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Research Article

Inhibitor Method Based Detection of AmpC Beta-Lactamases Producing *Escherichia coli* and *Klebsiella pneumoniae* among Clinical Isolates in Lahore, Pakistan

Muhammad Sarfraz*, Shahida Hasnain

Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

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MS and SH presented the concept and designed the study. MS did experiments and wrote the manuscript. SH supervised the study and critically reviewed the article.

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Plasmid-mediated AmpC beta-lactamases, *Klebsiella pneumoniae*, *Escherichia coli*, Resistance profile, Colistin-sulphate.

Abstract | Screening of plasmid mediated AmpC beta-lactamases (AmpC) has clinical significance due to developing resistance of clinical pathogens against several beta-lactam antibiotics. This study was conducted on 11,725 specimens collected from hospitalized and non-hospitalized patients from July 2013 to June 2016. API 20E system has been used to identify *K. pneumoniae* and *E. coli;* preliminary screening for AmpC beta-lactamase production was done by cefoxitin disc followed by inhibitor based confirmatory method. Antibiogram was performed following CLSI guidelines 2013. Of 11,725 clinical specimens, 29 % were culture-positive. 80% *K. pneumoniae* and 70% *E. coli* showed resistance to cefotaxime or ceftazidime. Only 63% *K.* pneumoniae and 58% *E. coli* and were resistant (<18 mm) to FOX. Phenotypic confirmation of pAmpC beta-lactamases was done using inhibitory based method and confirmed 46% *K. pneumoniae* and 8% *E. coli* pAmpC positive. A variable resistance pattern was seen in both pAmpC beta-lactamases producing *K. pneumoniae* and *E. coli* for Amikacin, Gentamicin, Levofloxacin, Ciprofloxacin, Imipenem, Meropenem, Pipracillin-Tazobactam, Cefoperazone-Sulbactam and Cefepime. The study elaborates recent trends in microbial profiles of pAmpC beta-lactamase producing pathogens in Lahore, Pakistan. This knowledge will enable the medical laboratories to report pAmpC β-lactamase detection accurately and support physicians to prescribe the appropriate antibiotics.

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Introduction

Plasmid-mediated acquisition of AmpC beta-lactamases is an important tool of antimicrobial resistance among the *Enterobacteriaceae*. Plasmid-mediated AmpC beta-lactamases (AmpC) have capability to hydrolyse most of β-lactams; has got attention since 1970 (Hanson, 2003). Treatment of nosocomial infections resulting from pAmpC carrying gram negative bacilli has become difficult as these pathogens present resistance to penicillins, cephalosporins, and, sometime, carbapenems (Jacoby, 2009). Epidemiological studies are important to develop the diagnostic screening protocols.

***Corresponding author: Muhammad Sarfraz**
sgaundal@hotmail.com

AmpC beta lactamase enzymes have been spread globally but not as much as extended-spectrum β-lactamases (Jacoby, 2009). These cephalosporinases hydrolyse the structural β-lactam ring of β-lactam drugs which is the common mechanism of bacterial antibiotic resistance (Bradford, 2001). AmpCs belong to class C according to the Ambler classification in 1980 and Group 1 as classified by Bush *et al.* (1995). AmpC are clinically significant as they may cause resistance to various numbers of beta-lactam antibiotics, like cefoxitin, cefotetan; narrow, expanded and broad spectrum cephalosporins (Martínez-Martínez *et al.*, 1999). *E. coli* and *K. pneumoniae* are important pathogens responsible for nosocomial infections in neonates (Younas *et al.*, 2018).

Occurrence and frequency of pAmpC is not properly

determined due to unavailability of Clinical and Laboratory Standard Institute (CLSI) guidelines protocol to detect AmpCs. CLSI has recommended, a cefoxitin disk with a three dimensional test to screen AmpC carrying isolates (Ingram *et al*., 2011; Lee *et al*., 2005). AmpC production is suspected when there is reduced susceptibility of third generation cephalosporins (despite cefepime) and cefoxitin (Jacoby, 2009). The method to screen AmpC positive is inhibitor based method using boronic acid (BA); this has been documented to be an effective inhibitor of AmpC enzymes (Younas *et al*., 2018). This study was carried out to screen the phenotypically AmpC positive *K. pneumoniae* and *E. coli* among various clinical specimens along with their antimicrobial resistance profile.

