes, *Klebsiella pneumoniae*, Saudi Arabia

INTRODUCTION

Plasmid mediated extended-spectrum β -lactamases (ES β Ls) were first detected in a *Klebsiella pneumoniae* isolate in Germany in 1983 (Kliebe *et al.*, 1985). The most common of the ES β Ls are those derived from broad-spectrum β -lactamases TEM-1/-2 and SHV-1 by acquisition of specific point mutations (Nukaga *et al.*, 2003). CTX-M-type is non-TEM and non-SHV ES β Ls, which have less than 40% homology with TEM and SHV (Canton and Coque, 2006). CTX-M β -lactamases are very rapidly disseminated and are now widely dispersed geographic areas, including many parts of Europe, Asia, Africa and America (Al-Agamy *et al.*, 2006; Canton and Coque, 2006). Epidemiological reports demonstrate that some enzymes are more frequently than others and also the predominant enzyme type varies with country and the diverse CTX-M types often exist within a single country

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(Livermore *et al.*, 2007; Coque *et al.*, 2002). Outbreaks of *K. pneumoniae* infections in neonates have been widely reported and are frequently associated with widespread colonization of babies, systemic infections and death (Hobson *et al.*, 1996). Hospital outbreaks of *K. pneumoniae*, especially in neonatal units, are often caused by ESBL-producing strains and have been increased over the past years (Carrèr *et al.*, 2009; Randrianirina *et al.*, 2009). No data on genotype of ESBL is available at regional level. Therefore, this study was undertaken to characterize ESBL genes in *K. pneumoniae* isolated from an outbreak in Neonatal Intensive Care Unit (NICU), Al-Qatif Hospital, Eastern Province, Saudi Arabia.

MATERIALS AND METHODS

Bacteria Strains

Sixteen isolates of *K. pneumoniae* were collected during an outbreak recorded between the 22 September 2007 to the 4 December 2007 from NICU during an outbreak the occurred in Al-Qatif Hospital, Eastern Province, Saudi Arabia. These isolates were sent to the college of Pharmacy, King Saud University, Riyadh. *K. pneumoniae* isolates were isolated from clinical specimens; 4 specimens were taken from cerebrospinal fluid, 9 specimens from blood, 2 specimens from umbilical swab. Laboratory strain, *E. coli* ATCC 25922, was included in all susceptibility testing. Two *K. pneumoniae* strains were used in phenotypic detection of ESBL; one of them harbours ESBL (positive control) and the other strain did not produce ESBL (negative control).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was determined by using E-test strips (AB Biodisk, Solana, Sweden) on Mueller-Hinton agar (Oxoid, Basingstoke, England) according to Clinical and Laboratory Standards Institute (Clinical Laboratory Standards Institute, 2006) recommendations.

Phenotypic Detection of ESBL

ESBL was detected phenotypically using ceftazidime/ceftazidime-clavulanic acid and cefotaxime/cefotaxime-clavulanic E-test strips. ESBL-producing *K. pneumoniae* isolate showed a Minimum Inhibitory Concentrations (MICs) by the antibiotics cefotaxime and ceftazidime in the presence of clavulanic acid of more than 8 fold.

Preparation of DNA Template

DNA templates for Polymerase Chain Reaction (PCR) process, were generated by suspending 5 colonies of overnight culture of *K. pneumoniae* isolate grown on tryptone soy agar (Winlab, UK) in 200 μ L of HPLC grade water (BDH, England). The suspension was boiled at 100°C for 10 min in thermal block (Techne, UK), then centrifuged at 15000 rpm for 5 min. An aliquot of 1 μ L of the supernatant was used as DNA template in the PCR.

PCR and DNA Sequencing Analysis

All 16 multiresistant *K. pneumoniae* isolates included in the study were screened for alleles encoding the most frequently reported ESBLs in Europe, Asia and USA, that is the *bla*_{CTX-M} phylogenetic lineage groups 1, 2, 8 and 9, the *bla*_{SHVs} and the *bla*_{TEMs}. The PCRs for genes encoding TEM, SHV and CTX-M β -lactamases were performed using primers and conditions described previously (Al-Agamy *et al.*, 2006; Bonnet *et al.*, 2001; Nüesch-Inderbinen *et al.*, 1997). Four PCR experiments that differentiated between CTX-M-1,

