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Original Article

Phenotypic Identification and Antibiotic Susceptibility Pattern of AmpC beta-Lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Urinary Tract Infections from a Tertiary Care Hospital of Rawalpindi, Pakistan

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Introduction: This study is aimed to compare phenotypic test methods and determine antibiotic susceptibility pattern of AmpC beta-lactamase producing uropathogenic *Escherichia coli* and *Klebsiella pneumoniae* in clinical isolates. **Method:** *E. coli* and *K. pneumoniae* were identified by standard microbiological procedures. Screening of AmpC beta-lactamase production was done by using cefoxitin disc (30 µg) showing inhibition zone diameter of <18 mm. Then, screen-positive isolates were subjected to Disc Approximation Test (DAT) and three dimensional extract test (3-DET) methods. Antibiotic susceptibility testing was performed by Kirby Bauer Disc diffusion technique. **Results:** A total of 120 Gram Negative Rods (GNRs) were included in the study. Amongst them cefoxitin resistant isolates were 68.33% (n=82/120). In these 82 isolates, *E. coli* were n=57 (69.51%) and *K. pneumoniae* were n=25 (30.48%). DAT identified 52.43% of AmpC beta-lactamase producing isolates, sensitivity of DAT was found to be 88% with specificity of 92%, Positive Predictive Value of 92.68%, Negative Predictive Value of 87.80%, and Diagnostic Accuracy of 90.24%. Antibiotic susceptibility testing by Kirby Bauer Disc diffusion technique showed that carbapenems (meropenem) and tigecycline were of higher therapeutic effects against these resistant pathogens. **Conclusion:** Introducing simple tests like DAT in the laboratories can control the spread of AmpC beta-lactamase harboring organisms. Carbapenems (meropenem) and tigecycline are of suitable therapeutic effect against these resistant pathogens. *J Med Microbiol Infec Dis, 2014, 2 (4): 143-146*

Keywords: Extended spectrum beta-lactamase, Disc approximation test, Three dimensional extract test.

INTRODUCTION

Emergence of antimicrobial resistance amongst uropathogenic *Escherichia coli* and *Klebsiella pneumoniae* has become a major public health concern of the 21st century [1]. According to World Health Organization (WHO) report 2014, alarming rates of resistance have been reported in all WHO regions due to resistant pathogens like *E. coli*, *K. pneumoniae* and *Staphylococcus saprophyticus* causing urinary tract infections (UTI), bloodstream infections, wound infections and pneumonia [2]. Hence, emergence of antimicrobial resistance due to AmpC beta-lactamase producing uropathogenic *E. coli* and *K. pneumoniae* are of accelerating, augmenting and increasing clinical concern accounting for 80% of community and hospital acquired UTI [3]. The appropriate selection of antibiotic for the treatment of UTI is inadequate and restricted by the increasing rates of antibiotic resistance due to AmpC beta-lactamase producing uropathogenic bacteria [4]. Hence, AmpC beta-lactamases are cephalosporinases, which are associated with in vitro resistance to all beta-lactam antibiotics except for carbapenems and cefepime [5]. These beta-lactamases are chromosomally encoded as well as plasmid encoded [6]. Uropathogenic *E. coli* and *K. pneumoniae*, producing plasmid-mediated AmpC beta-lactamases contribute towards nosocomial outbreaks of infection [7]. Detection and discernment of AmpC beta-lactamases is a challenge and trial for the clinical diagnostic laboratories [8]. Hence, absence and unavailability of an authentic method for identifying these resistant pathogens cause their rapid dissemination [9]. Currently there are no Clinical and Laboratory Standards Institute (CLSI) recommended guidelines for identification of AmpC beta-lactamase harboring pathogens [10]. Therefore, there is a great need to implement simple and authentic methods in routine laboratory investigations to accurately detect these resistant pathogens especially in developing countries. Researchers have used various test methods for AmpC beta-lactamase detection, like the three dimensional extract test (3-DET) method [11], inhibitor based method [12], cefoxitin agar method [13] and Disc Approximation Test (DAT) methods [14]. The prevalence of AmpC beta-lactamase producing bacteria increases the burden of implementing infectious disease management globally [15].

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Thus, their accurate and authentic detection is important from epidemiological and infection control point of view [16]. This prompted us to determine the frequency of AmpC beta-lactamase producing bacteria isolated from a tertiary care hospital of Pakistan by introducing simple tests like DAT in routine laboratory diagnosis. We found that DAT method is simple, easy, reliable and cost effective phenotypic confirmatory test. Carbapenems (meropenem) and tigecycline are effective therapeutic options against AmpC beta-lactamase producing uropathogenic bacteria [17].

