



Optimization of PCR for rapid detection of CTX-M gene in ESBL producing *Klebsiella pneumoniae* clinical isolates from Punjab, Pakistan

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ABSTRACT

Aims: The study was carried out firstly, to evaluate the prevalence of extended spectrum beta lactamase (ESBL), multidrug resistant *Klebsiella pneumoniae* isolates from Punjab, Pakistan and secondly, to characterize the genotypes of their beta lactamase producing enzymes and optimization of PCR based method for rapid and authentic detection of antibiotic resistant gene.

Methodology and results: Two hundred of *K. pneumoniae* strains were isolated from different clinical samples. Blood and MacConkey Agar were used to isolate and identify bacterial microorganisms while Muller Hinton Agar was used to evaluate the antimicrobial susceptibility against different antibiotics as per CLSI 2012 guidelines. ESBL producing bacteria were screened by double disk synergy /combination disk test. PCR was optimized and performed for resistant gene (CTX-M). The results showed that most of the isolates were resistant to multiple antibiotic including cephalosporin, aztreonam, sulphamethoxazole/trimethoprim, ciprofloxacin, doxycyclin and were sensitive to imipenam and amikacin. Frequency of CTX-M gene in ESBL producing *K. pneumoniae* was 94%.

Conclusion, significance and impact of study: Based on the finding of this study it is suggested that prevalence of CTX-M gene (95%) is very high among ESBL producing isolates. Therefore, PCR based method may help clinicians for rapid detection and treatment of patients by choosing right medication against the resistant bacteria as early as possible.

Keywords: Antibiotic resistance, CTX-M, ESBL bacteria, *Klebsiella pneumoniae*

INTRODUCTION

Klebsiella pneumoniae is a common cause of infection in human being and can be isolated from the hospitals and congested communities (Rampure *et al.*, 2013). The infectious nature of *K. pneumoniae* is one of the factor in its wide spread pathogenicity (Ramirez *et al.*, 2012). Initially it was associated with urinary tract and respiratory infections however, due to emergence of virulent capsular typing now it is associated with broad spectrum of infections i.e. bacteremia, septicemia etc. including *K. pneumoniae* syndrome (Nadasy *et al.*, 2007). In addition, factors like antibiotic misuse, poor infection control practices, mobility of genetic elements, unawareness and poverty also contribute toward the emergence of the resistant strains (Roshan *et al.*, 2011).

Extended-spectrum beta-lactamase (ESBL) is plasmid mediated enzymes are able to breakdown the structure of penicillins and third generation cephalosporins and monobactams. In the past, classical and dominant ESBLs enzymes were TEM, SHV, and OXA, while emergence of CTX-M was rarely reported (Livermore *et al.*, 2007). However, now CTX-M enzymes are more commonly reported among *K. pneumoniae* and other Gram negative

bacilli emerged in several countries worldwide (Patricia, 2001; Jacoby and Silvia, 2005). More than 80 ESBL enzymes belonging to CTX-M type have been reported till now (Paterson *et al.*, 2003).

Significant numbers of CTX-M enzymes producing *K. pneumoniae* were isolated from (Karachi) Pakistan (Khan *et al.*, 2010) indicating high prevalence of ESBL positive bacteria in Pakistani population (Shah *et al.*, 2004). It is very important to detect phenotype and genotype of ESBL-mediated resistance in *K. pneumoniae* in other regions of Pakistan i.e. Punjab. This is the first report about the molecular characterization of CTX-M gene in *K. pneumoniae* from Punjab, Pakistan.

In conventional methods phenotypic identification of resistance bacteria based on different clinical tests including bacterial growth inhibition in disk diffusion or dilution tests (E test), which often give not positive results as well as prolong time to complete and may lead to wrong prescription (Leverstein-van Hall *et al.*, 2002; Farber *et al.*, 2008; Feizabadi *et al.*, 2010; Hassan *et al.*, 2014).

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These factors stress to find out some new approach and or method. Polymerase chain reaction (PCR) is an important tool and can be used for the rapid detection of antibiotic resistance in bacteria for successful antibiotic therapy. In this paper, we report the optimization of PCR for rapid detection of *CTX-M* gene in ESBL producing *K. pneumoniae*.

