Original Article

Emergence of CTX-M Group 1-ESBL producing *Klebsiella pneumonia* from a tertiary care centre in Karachi, Pakistan

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Abstract

Background: Extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* have been reported previously from Pakistan but the genotypic characteristics of these enzymes is not known. Hence the aim of the study was first to characterise the genotypic content of these beta-lactamases and secondly to assess the clonal relationship of these isolates.

Methodology: We analysed 65 non-duplicate ESBL positive, *K. pneumoniae* isolates prospectively collected based on phenotype as detected using the two-disc method. Isolates were collected from different sources: blood cultures (46.15%; n = 30); tracheal aspirates (24.6%; n = 16); urine (10.7%; n = 7); wound swabs, pus and tissue (18.4%; n = 12). ESBL production was confirmed by the ESBL E-test method and the presence of the $bla_{\text{CTX-M}}$ encoding genes was confirmed by polymerase chain reaction. The clonal relationship of clinical isolates was studied by Pulsed Field Gel Electrophoresis.

Results: The results showed that 93.84% (n = 61) isolates of *K. pneumoniae* were positive for the $bla_{\text{CTX-M-1}}$ group. One isolate showed PCR signals for $bla_{\text{CTX-M-25}}$ group**.** None of our isolates were positive for CTX-M groups 2, 8 and 9. The majority of $bla_{\text{CTX-M}}$ positive isolates were genetically unrelated and no epidemic clones were identified. Conclusion: This study reports the emergence of CTX-M groups 1 and 25 producing isolates of *K. pneumoniae* with genetic diversity in Karachi, Pakistan.

Key Words: Gram negative, extended spectrum beta-lactamases, multidrug resistance, hospital acquired infections, non-clonal isolates

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Introduction

Extended-spectrum β-lactamase (ESBL) producing strains of *Klebsiella pneumoniae* were first detected in Germany in 1983 [1]. Today multi drug resistant *K. pneumoniae* is recognised as a global challenge, with the potential to develop resistance to all available antibiotic armamentarium [2].

Most ESBLs are mutants of classical TEM and SHV genes. These mutations allow them to hydrolyze extended spectrum antibiotics. More than 150 TEM enzymes and more than 50 different mutants of SHV have been reported [3]. A recently discovered family of plasmid mediated ESBLs called CTX-Ms, which preferentially hydrolyze cefotaxime, has emerged in several continents around the world [3,4]. More than 40 different variants of CTX-M enzymes are currently known.

ESBL producing isolates of *K. pneumoniae* have been reported from Pakistan previously by Shah *et al*. [5]. In this study an analysis of 400 isolates of Enterobacteriaceae for ESBL production identified that 70% of *K. pneumoniae* isolates were found positive for ESBL production [5]. However, another study by *Mirza et al*. identified only 4 out of 50 clinical isolates of *E. coli* as positive for CTX-M phenotype using the double disc method and MIC break points [6]. Neither study, however, performed genetic characterization of CTX-M enzyme types produced by these isolates.

At the clinical microbiology laboratory of the Aga Khan University Hospital (AKUH), *K. pneumoniae* is the most common Enterobacteriaceae and is associated with multiple drug resistance, including the third-generation cephalosporins. In *K. pneumoniae*, resistance to cephalosporins mediated by ESBL has dramatically increased over the years from 22.9% in 2002 to 37% in 2007 (AKU unpublished data). Hence in our current study we evaluated the genetic characterization of CTX-M enzymes in clinical isolates of *K. pneumoniae* and sought to determine their clonal relationship as an endemic strain to ascertain the possible emergence of a clonal strain.

Table 1. Primers used for amplification

Primer [9]	Sequence	Amplicon size
CTXM GP 1 F CTXM GP 1 R	5′-AAAAATCACTGCGCCAGTTC-3′ 5′-AGCTTATTCATCGCCACGTT-3′	415bp
CTXM GP 2 F CTXM GP 2 R	5′-CGACGCTACCCCTGCTATT-3′ 5′-CCAGCGTCAGATTTTTCAGG-3′	552bp
CXTM GP 9 F CTXM GP 9 R	5′-CAAAGAGAGTGCAACGGATG-3 5′-ATTGGAAAGCGTTCATCACC-3′	205bp
CTXM GP 8 F	5′-TCGCGTTAAGCGGATGATGC-3	666bp
CTXM GP 25 F CTXM GP 8/25* R	5′-GCACGATGACATTCGGG-3′ 5′-AACCCACGATGTGGGTAGC-3′	327bp

F= Forward Primer, R=Reversed Primer, *: GP 8/25 shares reversed primer. [9]

Materials and methods

AKUH is a 550-bedded university hospital that includes a 12-bed adult ICU and an 8-bed neonatal ICU. All ESBL producing isolates of *K. pneumoniae* that were identified on a two disc screening method were confirmed biochemically using API-20E analytical system [7]. These isolates were prospectively collected over period of one year (2007-08) and stored at -80^{o}C.