MATERIAL AND METHODS

This cross-section validation study was carried out from November 2014 to April 2015 at the Department of Microbiology, Army Medical College, National University of Sciences and Technology (NUST), Islamabad, Pakistan. The samples were collected after approval from the institutional ethics committee. Non Probability, convenience sampling technique was used.

A total of 120 Gram negative rods (GNRs) were isolated from the clinical samples of urine and cultured on Cystine Lactose Electrolyte Deficient (CLED) agar. Organisms were identified by standard microbiological procedures (Gram's stain appearance, colonial morphology, catalase test, cytochrome oxidase reaction, motility, routine biochemical tests) and by using API 20 E (Biomerieux, France).

Antibiotic sensitivity was performed by using modified Kirby Bauer disc diffusion technique. Phenotypic detection of Extended-spectrum-beta-lactamase (ESBL) was carried out by disc diffusion test method as per CLSI screening criteria [10]. Isolates were screened for AmpC beta-lactamase production by disc diffusion method using cefoxitin disc (Oxoid, UK). A 30 µg cefoxitin disc was placed on inoculated Mueller-Hinton agar plates (Oxoid, UK). According to CLSI [10] criteria, isolates with zone diameter less than l8 mm were considered to be an AmpC beta-lactamase producer. *E. coli* 25922 was used as a control strain.

3-DET method as described by Coudron and colleagues was used as a standard phenotypic method to detect AmpC beta-lactamase production [11]. In this method, 0.5 McFarland bacterial suspensions were prepared from an overnight culture. Then 50 µl of each was inoculated in 10 ml of trypticase soy broth (TSB, Oxoid, UK). TSB was incubated at 37°C for 4 h. Bacterial cells were concentrated by centrifugation and enzyme preparations were made by freeze thawing the cell pellets five times. The surface of a Mueller-Hinton agar plate was inoculated with control strain of *E. coli* ATCC25922. A cefoxtin disc (30 µg) was placed in the center of inoculated agar plates. A sterile scalpel blade was used to make a slit of 5 mm in the agar near the disc in outward radial direction. After that, 30 µl of the enzyme preparation was dispensed into the slit, by using a micropipette. The inoculated agar plates were incubated at 37°C for 24 h. The enhanced growth of surface organism at the point where the slit intersected the zone of inhibition was considered as a positive 3-DET test.

All isolates subjected to 3-DET method were at the same time also tested by DAT method as described by Gupta *et al*. [13]. In this method, 0.5 McFarland bacterial suspension was prepared from an overnight blood agar plate. Then MHA plate was inoculated using this suspension as per standard disk diffusion method. A 30 µg ceftazidime disk was placed at the center of MHA plate. Also 10 µg imipenem, 30 µg cefoxitin, and 20/10 µg amoxicillin-clavulanate disks were placed at a distance of 20 mm from ceftazidime disk. The plate was incubated at 35°C for 24 h. After overnight incubation, the plate was examined for any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the imipenem, cefoxitin and amoxicillin-clavulanate disks. Result was interpreted to be positive for AmpC beta-lactamase production if blunting or flattening of the zone was observed.

Antimicrobial susceptibility of isolates against aminoglycosides, fluoroquinolones, cotrimoxazole, carbapenems, tetracyclines, nitrofurantoin and beta-lactam/beta-lactamase inhibitor combination was tested by using Kirby Bauer disc diffusion technique, according to the CLSI guidelines.

The comparative statistical analysis was done by using SPSS version 20, 2 x 2 table. Data obtained from 3-DET method was considered as the gold standard [11] for this study and compared with data from DAT methods.

Parameters like sensitivity, specificity, negative predictive value, positive predictive value and diagnostic accuracy were determined. True positives were AmpC beta-lactamase producers by both 3-DET and DAT methods. False positive were AmpC beta-lactamase producers by DAT and not by 3-DET. False negative were the isolates which were non-AmpC beta-lactamase producers by DAT but were producing AmpC beta-lactamases by 3-DET. True negatives those which were non-AmpC beta-lactamase producers by both methods.

RESULTS

A total of 120 GNRs were included in the study. Out of which the screening test with cefoxitin disk (30 µg) identified n=82 (68.33%) isolates as possible AmpC beta-lactamase producers. Amongst these isolates uropathogenic *E. coli* were n=57 (69.51%) and *K. pneumoniae* were n=25 (30.48%).

These 82 isolates were subjected to 3-DET method and DAT method for AmpC beta-lactamase detection. DAT method identified 52.43% of AmpC beta-lactamase producing isolates, sensitivity of DAT was found to be 88%, specificity was 92%, Positive Predictive Value was 92.68%, Negative Predictive Value was 87.80% and Diagnostic Accuracy was 90.24% as shown in table 1.

