

Evaluation of phenotypic tests for detection of Amp C beta-lactamases in clinical isolates from a tertiary care hospital of Rawalpindi, Pakistan

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Abstract

Objective: To evaluate sensitivity and specificity of disc approximation test compared to three-dimensional extract test as a phenotypic gold standard test for detection of AmpC beta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*.

Method: The cross-sectional validation study was conducted from November 2014 to April 2015 at Army Medical College, Rawalpindi, Pakistan. Extended spectrum beta lactamases (ESBLs) were isolated from various clinical specimens. Screening for AmpC beta-lactamases was done by using cefoxitin disc (30µg) showing inhibition zone diameter of <18mm. Screen-positive isolates were subjected to disc approximation test (DAT) and three-dimensional extract test (3-DET). SPSS 20 was used for statistical analysis.

Results: A total of 120 ESBL producing Gram negative rods were included in the study. Out of these 120, 82 (68.33%) were found to be AmpC beta-lactamase producing on screening with cefoxitin disc. Amongst these 82 isolates, *Escherichia coli* were identified in 57 (69.51%) and *Klebsiella pneumoniae* in 25 (30.48%). Phenotypic confirmation by disc approximation test (DAT) identified 43 (52.43%). AmpC beta-lactamase producing isolates, whereas gold standard 3-DET showed 38 (46.34%) of AmpC beta-lactamase producing isolates. Hence, sensitivity of disc approximation test (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%.

Conclusion: Implementation of disc approximation test in the laboratories can help in identifying AmpC beta-lactamase harbouring organisms.

Keywords: AmpC beta lactamases, Extended-spectrum-β-lactamase (ESBLs), Gram negative rods (GNRs). (JPMA 66: 658; 2016)

Introduction

Multiple drug resistance is a very serious and pervasive phenomenon in contemporary medicine and has arisen as one of the leading public health issues of the 21st century.¹ Emergence of antimicrobial resistance among Gram Negative Rods (GNRs) due to AmpC beta-lactamases are of accelerating, augmenting and increasing clinical concern since the late 1970s. Amp C beta-lactamases belong to molecular class C in the Ambler structural classification of beta-lactamases, whereas in the functional classification scheme of Bush et al., they were assigned as group-I.² These are cephalosporinases which confer resistance to cephamycins, cephalosporins, aztreonam and beta-lactam/beta-lactamases inhibitor combinations like ampicillin-clavulanic acid and piperacillin-tazobactam.³ Predominance of Amp C beta-lactamases has accelerated tremendously over the past decade and are of meticulous concern and leading to inconsequential

therapeutic outcome.⁴ The genes for Amp C beta-lactamases are usually found on chromosomes of various members of family Enterobacteriaceae like *Enterobacter* species, *Shigella*, *Providencia*, *Citrobacter freundii*, *Morganella morganii*, *Serratia*, and *Escherichia coli*.⁵ Since 1989 these inducible chromosomal genes have also been determined on plasmids.⁶ These plasmid mediated Amp C beta-lactamases set hindrance and are stumbling blocks for the clinical microbiologists working in hospital laboratories.⁷ Amp C beta-lactamase production is associated with in vitro resistance to all beta-lactam antibiotics except carbapenems and cefepime. Detection and discernment of such devious beta-lactamases is a challenge and trial for the laboratories. Absence and unavailability of an authentic method for identifying these resistant pathogens cause their rapid and dissemination, leaving the patients and institutions at health risk. Currently there are no Clinical and Laboratory Standards Institute (CLSI) recommended guidelines for identification of Amp C beta-lactamases and it is the need of the hour to introduce and implement simple, rapid, valid and authentic methods in routine laboratory investigations, especially in developing countries.⁸ Researchers with their team of

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scientists have used various test methods for Amp C beta-lactamase detection, for example the three dimensional extract test (3DET),⁹ inhibitor-based method using inhibitors like boronic acids,¹⁰ cefoxitin agar method,¹¹ and disc approximation test (DAT) methods.¹²

With the worldwide increase in the occurrence, types and rate of dissemination of these enzymes, their early detection is crucial and critical since AmpC beta-lactamases show marked variation in geographic distribution.¹³ Detection of pathogens producing Amp C beta-lactamases are often associated with potentially fatal laboratory reports of false susceptibility to beta-lactams phenotypically.¹⁴ Thus, their accurate, authentic and valid detection are important from epidemiological, clinical, laboratory, and infection control methods.¹⁵

The prevalence of AmpC producing clinical isolates increases the burden of implementing infectious disease management globally, especially in developing countries. The current study was planned to evaluate sensitivity and specificity of Disc Approximation Test (DAT) and compared it to phenotypic gold standard 3-DET test for the identification of AmpC beta-lactamases.