Materials and Methods

This study was conducted in the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan, from July 2013 to June 2016. A total number of 11,725 clinical samples were collected from patients. All the samples including blood, urine, cerebrospinal fluid (CSF), pus, sputum, tracheal secretions and pleural effusion were processed to detect AmpC positive *K. pneumoniae* and *E. coli*. These samples were inoculated on Blood agar, MacConkey agar; whereas urine samples were proceeded on CLED agar. The API (analytical profile index) 20E (bioMerieux) was used for phenotypic identification. Antimicrobial susceptibility testing was performed using Kirby Bauer disc diffusion method for all isolates of *E. coli* and *K. pneumoniae* (Cheesbrough, 2006). The antibiotic discs of Amikacin (30 µg), Gentamicin (10 µg), Co-Amoxiclav (20/10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Cefuroxime (30 µg), Cefixime (5 µg), Cefepime (30 µg), Cefoxitin (30 µg), Ciprofloxacin (5 µg), Moxifloxacin (5 µg), Levofloxacin (5 µg), Piperacillin-Tazobactam (100/10 µg), Cefoperazone-sulbactam (10/5 µg), Imipenem (10 µg), Colistin sulphate (25 µg) and Meropenem (10 µg) were used for antimicrobial susceptibility testing. Clinical and Laboratory Standard Institute guidelines were followed to measure and report zone of inhibition of each isolate as sensitive, intermediate or resistant (CLSI, 2013). ATCC 25922 strains of *E.coli* and ATCC 700603 strains of *K. pneumoniae* have been used as control.

All Cefotaxime and/or Ceftazidime resistant isolates of *E. coli* and *K. pneumoniae* primarily tested for AmpC production using the cefoxitin disc (FOX, 30 µg).

All Cefoxitin resistant *K. pneumoniae* and *E. coli* tested for phenotypic confirmation of AmpC production using cefoxitin discs containing boronic acid. A disc of FOX alone and one with phenylboronic acid (400 µg) was placed on the Muller Hinton ager plate and was incubated at 37°C. A zone size of ≥5 mm around the disc of FOX containing boronic acid in comparison to Cefoxitin alone was reported positive for AmpC (Younas *et al*., 2018).

Results

Of 11,725 various clinical specimens 29 % (3,400/11,725) were positive for bacterial growth. Of 3,400 pathogens 24% (816/3,400) were *E. coli* and 15% (510/3,400) were *K. pneumoniae*. CTX or CAZ resistant were observed in 70% (570/816) *E. coli* and 80% (408/510) *K. pneumoniae*. All CAZ or CTX resistant strains were subjected for FOX screening, 58% (330/570) *E. coli* and 63% (257/408) were resistant (<18 mm) to FOX. AmpC beta-lactamase was confirmed in 8% (26/330) *E. coli* and 46% (124/257) *K. pneumoniae* by inhibitory based AmpC beta-lactamase method.

Gender wise distribution of AmpC positive isolates was 90 (60%) in male patients and 60 (40%) in female patients. Positive AmpC isolates were recovered from blood 64 (43%) followed by 42 (28%) from urine, 17 (11%) from abscess, 12 (8%) from endotracheal tube (ETT) while few were isolated from other specimens (Figure 1).

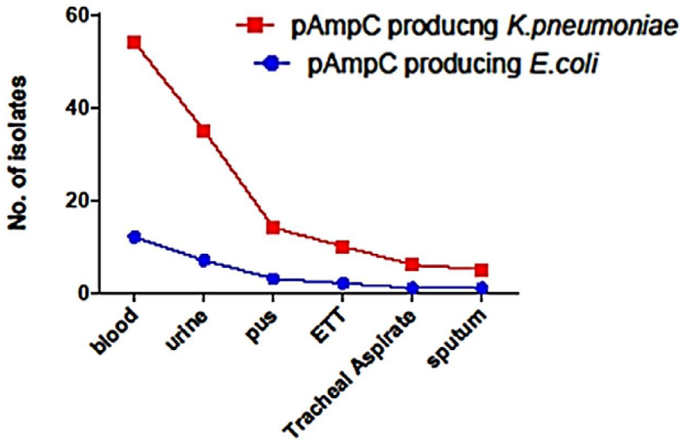

Figure 1: Distribution of pAmpC positive isolates in various clinical samples.

Table I: Prevalence of pAmpC among *E. coli* and *K. pneumoniae*.

	Number	Percentage
***E. coli* (n=816)**		
pAmpC positive	26	3.0
Non pAmpC	790	97.0
***K. pneumoniae* (n=510)**		
pAmpC positive	124	23.5
Non pAmpC	386	76.5

The production of AmpC was phenotypically confirmed in 11% (150/1326) isolates of *K. pneumoniae* and *E. coli*. Only 3% (26/816) *E. coli* and 24% (124/510) *K. pneumoniae* were positive for AmpC production; whereas non AmpC producing strains were 97% (790/816) *E. coli* and 76% (386/510) *K. pneumoniae* (Table I).