Table 1: The primers used in amplification of β -lactamases genes

Gene	Primers	Nucleotide sequence	Position
EM	T1	5'-ATT CTT GAA GAC GAA AGG GCC TC-3'	F
	T3	5'-TTG GTC TGA CAG TTA CCA ATG C-3'	B
SHV	NI1	5'-GCC CGG GTT ATT CTT ATT TGT CGC-3'	F
	NI2	5'-TCT TTC CGA TGC CGC CAG TCA-3'	B
CTX-M	CTX-MA	5'-CGCTTTGCGATGTGCAG-3'	F
	CTX-MB	5'-ACCGCGATATCGTTGGT-3'	B
CTX-M-1	ALA2	5'-ATGGTTAAAAAATCACTGCG-3'	F
	P2D	5'-CAGCGCTTTTGCCGTGTAAG-3'	B
CTX-M-2	CTX-M2GF	5'-TTA ATG ACT CAG AGC ATT C-3'	F
	CTX-M2GR	5'-GAT ACC TCG CTC CAT TTA TTG-3'	B
CTX-M-8	CTX-M8GF	5'-TGA ATA CTT CAG CCA CAC G-3'	F
	CTX-M8GR	5'-TAG AAT TAA TAA CCG TCG GT-3'	B
CTX-M-9	C-1	5'-AACACGGATTGACCGTCTTG-3'	F
	C-2	5'-TTACAGCCCTTCGGCGAT-3'	B

F: Forward, B: Backward

-2, -8 and -9 groups of enzymes were performed for CTX-M-positive isolates using the primers and the methods described by Baraniak *et al.* (2002), Chanawong *et al.* (2002) and Villegas *et al.* (2004). The primers used in this work are shown in Table 1. The PCR products were purified using PCR purification kit (Qiagen, Hilden, Germany) and those products were sequenced on both strands using 3130XL genetic analyser, (Applied Biosystem) according to Sanger *et al.* (1977). PCRs used to detect *bla* genes were done in a thermocycler (Fexigene, Techne, UK). However, PCR machine used in sequencing was PCR system 9700, (Applied Biosystem).

Conjugation Experiments

Transferability was tested by Broth Matting Method using rifampicin-resistant mutant *E. coli* JM105 (Pharmacia Biotech, USA) as a recipient at 1:2 donor/recipient ratios (Vatopoulos *et al.*, 1990). Conjugation mixtures were plated on MacConkey agar containing 100 $\mu\text{g mL}^{-1}$ rifampicin and 10 $\mu\text{g mL}^{-1}$ cefotaxime and incubated for 24-48 h at 37°C to select transconjugants. The MIC and PCRs were done to the transconjugants.

RESULTS

Antimicrobial Susceptibility

The antimicrobial resistance pattern of *K. pneumoniae* is shown in Table 2. The results indicate that all isolates of *K. pneumoniae* were multidrug resistant. All isolates were resistant to amoxicillin, aztreonam, cefotaxime, ceftazidime, ceftriaxon at MIC > 256 $\mu\text{g mL}^{-1}$, ciprofloxacin (MIC > 32 $\mu\text{g mL}^{-1}$), amikacin (MIC 128->256 $\mu\text{g mL}^{-1}$) and gentamicin (MIC 196->256 $\mu\text{g mL}^{-1}$). They were susceptible to cefoxitin (MIC 1-2 $\mu\text{g mL}^{-1}$) and imipenem (MIC 0.0625-0.125 $\mu\text{g mL}^{-1}$). The susceptibility to cefepime varied with MICs ranged from 16 to >256 $\mu\text{g mL}^{-1}$, while 50% of the isolates had MIC of >256 $\mu\text{g mL}^{-1}$. The resistance to third generation cephalosporins was markedly inhibited by clavulanic acid.

Characterization of ES β L

The results of phenotypic detection of ES β L revealed that all isolates were found to be having ES β L. TEM, SHV and CTX-M β -lactamase genes in ESBL producing *K. pneumoniae* isolates by PCR. CTX-M β -lactamase gene was further investigated by PCR using the specific primers which amplify CTX-M-1 group, CTX-M-2 group, CTX-M-8 group and CTX-M-9 group. The PCR results showed that all isolates harbored CTX-M-1 like

Table 2: Susceptibility testing results of ESBL-producing *Klebsiella pneumoniae* isolates