Bacterial isolate and susceptibility testing

Antimicrobial susceptibility testing was performed by the Kirby Bauer method per the Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. Antimicrobial discs tested were ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), piperacillin/tazobactam (100/10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg) aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), ofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), and imipenem (10 µg). Resistance to tigecycline was ascertained using the disc testing as outlined by the British Society for Antimicrobial Chemotherapy (BSAC) guidelines. *Escherichia coli* ATTC 25922 was used as the quality control strain.

ESBL screening

Isolates were screened for ESBL production using the double disc synergy test using aztreonam (30 µg) and amoxicillin clavulanate (10 µg). ESBL production was deduced when the zone of aztreonam was expanded by clavulanate. In order to confirm this ESBL production, the ESBL E-test method (AB-Biodisk, Solna, Sweden) was conducted. In this test, ESBL detection was concluded if the MIC ratio for cefotaxime alone (cefotaxime + clavulanate) was greater than 8, following the manufacturer's instructions.

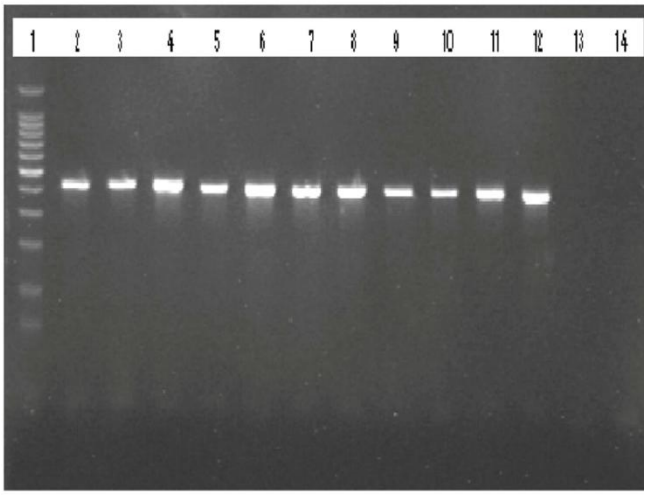
Detection of bla$_{CTX-M}$ *gene by PCR*

Isolates with an ESBL phenotype were further investigated for *bla*$_{CTX-M}$ alleles using primers specific for each of the five groups of CTX-M primers as described by Woodford *et al*. [9]. *E. coli* NCTC 13353 was used as a positive control. Table 1 shows details of the primer sequences used for each group. Amplification conditions for CTX-M groups (1, 2, 8 and 9) were as follows: early denatured at 94°C for 10 minutes followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute, with a final elongation step of 10 minutes at 72°C. For CTX-M group 25 the PCR conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 40 cycles of 94°C for 25 seconds, 60°C for 40 seconds, and 72°C for 50 seconds, with ultimate extension step of 10 minutes at 72°C. Results of PCR analysis were reconfirmed at the Centre of Infectious Disease, University of Edinburgh, United Kingdom.

Pulsed field gel electrophoresis (PFGE):

The clonal relationship of the clinical isolates was studied by PFGE, as previously reported [10]. Briefly, 1 ml of overnight culture broth was taken and resuspended in Pett IV solution (1 M NaCl, 10mM Tris [pH 7.5]). The suspensions were mixed with an equivalent volume of 1.3%. Low melting point agarose (Sigma, Saint Louis, Missouri, USA) was solidified in moulds (BioRad, California, USA) to make plugs. These plugs were transferred to 2 ml of lysis buffer (0.1M EDTA, 1M NaCl, 0.5% brij 58, 0.2% deoxycholate and 05% of sarcosine) containing 1 mg/ml of lysozyme and 10 mg/ml of RNase A (Invitrogen, NY, USA) and incubated at 37˚C for 18-20 hrs. The next day, lysis buffer was removed and plugs were incubated at 55˚C for 24 hours in 2 ml of ESP buffer (0.5M EDTA, 1% sarcosine) along with 100mg/ml of Proteinase K (Promega, Madison, USA). The plugs were then washed four