The frequency of AmpC positive isolates recovered from adults and children was 8% (12/150) and 92% (138/150), respectively.

All AmpC positive *E. coli* and *K. pneumoniae* were resistant to amoxicillin/clavulanic acid, ceftazidime and cefotaxime. Positive AmpC *E. coli* were resistant to amikacin 6/26 (24%), imipenem 7/26 (29%), cefoperazone-sulbactam 11/26 (45%), meropenem 12/26 (47%), piperacillin-tazobactam 13/26 (50%), cefepime 16/26 (63%), ciprofloxacin 20/26 (79%), gentamicin 23/26 (89%), levofloxacin 24/26 (92%) and cefixime 25/26 (95%). Positive AmpC *K. pneumoniae* were resistant to amikacin 72/124 (58%), imipenem 33/124 (27%), cefoperazone-sulbactam 68/124 (55%), meropenem 64/124 (43%), piperacillin-tazobactam 71/124 (57%), cefepime 92/124 (74%), ciprofloxacin 109/124 (88%), gentamicin 115/124 (93%), levofloxacin 105/124 (85%) and cefixime 118/124 (95%) (Figure 2).

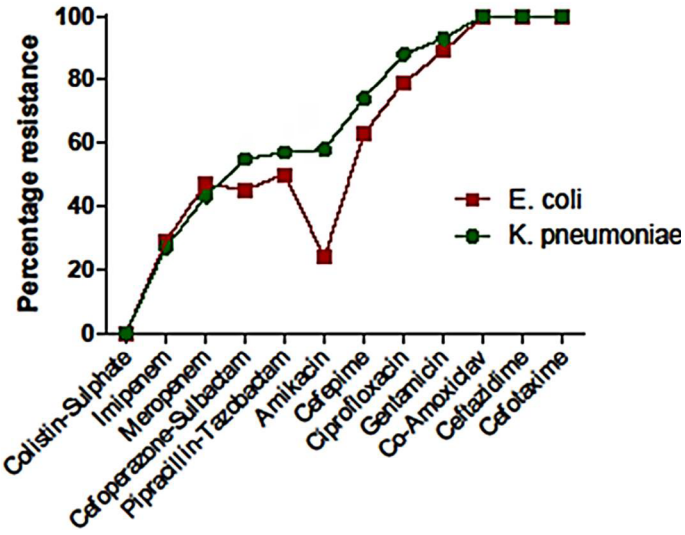

Figure 2: Antimicrobial resistance pattern of pAmpC positive isolates against different antibacterial drugs recovered from clinical specimens.

The minimum inhibitory concentration of cefoxitin for positive AmpC *K. pneumoniae* and *E. coli* were 256 to >512 µg/ml. Both pAmpC positive *K. pneumoniae* and *E. coli* isolates showed high MIC against ceftazidime, cefotaxime and cefoxitin >512 µg/ml (Table II).

Table II: Minimum inhibitory concentration in plasmid mediated AmpC positive *E. coli* and *K. pneumoniae*.

Isolates	Antibiotics (MICs) µg/ml			
	Cf	Cz	Cx	Ct-Sp
***E. coli* (n=26)**	256 to >512	>512	>512	0.25 to 0.75
***K. pneumoniae* (n=124)**	256 to >512	>512	>512	0.38 to 2.0

Cf, Cefoxitin; Cz, Ceftazidime; Cx, Cefotaxime; Ct-Sp, Colistin-Sulphate.

Discussion

AmpC beta-lactamases is a significant class of beta-lactamases isolated from several gram negative bacilli and are not inhibited by clavulanic acid. *E. coli* and *K. pneumoniae* are the predominant pathogens causing nosocomial infections in hospitalized patients which harbour pAmpC beta-lactamases and produces resistance to various clinically important antibiotics like cephalosporins.

In current study, 26 (3%) out of 816 *E. coli* and 124 (24%) out of 510 *K. pneumoniae* were AmpC beta-lactamase positive. Results are similar to the study conducted in Pakistan in 2016 (Salamat *et al.*, 2016). Ding *et al.* (2008) carried out study in 5 hospitals of China, where 8.5% *E. coli* were found as AmpC beta-lactamase producers. A study was conducted at Veteran's Medical Centers in Omaha, only 13 (1.9%) out of 683 *E. coli* were found as AmpC beta-lactamase producers (Coudron *et al.*, 2000). In another study, the occurrence of AmpC producing *E. coli* from 10 Greek hospitals was published; there were 55 (2.6%) AmpC beta-lactamase positive *E. coli*. Prevalence rate of AmpC beta-lactamases producing *E. coli* at a tertiary care center in US was observed to be 1.2% (Gazouli *et al.*, 1998). Mulvey *et al.* (2005) in their work at Canada Hospital found out a high rate of 123 (53%) AmpC beta-lactamase positive *E. coli*. High prevalence of AmpC beta-lactamase positive *E. coli* was also found at Medical Centers in Taiwan, which was 43.6% (Kaye *et al.*, 2004; Yan *et al.*, 2006). Above studies do not agree with our findings. There is high and low occurrence of AmpC positive *E. coli* reported in literature as compared to the present study.