Materials and Methods

The cross-sectional 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, which is affiliated with an 1100-bed tertiary care hospital. Non-probability, convenience sampling technique was used. The samples were collected after approval from the institutional ethics committee. Gram-negative bacteria were isolated from the clinical samples of urine, pus, blood, nasobronchial lavage, intravenous catheter tips and urinary catheter tips during the study period.

The sample size was calculated using World Health Organisation (WHO) calculator thus: Anticipated population proportion= 12%;¹⁶ Confidence Level= 95%; and Absolute precision=6%. The minimum sample size required was 120.

Gram-negative bacteria were identified by standard microbiological procedures (Gram's stain appearance, colonial morphology, catalase test, cytochrome oxidase reaction, motility, routine biochemical tests) and by using analytical profile index (API) 20E (Biomérieux, France). Duplicate samples from the same patients were excluded. Antibiotic sensitivity was done using modified Kirby Bauer disc diffusion technique. Screening for

extended-spectrum- β -lactamase (ESBL) production was carried out by disc diffusion method using indicator drug as per CLSI screening criteria and confirmation was performed using CLSI phenotypic confirmatory test.⁸

Isolates were screened for AmpC beta-lactamase production by disc diffusion method using cefoxitin disc (Oxoid, UK). A 30 μ g cefoxitin disc was placed on inoculated Mueller Hinton agar plates (Oxoid, UK). By following the CLSI criteria, isolates with zone diameter less than 18mm were selected for AmpC beta-lactamase testing. *E.coli* 25922 was used as control strain. Positive isolates were then subjected to phenotypic confirmatory tests.

Three Dimensional Extract Test (3DET) was used as standard phenotypic method to detect Amp C beta lactamase production.⁹ First, 0.5 McFarland bacterial suspensions were prepared from an overnight culture. Then 50 μ l of each was inoculated in 10ml of trypticase soy broth (TSB) (Oxoid, UK). The inoculated TSB was incubated at 37°C for four hours. Then bacterial cells were concentrated by centrifugation. The enzyme was prepared by freeze-thawing the cell pellets five times. The Mueller-Hinton agar (MHA) plate was inoculated with control strain of *E.coli* ATCC 25922. Then a cefoxitin disc (30 μ g) was placed in the centre of inoculated agar plates and by using a sterile scalpel blade, a slit starting 5mm from the edge of the disc, was cut in the agar in outward radial direction. After that 30 μ l of the enzyme preparation was dispensed into the slit, beginning near the disc and moving in an outward direction by using a micropipette. The inoculated agar plates were incubated at 37°C for 24 hours. "The enhanced growth of surface organism at the point where the slit intersected the zone of inhibition was considered a positive 3-dimensional extract test and was interpreted as evidence for the presence of Amp C beta-lactamases".

All isolates subjected to 3DET were at the same time also tested by DAT.¹³ First, 0.5 McFarland bacterial suspension was prepared from an overnight blood agar plate and surface of MHA plate was inoculated using this suspension as per standard disk diffusion method. A 30 μ g ceftazidime disk was placed at the centre of MHA plate. Then, 10 μ g imipenem, 30 μ g cefoxitin, and 20/10 μ g amoxicillin-clavulanate disks were placed at a distance of 20mm from the ceftazidime disk. The plate was incubated at 35°C for 24 hours. Next the plate was examined for any blunting or flattening of the zone of inhibition between the ceftazidime disk and the imipenem, cefoxitin and amoxicillin-clavulanate disks.

Result was considered to be positive if there was any blunting or flattening of the zone.

SPSS 20 was used for statistical analysis. Data obtained from 3-DET was considered the gold standard⁹ for this study and compared with DAT data. Parameters like sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and accuracy were determined. True positives were AmpC beta-lactamase producers by both 3-DET and DAT. 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 were those which were non AmpC beta-lactamase producers by both methods.