Figure 1. PCR gel picture of *bla*$_{CTXM-1}$ alleles of *K. pneumoniae* isolates



Lanes: 1. 100bp ladder, 2. Positive control *bla*$_{CTXM-1}$, 3 to 12. Clinical isolates, 13. Negative control (Ecoli j53), 14. Blank

times with 20 ml of TE buffer and digested with 20 U of Xba 1 for 24 hours at 37˚C. Fragments of DNA were separated in 1% agarose gel (Pulse Field Certified Agarose-Bio Rad) using CHEF DRIII apparatus (Bio Rad) under the following conditions: 6V/cm^2 for 22 hours at 14˚C, with initial pulse time of 2 seconds and final pulse time of 35 seconds. Photographs of ethidium bromide stained gels were examined visually. DNA band patterns were read according to criteria defined by Tenover *et al*. as follows: indistinguishable; no variation in DNA band fragment; closely related: variation in 2-3 bands and unrelated; variation > 3 bands [11].

Results

A total of 65 non-duplicate ESBL positive, *K. pneumoniae* were prospectively collected based on their phenotype as detected using the two disc method. The majority of the isolates were from blood cultures (46.15%; n = 30) followed by tracheal aspirates (24.6%; n = 16), urine (10.7%; n = 7) while cultures from catheter tips, wound swabs, tissue and pus together constituted 18.4% (n = 12). The majority of isolates were yielded from patients admitted to the neonatal intensive care unit 18.46% (n = 12) followed by the medical wards 15.38% (n = 10), adult ICU 13.84% (n = 9) and the pediatric unit 10.76% (n = 7). The exact hospital location for 12 patients could not be ascertained as patients were shifted to different wards during the duration of their hospital stay.

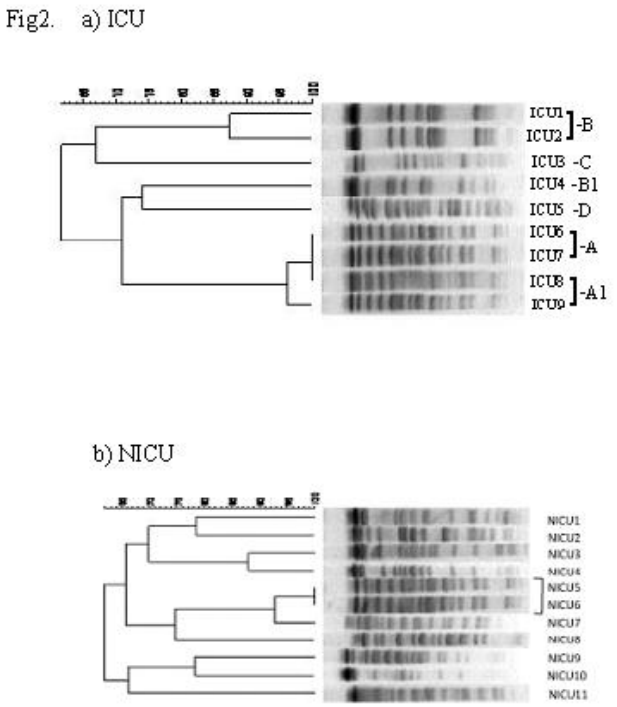
Detection of bla$_{CTX-M}$ *gene*

Sixty-one (93.84%) isolates of *K. pneumoniae* were positive for *bla*$_{\mathbf{CTX\text{-}M\text{-}1}}$ group alleles. Only one isolate showed PCR signals for *bla*$_{\mathbf{CTX\text{-}M\text{-}25}}$ group alleles**.** None of our isolates were positive for CTX-M groups 2, 8 and 9 (Figure 1)

Pulsed field gel electrophoresis

In general, molecular typing revealed considerable genetic diversity among the clinical isolates (Figure 2); however, some PFGE patterns were found to be associated with isolates obtained from the adult ICU. This trend was not apparent with isolates obtained from NICU. Among the ICU cohort strains, ICU$_{1-2}$ and ICU$_{6-9}$ were found to be indistinguishable; hence they were deemed to be closely related (Figure: 2A). In the case of the NICU strains, two isolates NICU$_{5-6}$ (Figure: 2B) appeared indistinguishable while the others showed more than three band variations and as such were considered unrelated.