Mulvey *et al.* (2005) in their study found 53.5% cases of AmpC positive *E. coli* among females and 46.5% in males. Similarly, a high incidence of 78% AmpC positive *E. coli* was found among females, in a study conducted at different hospitals in Canada (Kaye *et al.*, 2004). These findings disagree with our results where a high frequency of 60% and 40% was found in males and females respectively for both *K. pneumoniae* and *E. coli*.

Higher prevalence of 91.9% AmpC positive *K. pneumoniae* and *E. coli* found among children in present study. Mulvey *et al.* (2005) reported 10.5% pAmpC positive *K. pneumoniae* and *E. coli* in the patients 0 to 15 years of age. 24.4% AmpC producing *K. pneumoniae* and *E. coli* among neonates have also been recorded (Ding *et al.*, 2008). Bell *et al.* (2007) found high incidence of paediatric septicemia caused by AmpC positive isolates in Dar'es Salaam, Tanzania. High rate of infections was found in children less than 1 year (45%) and 15% AmpC producing strains was reported in children 1-5 years of age (Bell *et al.*, 2007). These figures agree with the present study where high percentage of AmpC positive bacterial infections has been

documented among the paediatric patients.

Antibiotic sensitivity testing results demonstrated that most of AmpC producing *E. coli* and *K. pneumoniae* were multidrug resistant. Those isolates, which produced only pAmpC or co-produced ESBL and AmpC, showed resistance to cefoxitin, cefotaxime, cefuroxime, ceftazidime, whereas more susceptibility was observed to imipenem, colistin sulphate, cefoperazone-sulbactam and meropenem. *K. pneumoniae* and *E. coli* showed 79% and 92% resistance to ciprofloxacin, 29% and 64% to amikacin, 90% and 93% to gentamicin, respectively. Whereas, co-amoxiclav and cefoxitin displayed 100% resistant to all isolates. Same results were reported in a study conducted at a paediatric Hospital in Pakistan in 2014 (Noor-ul-Ain Jameel *et al*., 2014). In Spain, a study was performed among hospitalized patients to observe the occurrence and antibiotic resistance of AmpC positive *E. coli;* high resistance was found against ceftazidime (100%) and cefotaxime (100%) (Martínez-Martínez *et al*., 1999). A study was conducted in 5 children hospitals of China, which showed antimicrobial resistance pattern of AmpC producing *E. coli*; these strains displayed high resistance to ciprofloxacin (70%), amikacin (30%) and gentamicin (70%) (Ding *et al*., 2008). In another study, it was documented that *Klebsiella* species showed greater sensitivity to the antimicrobial drugs than the *E.coli* isolates (Akujobi *et al*., 2012). This also disagrees with our study, where *Klebsiella* species were more resistant to the antimicrobial drugs tested than the *E.coli*.

The AmpC genes have multidrug-resistant plasmids which are acquired by bacterial pathogens and lead to the limited treatment options (Kanamori *et al*., 2011). The standardized laboratory guidelines for pAmpC screening are not available, so infections cause by pAmpC-positive *E. coli* and *K. pneumoniae* may become a greater threat for hospitalized patients. Furthermore, multidrug-resistant carrying AmpC genes have been documented which may spread among bacteria leading to a new emerging threat (CLSI, 2013; Kanamori *et al*., 2011).

To stop the spread of AmpC positive strains, the hospitals must have functional infection control committee with updated hospital antibiotic policy. As the AmpC positive organisms are also present in outdoor patients, they should also be screened to avoid the dissemination beta-lactamases in community.

Conclusion

Plasmid-induced AmpC β-lactamases positive *K. pneumoniae* and *E. coli* are major concerns of infection control and treatment strategies. This study will enable the medical laboratories to report AmpC β-lactamase detection accurately and assist physicians to prescribe the appropriate antibiotics. Standard infection control practices can help to control the spread of AmpC β-lactamase positive *K. pneumoniae* and *E. coli* in hospitalized patients.

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Conflicts of interest

The authors declare no conflicts of interest.

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