Antibiotic susceptibility testing by Kirby Bauer Disc diffusion technique showed that AmpC beta-lactamase producing bacteria were more sensitive to carbapenems (meropenem) and tigecycline. On the other hand they showed marked resistance to aminoglycosides, fluoroquinolones, cotrimoxazole and tetracyclines as shown in table 2.

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Table 1. Comparison of DAT with 3-DET

Statistic	Formula	Value	95% CI
Sensitivity	$\frac{a}{a+b}$	88.37%	74.92% to 96.11%
Specificity	$\frac{d}{c+d}$	92.31%	79.13% to 98.38%
Positive Likelihood Ratio	$\frac{Sensitivity}{100-\text{Specificity}}$	11.49	3.85 to 34.26
Negative Likelihood Ratio	$\frac{100-Sensitivity}{\text{Specificity}}$	0.13	0.05 to 0.29
Disease prevalence	$\frac{a+b}{a+b+c+d}$	52.44% (*)	41.11% to 63.59%
Positive Predictive Value	$\frac{a}{a+c}$	92.68% (*)	80.08% to 98.46%
Negative Predictive Value	$\frac{d}{b+d}$	87.80 % (*)	73.80% to 95.92%

Note. Diagnostic Accuracy = 90.24%

Table 2. Resistance pattern of positive AmpC beta-lactamase isolates n=82

Drugs	Resistance (n)	Percentage (%)
Amikacin	36	43
Gentamycin	28	34
Ciprofloxacin	20	24
Minocycline	37	45
Cotrimoxazole	45	54
Piperacillin Tazobactam	48	58
Tigecycline	4	4
Meropenem	0	0

DISCUSSION

Accurate identification of AmpC beta-lactamase production is significant in order to establish adequate antibiotic therapy [18]. It is urgent to introduce an appropriate phenotypic method that can be integrated into diagnostic clinical laboratories for detection of antimicrobial resistance due to AmpC beta-lactamase producing bacteria [19]. Perplexity and confusion exists about the implication of these resistance mechanisms, proper reporting conventions and most appropriate routine test methods. Failure to detect AmpC beta-lactamases has led to their unrestrained spread and therapeutic failures [20]. In this study, DAT (phenotypic confirmatory test) was tested against a standard phenotypic test (3-DET). DAT detected the AmpC beta-lactamase carrying bacteria accurately, as sensitivity of this method was 88% and specificity was 92%. Regional data from India showed that percentage of AmpC beta-lactamase producing bacteria as detected by DAT was 23% by Singhal *et al.* [21]. Another study from Ahmedabad, India by Modi *et al.* [22] detected 81% of AmpC beta-lactamases by DAT method. Similarly in a study by Michael *at al.* [23] DAT detected 85% of *AmpC* beta-lactamases, which is comparable with our study results. In a study by Tan *et al.* [24] DAT detected 25.2% AmpC beta-lactamases, unlike our study results.

Other phenotypic methods like the Kirby-Bauer disk potentiation method, cefoxitin-Hodge test, AmpC saline disc test method, Combined disc diffusion test and AmpC E test methods are labour-intensive, technically intricate, expensive and inappropriate for routine screening in clinical microbiology laboratories. For example, AmpC saline disc test method is difficult to perform with maximum chance of human error since it is tricky to adjust the pH of EDTA buffer, if it is being prepared in-house hence, showing false negative results. Boronic acid disc test method is relatively easy to perform and test results are encouraging, but it is expensive, costing eleven thousand rupees for only boronic acid, with additional seven thousand rupees for DMSO. Therefore it is not suitable for a low income country to carry out these tests in routine microbial testing. Similarly, AmpC E-test method is reliable, results are encouraging but it is also very expensive, costing thirty seven thousand rupees for only twenty tests. Besides that trained staff is required to interpret E-test results. PCR test remains the gold standard test for identifying AmpC beta-lactamases [25]. This test is also very expensive and can be performed only in well-equipped laboratories. Besides that a highly trained staff is required to perform and interpret PCR test results.

However, implementation of simple, accurate, and cost effective diagnostic tests like DAT in routine laboratory investigations in developing countries like Pakistan, India and Afghanistan can help to eradicate and control antimicrobial resistance due to AmpC beta-lactamases.

Antimicrobial sensitivity testing by Kirby Bauer Disc Diffusion technique showed carbapenems (meropenem) to be better therapeutic option and these results are comparable to a regional study by Afreenish *et al.* who showed

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carbapenems to be 100 percent sensitive against these resistant pathogens [17]. Similarly in a study by Delgado-Valverde *et al*. carbapenems showed good invitro susceptibility against these resistant pathogens [26].

Introducing simple tests like DAT in the laboratories can control the spread of AmpC beta-lactamase harboring uropathogens. Carbapenems (meropenem) and tigecycline can be good therapeutic options against these resistant pathogens.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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