Isolates	ESBL-producing <i>K. pneumoniae</i> (n = 16), MIC $\mu\text{g mL}^{-1}$			Resistance
	Range	MIC ₉₀ ($\mu\text{g mL}^{-1}$)	MIC ₅₀	
Amoxicillin	0.016-256	≥ 256	≥ 256	16(100)
Amoxicillin/clavulanate	0.016-256	16/4	4/4	2(12.5)
Piperacillin/tazobactam	0.016-256	16/4	8/4	0(0)
Cefotriaxon	0.016-256	≥ 256	≥ 256	16(100)
Ceftazidime	0.016-256	≥ 256	≥ 256	16(100)
Ceftazidime/clavulanate	0.016-256	6/4	4/4	0(0)
Cefotaxime	0.016-256	≥ 256	≥ 256	16(100)
Cefotaxime/clavulanate	0.002-32	6/4	4/4	0(0)
Aztreonam	0.016-256	≥ 256	≥ 256	16(100)
Cefepime	0.016-256	≥ 256	16	8(50)
Cefoxitin	0.016-256	2	1	0(0)
Imipenem	0.002-32	0.125	0.0625	0(0)
Ciprofloxacin	0.002-32	≥ 32	≥ 32	16(100)
Amikacin	0.016-256	≥ 256	128	16(100)
Gentamicin	0.016-256	≥ 256	196	16(100)

Value in bracket are percentage

genes and no amplicons were obtained for other three tested CTX-M groups. The purified PCR products of TEM, SHV and CTX-M-1 like genes were sequenced on both strands. The DNA sequencing of 16 isolates showed that they carried *bla*_{TEM-1b}, *bla*_{SHV-1} and *bla*_{CTX-M-15} genes.

Transferability of the Antibiotic Resistant Determinants

Matting out assay showed that transconjugants were obtained from the donor strains when selected on MacConkey agar containing 100 $\mu\text{g mL}^{-1}$ rifampicin and 10 $\mu\text{g mL}^{-1}$ cefotaxime. The MIC of the transconjugants revealed that they were resistant to cefotaxime and ceftazidime. However, the transconjugants were sensitive to ciprofloxacin (MIC 0.06 $\mu\text{g mL}^{-1}$). The PCRs showed that all transconjugants carry TEM-1, SHV-1 and CTX-M-15 genes.

DISCUSSION

The dissemination of ESBL-producing Enterobacteriaceae in the hospital setting is a problem with major therapeutic and epidemiological consequences, particularly when the affected wards involve those caring for critically ill patients such as the ICU. Multiple outbreaks of ESBL-producing Enterobacteriaceae have been reported over the past 2 decades and *K. pneumoniae* has been shown to be the most frequently involved organisms (Rebuck *et al.*, 2000; Quale *et al.*, 2002). In the present study, we have characterized the strain causing the first hospital-associated outbreak of an ESBL-producing member of the Enterobacteriaceae family in Eastern province. Sixteen multiple resistant *K. pneumoniae* strains were isolated in 2007 when an outbreak occurred in NICU, Al-Qatif Hospital. *Klebsiella pneumoniae* isolates were found to be producing ESBL and multiple resistant to most of the tested antibiotics. However, they were susceptible to imipenem and cefoxitin. Imipenem is frequently the only therapeutic options available for treatment of hospital-acquired severe infections caused by multiresistant ESBL-producing *K. pneumoniae* such as the strain described in the present study. Nevertheless, universal susceptibility to these last-line antimicrobials in *K. pneumoniae* is no longer guaranteed. Indeed, several carbapenemases have been described as occurring in *K. pneumoniae*, including representatives from classes A, such as the highly disseminated KPC enzymes; B, such as

IMP, VIM, GIM, SIM or SPM metallo- β -lactamases; or class D, such as OXA-48 (Yigit *et al.*, 2001; Nordmann and Poirel, 2002; Poirel *et al.*, 2004). Fortunately our strains were susceptible to imipenem and to ceftazidime and were devoid of carbapenemases and AmpC plasmid-mediated β -lactamases (Data not shown).