Figure 2. A representative pulse field gel electrophoresis (PFGE) profile of Xbal-digested genomic DNA from CTXM ESBL producing isolates



A) Corresponds to isolates from ICU. Four Isolates in this cohort (lanes 6,7,8,9) showed identical patterns
B) Shows gel pictures of isolates from NICU, two strains (lanes 5 and 6) showed identical PFGE patterns

Susceptibility patterns

Table 2 shows details of the susceptibility profiles of the *bla*$_{CTX-M-1}$ producing *K. pneumoniae* to different antibiotics. Among the beta-lactam drugs 96% were found resistant to ampicillin. Resistance to beta-lactamase inhibitor combinations such as Augmentin and Pipracillin Tazobactam was also significant (see Table 2). Among the non-beta-lactam group of antibiotics, 83.6% were resistant to cotrimoxazole, while 85.24% and 36% were resistant to gentamicin and amikacin respectively. Resistance to ciprofloxacin was noted as 49%. None of the isolates were found resistant to imipenem; however, 21 % were found resistant to tigecycline, which has been recently introduced in our hospital formulary.

Table 2. Percent resistance to different groups of antibiotics of 61 K.P Isolates positive for bla$_{CTXM-1}$ allele, detected at Clinical Laboratory Aga Khan University Hospital, Karachi, Pakistan.

Antibiotic tested	% Resistance
Amikacin	36.05
Aztreonam	77.00
Gentamicin	85.24
Ciprofloxacin	49.18
Ampicillin	96.00
Augmentin	60.00
Cotrimoxazole	83.60
Piperacillin-tazobactam	39.34
Imipenem	00.00
Tigecycline	21.30

Discussion

ESBL producing isolates of *K. pneumoniae* have been reported from different hospitals in Pakistan [5]; however, the molecular characterization of the types of ESBL enzymes has not been performed previously. This is the first study from Pakistan that reports the emergence of CTX-M producing isolates of *K. pneumoniae* in a tertiary care centre.

The majority of the isolates in our study were acquired from blood samples followed by the respiratory specimens, signifying true infections. This is significant as most of these samples were from patients in the intensive care units of Aga Khan Hospital, putting them at high risk for ventilator-associated pneumonia and bacteremia due to Gram-negative rods.

Ninety-three percent of ESBL positive isolates were positive for CTX-M group 1 by PCR, whereas only one isolate was positive for CTX-M group-25. CTX-M-1 group includes six plasmid mediated variants (CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15 and FEC-1) which have been identified in Enterobacteriaceae globally, including in Asia [12-14].

The spread of ESBL isolates within hospitals is of particular concern. The *bla*$_{CTX-M}$ positive isolates were tested by PFGE to determine clonal relationships. Most of the isolates tested were found to be genetically unrelated and no definite epidemic strains were found within the hospital. Few isolates, however, did show genetic relatedness. These were collected as a part of outbreak investigations during the study period.

Dissemination of genetically diverse strains of ESBL producing Enterobacteriaceae is not uncommon, as similar findings have been reported previously. Thailand has reported the spread of CTX-M-14 type ESBL in *K. pneum*oniae isolates with high genetic diversity [14]. Plasmid conjugation studies have confirmed the potential involvement of IS*Ecp*1 in the mobility of *bla*$_{CTX-M}$ between different bacterial strains [15]. However, the prevalence of these enzymes in unrelated strains implicates the role of mobile genetic elements, such as plasmids, and insertion sequences, such as IS*Ecp*1, and suggests a potential for accelerated dissemination of these resistance determinants [15] to other bacteria, such as *Salmonella typhi*, *Vibrio cholerae* and *Shigella* species which are endemic in our country. Further studies are underway to detect the presence of IS*Ecp*1 in our isolates.

The expression of co-resistance and/or the co-transfer of resistant determinants is probable in our isolates as majority of the CTX-M-1 group positive isolates were resistant to non-beta-lactam antibiotics as reported by others [10,12-13]. Of particular interest was the resistance to antibiotics such as tigecycline, which has been introduced recently in our hospital formulary. None of the isolates were found resistant to carbapenem suggesting its importance in the treatment of these newly emerging CTX-M-1 group positive strains in our hospital.

In conclusion, this study reports the emergence of CTX-M groups 1 and 25 producing isolates of *K. pneumoniae* with genetic diversity in a major tertiary care facility in Pakistan. This finding underscores the need for the judicial use of antibiotics, especially third-generation cephalosporin, as well as good hospital infection control practices to curtail the spread of these resistant organisms.

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