MATERIALS AND METHODS

Ethical approval was obtained from the institution review board (IRB) at Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan. Informed consent was obtained from all patients. All clinical samples were processed for identification of *K. pneumoniae* at Department of Microbiology, Chughtai Lab Medical Pathology Laboratory, Lahore, Pakistan, during January 2014 - June 2015, and antibiotic resistance *CTX-M* gene characterization was carried out at Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.

A total of 200 *K. pneumoniae* isolates were isolated from blood, fluid, pus, tissue, indwelling device tips, respiratory tract and urogenital tract of patients having different kinds of infections. Repeated samples from same patients were excluded for this study. Samples were marked with unique number for identification purpose and processed for microbiological analysis within two hours after receiving at lab.

Identification of isolates

Each sample was inoculated separately on Blood Agar and MacConkey Agar plate. Inoculated plates were aerobically incubated at 37 °C for 24 h. After incubation, bacterial colonies were morphologically and biochemically characterized. Colonies having character like, pink coloration, mucoid nature, non-motile at MacConkey agar (suspected to be *Klebsiella* spp.) were further analyzed by API 20E (BioMérieux, France) system for the identification up to specie level.

Antimicrobial susceptibility testing

The antimicrobial susceptibility test for *Klebsiella* isolates was performed in accordance to the guideline of Clinical and Laboratory Standards Institute (CLSI 2012). Multiple antibiotics (at least one from each group) were tested to detect the isolates resistance to antibiotics.

For antimicrobial susceptibility test an isolated bacterial colony from the Mac-Conkey agar plate was plucked with a sterilized loop and 0.5 McFarland suspension was prepared. Then spreading of inoculum was done in four dimensions by rotating the plate at 90° after swabbing half plate with the help of sterilized swab stick. Then with the help of multi-disc dispenser, antibiotic discs were applied on the Mueller-Hinton agar plate. The plates were aerobically incubated at 37 °C in incubator for 24 h. After incubation, the diameter of the zones of inhibition of these

antibiotics was calculated in millimeters with the help of metric ruler, according to the "Clinical and Laboratory Standards Institute" guidelines.

ESBL screening

Phenotypic detection of ESBL enzyme was performed in two steps. First was the screening test by using indicator 3rd generation cephalosporin disk to observe the decreased susceptibility of test bacteria against that indicator disk using disk diffusion method while 2nd was the confirmation test for presence of synergy between any of 3rd generation cephalosporin and clavulanic acid (Manchanda *et al.*, 2005; Drieux *et al.*, 2008). Those isolates, which showed resistance against 3rd generation cephalosporins were phenotypically confirmed for ESBL enzyme production by two tests.

In double disk synergy test disk containing clavulanic acid was placed in the center of Muller-Hinton plate, inoculated with test organism. A disk of cefotaxime (30 µg), ceftazidime (30 µg) and aztreonam (30 µg) was placed at a distance of 30 mm center to center from disk containing clavulanic acid. Expansion of zone of inhibition towards disk containing clavulanic acid indicated presence of ESBL enzyme (Tankhiwale *et al.*, 2004). However, in combination disk test, Muller-Hinton agar plate was inoculated with the test organism. A disk of cefotaxime (30 µg) or Ceftazidime (30 µg) with and without clavulanic acid (10 µg) were placed at the plate. If the difference in the zone diameters of either of the cephalosporin disks without clavulanic acid and cephalosporin disks with clavulanic acid came to be ≥5 mm, then the organism was considered as ESBL producer (Carter *et al.*, 2000).

PCR amplification of *CTX-M* gene

After ESBL screening genotypic characterization of resistance mechanisms was further investigated for *CTX-M* gene by using primers described by (Kiratisin *et al.*, 2008). DNA extraction was performed by lysis with lysozyme and 10% SDS followed by phenol: chloroform treatment and precipitated by chilled absolute ethanol (Sambrook *et al.*, 2012). PCR for *CTX-M* gene was optimized at following condition. Early denaturation at 94 °C for 3 min followed by 36 cycles of 94 °C for 45 sec, 52 °C for 45 sec and 72 °C for 45 sec, with a final extension step of 10 min at 72 °C.