Genotypic characterization of β -lactamase genes revealed that ESBL producing *K. pneumoniae* isolates carry the bla_{TEM} , bla_{SHV} and bla_{CTX-M} genes. Over the past decade, CTX-M genes have become the most common ESBLs in many countries all over Europe, Asia, Africa and America and most recently in Kuwait (Al-Agamy *et al.*, 2006; Livermore *et al.*, 2007; Ensor *et al.*, 2009). All isolates carry CTX-M-1 like gene group whereas, no PCR amplified products were detected with other three CTX-M groups. The results of the PCR showed that these isolates harbored $bla_{CTX-M-1-like}$ gene, which includes $bla_{CTX-M-1}$, $bla_{CTX-M-3}$, $bla_{CTX-M-10}$, $bla_{CTX-M-12}$, $bla_{CTX-M-15}$, $bla_{CTX-M-22}$, $bla_{CTX-M-23}$ and $bla_{CTX-M-28}$ (Livermore and Woodford, 2007). DNA sequencing of TEM, SHV and CTX-M-1 like genes revealed that presence of bla_{TEM-1} , bla_{SHV-1} and $bla_{CTX-M-15}$. CTX-M-15, which was first detected in India in 2001 (Karim *et al.*, 2001) and it is now recognized as the most widely distributed CTX-M enzyme (Livermore *et al.*, 2007). The rapid and massive spread of CTX-M-type ESBLs is rapidly changing the ESBL epidemiology and, in some geographical areas, these enzymes are now the most prevalent ESBLs in Enterobacteriaceae. Outbreaks of CTX-M-15-producing Enterobacteriaceae have also been reported in France, Italy, Spain, Portugal, Austria, Norway, the United Kingdom, Tunisia, South Korea, Canada, Egypt and Kuwait (Conceição *et al.*, 2005; Abbassi *et al.*, 2006; Al-Agamy *et al.*, 2006; Soge *et al.*, 2006; Livermore *et al.*, 2007; Ensor *et al.*, 2009). In a recent study was conducted in Saudi Arabia, *K. pneumoniae* LO10 producing ESBL SHV-12 isolated during outbreak occurred in the neonatal unit at Security Forces hospital (Al-Obeid *et al.*, 2008). However, in our study, *K. pneumoniae* producing ESBL CTX-M-15 isolated during outbreak occurred in NICU, Al-Qatif Hospital. The study performed by Al-Obeid *et al.* (2008) and the present study are the only two studies conducted that have describes the outbreaks in ICU due to ESBL-producing *K. pneumoniae* in Saudi Arabia. The variation in two studies is based on the type of ESBL while in the previous study it was the bla_{SHV-12} genes. However, in the current study the variation is in $bla_{CTX-M-15}$ genes. This variation is difficult to explain, but may be due to differences in the geographical area, differences in the time of collection of isolates and differences in the type and volume of consumption of antibiotics. Outbreaks of *Klebsiella* infections in neonatal units associated with widespread colonization of babies have been reported (Hobson *et al.*, 1996). Outbreaks of *K. pneumoniae* usually occurred by monoclonal however in fewer studies outbreak occurs by multiclonal (De-Oliveira *et al.*, 2008; Carrer *et al.*, 2009). In the current study, we can not confirm this outbreak due to single clone or multiple clones due to the lack of a genomic epidemiological marker. Techniques such as pulsed field gel electrophoresis and random amplified polymorph DNA has not been done to investigate this outbreak. However, there are great evidences to indicate that an outbreak of CTX-M-15 producing *K. pneumoniae* may be due to single clone. Some evidences were taken such as all isolates had the same resistance pattern, all isolates were able to transfer ceftazidime resistant determinants, antibiotic resistance of the transconjugants is similar and the isolates and their transconjugants had TEM-1, SHV-1 and CTX-M-15.

General considerations were taken by infection control practice in the NICU to stop the outbreak. Some of the important consideration; the isolation of all colonized and diseased patients as a cohort nursing and admission has stopped but unavoidable cases were sent to another unit; dedication of nurses to colonized and diseased patients; environmental and

clinical samples was obtained. In addition, environmental specimens including bed, milk, milk bottles, equipment, thermometer, blood pressure cuff, intravenous saline, ventilators, respiratory equipment soap and water were taken. All environmental specimens were negative except blood pressure cuff was positive for *K. pneumoniae* which is sensitive to antibiotics. On the other hand, clinical specimens including umbilical, rectal, intravenous swab, finger print of nurses and those who were positive umbilical and rectal swab rescreened after decolonization with chlorohexadine and alcohol. Frequent meeting with pediatric head, NICU supervisor and house keeping department was on daily babies; decontamination of environment was routinely done; infection control course for all hospital nurses for a period of 2 months was mandatory.

In conclusion, this is the second study that describes the outbreak of ES β L-producing *K. pneumoniae* in Saudi Arabia and the first report of outbreak in Saudi Arabia due to *bla*_{CTX-M-15} genes. It is evident that the *bla*_{CTX-M} genes are now increasingly detected in patients throughout Saudi Arabia and to define the extent of this spread, more studies on the cefotaxime-resistant *Klebsiella* sp. and other Enterobacteriaceae needs to be carried out in the different geographical area around the kingdom. Furthermore, outbreaks in Saudi hospitals must be investigated to define the predominate ES β L gene in the Enterobacteriaceae strains that are responsible for the outbreaks.

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