Results

A total of 120 ESBL producing Gram Negative Rods (GNRs) were included in the study. Amongst these 120 ESBL producers, screening test with cefoxitin disk (30µg) detected 82(68.3%) isolates as possible AmpC beta-lactamase producers. Of these 82 isolates, E.coli were 57(69.51%) and Klebsiella pneumoniae were 25 (30.48%). The 3-DET detected 38(46.34%) isolates as Amp C beta-lactamase producers, whereas DAT detected 43 (52.43%) of isolates as Amp C beta-lactamase producers. Sensitivity and specificity of DAT was found to be 88% and 92%, respectively. Positive Predictive Value was 92.68%, Negative Predictive Value was 87.80% and diagnostic accuracy was 90.24% (Table-1 and 2).

Table-1: True Positive, False Positive, False Negative and True Negative isolates.

Test	Disease Present		Disease Absent		Total
	n		n		
Positive	True Positive	a= 38	False Positive	c= 3	a+c = 41
Negative	False Negative	b= 05	True Negative	d= 36	b+d = 41
Total		a+b = 43		c+d = 39	82

Table-2: Comparison of Disc Approximation Test with 3-Dimensional Extract Test.

Statistic	Value	95% CI
Sensitivity	88.37%	74.92% to 96.11%
Specificity	92.31%	79.13% to 98.38%
Positive Likelihood Ratio	11.49	3.85 to 34.26
Negative Likelihood Ratio	0.13	0.05 to 0.29
Positive Predictive Value	92.68% (*)	80.08% to 98.46%
Negative Predictive Value	87.80% (*)	73.80% to 95.92%

Diagnostic Accuracy = 90.24%

CI: Confidence interval.

Discussion

Detection of Amp-C beta lactamase producing bacteria is essential in order to establish adequate antibiotic therapy and to achieve encouraging clinical outcomes. Failure to accurately identify these devious enzymes has led to their uninhibited spread resulting in therapeutic failures.¹⁷ Hence there is a need for a reliable phenotypic method that should be integrated into diagnostic clinical laboratories for the detection of these resistant pathogens. The prevalence of AmpC-mediated resistance globally is unknown because of insufficient number of surveillance studies and also due to complexity of confirmatory detection methods.¹⁸ Perplexity and confusion exists about the importance of these resistance mechanisms, suitable reporting conventions and optimal test methods. Unfortunately, AmpC beta-lactamases are not reliably detected by standard susceptibility testing methods in the clinical microbiology laboratory.¹⁹ In our study, DAT, a phenotypic test, was tested against 3DET, a standard phenotypic test. DAT detected Amp C beta-lactamase carrying bacteria more reliably, as sensitivity came out to be 88% and specificity was 92%.

Regional data from India by Modi et al., 2012, showed that diagnostic accuracy of DAT was 81%,²⁰ which is comparable to our study results showing diagnostic accuracy of 90.24%. Similarly, in a study by Dunne et al.,²¹ DAT detected 85% of AmpC beta-lactamases. But unlike our study results, study by Tan et al.,²² showed that DAT detected 25.2% AmpC beta-lactamases and 23% by Singhal et al., from India detected AmpC beta-lactamases.²³

Other phenotypic methods like the "Kirby-Bauer disk potentiation method with some β-lactamase inhibitors, or the cefoxitin-Hodge test, AmpC disc test, combined disc diffusion test and AmpC E test methods" are labour-intensive, technically complex, expensive and unsuitable for routine screening in clinical microbiology laboratories and may not detect all AmpC beta-lactamases. 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 control the spread of antimicrobial resistance due to AmpC beta- lactamases.

Conclusion

Introducing and implementing simple tests like Disc Approximation Test (DAT) in the clinical laboratories can alleviate the spread of AmpC beta-lactamase harbouring organisms. The potential benefits would include better patient outcomes in terms of avoiding inappropriate therapy, a reduction in the rise of antibiotic resistance and

better infection control strategies by detection of Amp C beta lactamase harbouring organisms. Developing country like Pakistan has inadequate and restricted resources with a limited healthcare budget. Hence, such resistant pathogens will be an additional drain on the existing inadequate resources and extra cost for patients in the public sector. However adequate infection control measures can help in over coming this solution.

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