RESULTS AND DISCUSSION

Results showed that majority [96 (48%)] of the *K. pneumoniae* isolates were from blood cultures, followed by respiratory tract infection [46 (23%)], UGT infections [23 (11.5%)], pus [18 (9%)], tip [9 (4.5%)], and body fluid [8 (4%)]. Out of 200 *Klebsiella* isolates 63% (n=126) were from male and 37% (n=74) were from female patients of all ages range between 1 to 70 years.

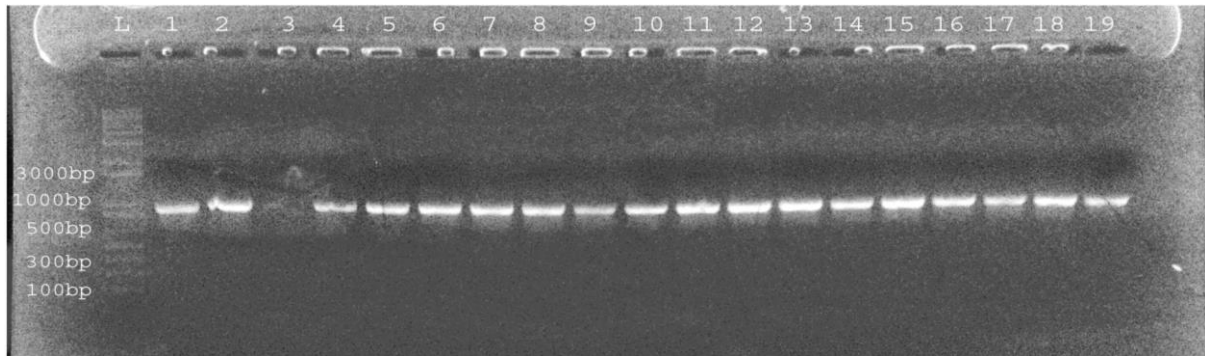


Figure 1: Agarose (1.5%) gel-electrophoresis of PCR amplified fragment (909 bp) of *CTX-M* gene. All representative samples are positive samples of *CTX-M* gene of 909 base pair product except sample 3. “L” is a 1 kb plus DNA ladder from Invitrogen.

Antimicrobial susceptibility of ESBL producing *K. pneumoniae* isolates showed that (Table 1) it is 87% resistant to amoxicillin/clavulanic acid (AMC), 94.59% to cephadrine (CE), 76.76% to cefoperazone (CFP), 76.76% to caftazidime (CAZ), 74.05% to cefipime (FEP), 74.59% to cefotaxime (CTX), 77.33% to aztreonam (ATM) and in the carbapenam group of drugs there is 22.16% resistance to imipenam (IPM) while observing aminoglycosides there is 22.70 % resistance to amikacin (AK). In addition, 67.03% isolates were resistant to trimethoprim/sulphamethoxazole (SXT). Whereas, 57% and 56% isolates were resistant to doxycycline (DO) and ciprofloxacin (CIP), respectively.

Table 1: Frequency of antimicrobial resistance *K. pneumoniae* isolates from Punjab region of Pakistan.

Antibiotic tested	% Resistance
Amoxicillin/Clavulanic Acid	87.00
Cephadrine	94.59
Caftazidime	76.76
Cefipime	74.05
Cefotaxime	74.59
Aztreonam	77.33
Imipenam	22.16
Amikacin	22.70
Doxycycline	57.84
Ciprofloxacin	56.76
Trimethoprim/sulphamethoxazole	67.03

In *K. pneumoniae* 49% (n=98) isolates were ESBL positive. PCR of positive ESBL isolates by specific primers (*CTX-M* gene) showed (Figure 1) among 98 samples of ESBL positive *K. pneumoniae*, 92 (94.5%) were positive and 5 (5.5%) were negative for this gene.

Results of our study concluded overall frequency of ESBL positive *K. pneumoniae* is 49%. It is reported that in a teaching hospital of China, *K. pneumoniae* isolates (n=153) were recovered from clinical samples during Jan-Dec 2010 and found 39.2 % prevalence of ESBL isolates (Du *et al.*, 2014). Similarly 23% and 19% prevalence of ESBL producing *K. pneumoniae* was observed in Italy and Germany populations (Girometti *et al.*, 2014.,

Gürntke *et al.*, 2014). While results of our study showed, frequency is nearly 50% which is 2x time higher than of above mentioned studies. However, in neighbouring and underdeveloped countries, the ESBL frequency is relatively high as 66.7% in India (Hawkey *et al.*, 2008) 72% in Tehran, Iran (Feizabadi 2010), 55% in Riyadh, Saudi Arabia (Al-Agamy *et al.*, 2009) and 54% in Turkey (Gur *et al.*, 2009) etc. However, lower frequency of ESBL is reported in South American and in European countries (Perez *et al.*, 2007; Khanfar *et al.*, 2009). This difference may be due to the unavailability of the standard medical facilities as well as low socio-economic conditions of the underdeveloped countries e.g. India and Iran etc.

Results of this study described *K. pneumoniae* resistance against commonly used antibiotics (amoxicillin /clavulanic acid, cephadrine, caftazidime, ciprofloxacin, cefotaxime, cefipime and trimethoprim/sulphamethoxazoleamikacin) is high, while imipenam and amikacin showed least resistance (Table 1). This resistance pattern to different kinds of antibiotic is closely related to the MDR *K. pneumoniae* isolates already reported in Pakistan, India and Iran (Ullah *et al.*, 2009; Langarizadeh *et al.*, 2011; Rampure *et al.*, 2013; Jamil *et al.*, 2014). Based on the findings of these studies, it could be suggested that antibiotic resistance is very high among ESBL producing *K. pneumoniae*. This high resistance to antibiotics in Pakistan is alarming and may be due to misuses and prolong exposure of antimicrobial drugs, contaminated intravenous catheters, hygiene of health care staff and other environment related factors. Beside this, irrational use of antibiotics which is not according to WHO criteria is also major factor to acquire resistance in our population (Hannan *et al.*, 2013).

In Punjab region of Pakistan, the frequency of ESBL producing *K. pneumoniae CTX-M* genotype is (94.5%), which is nearly similar to 93.8% already reported in Karachi (Khan *et al.*, 2010). A seven-year study (2005-2012) at a tertiary care hospital in suburban New York City, USA concluded rapid spread of *CTX-M* enzyme from 0% (2005-2008) to 5% (2009) to 34.3% (2011) (Wang *et al.*, 2013). Similarly, 25% prevalence of *CTX-M* gene in Japan (Harada *et al.*, 2013), 41% in China (Du *et al.*,

2014) has been reported. These figures are much low as compared to our study (94.5%). On the other hand, prevalence of CTX-M is high as in Thailand 99.2% (Kiratisin *et al.*, 2008) and Saudi Arabia 71% strain of *K. pneumoniae* harbour CTX-M gene (Hassan *et al.*, 2014). This is much similar to our findings.

To our knowledge, this is the first report about the emergence of CTX-M gene in *K. pneumoniae* clinical isolates from Punjab region of Pakistan. It is possible that prevalence of CTX-M may be differing from region to region in Pakistan or it can be same, therefore it is necessary to screen ESBL bacteria for CTX-M in different regions of Pakistan. In future, studies are required to explore MDR, Gram negative bacteria and molecular characterization of these resistance genes from other regions of the Pakistan.

CONCLUSION

In conclusion, this study described that a high level of CTX-M positive ESBL strains are circulating in the Pakistan. In most microbiological laboratories, the ESBL E-test is the most commonly employed confirmatory test; but, it takes time; therefore, PCR based assay is need of hour; because, it is authentic and relatively fast. In addition, it can be cost effective if multiplex PCR is optimized for many antibiotic resistance genes (*TEM*, *SHV*, *OXA* CTX-M etc.). Classical tests like E or others test some time give not positive results while PCR is positive for that sample. It showed that PCR is more authentic and reliable test for the genotypic determination of the antibiotic resistant gene families.

Presence of active gene is alarming situation in society like Pakistan. This fact emphasized to develop a strong and comprehensive mechanism for the regular surveillance of antibiotic resistant strains in a population.

CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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