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Evaluation of Two Phenotypic Methods for the Detection of Plasmid-Mediated AmpC β-Lactamases among Enterobacteriaceae Isolates

Ronni Mol P[1,2] Khalid Mubarak Bindayna[2] Ganesan Shanthi[1]

[1]Division of Microbiology (Medicine), Rajah Muthaiah Medical College, Annamalai University, Chidambaram, Tamil Nadu, India
[2]Department of Microbiology, Immunology and Infectious Diseases, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Kingdom of Bahrain

Address for correspondence Ronni Mol P, MBBS, MD, PhD Research Scholar,Division of Microbiology (Medicine), Rajah Muthaiah Medical College, Annamalai University, Chidambaram 608002, Tamil Nadu, India (e-mail: ronnimj@agu.edu.bh).

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Abstract

Objectives AmpC β-lactamases are cephalosporinases that confer resistance to cephalothin, cefazolin, cefoxitin, penicillin, and β-lactamase inhibitor-β-lactam combinations. Even though the AmpC resistance is reported, but the accurate occurrence of AmpC β-lactamases in *Enterobacteriaceae* members is still unknown. Techniques to identify AmpC producers are still evolving but not yet optimized for the clinical laboratory. Here we aimed to compare the test performance of two different phenotypic methods, that is inhibitor-based assay using boronic acid and disk approximation test for AmpC detection in *Enterobacteriaceae* isolates from a tertiary hospital microbiology laboratory.

Materials and Methods The study includes 137 nonrepeat *Enterobacteriaceae* strains. Bacterial isolates, that yielded a zone diameter of less than 18 mm for cefoxitin by disk diffusion method were considered potential AmpC producers and further confirmed by phenotype methods—inhibitor-based assay using boronic acid and disk approximation test. A multiplex polymerase chain reaction was used to detect the most common plasmid-mediated AmpC genes: ACC, FOX, MOX, DHA, CIT, and EBC.

Results Of the 137 clinical isolates, 58 (42.33%) were cefoxitin resistant, while 53.4 and 18.9% of the cefoxitin-resistant isolates were positive by inhibitor-based assay and disk approximation test. Multiplex PCR detected 42 (30.6%) isolates with AmpC genes. Of the 42 isolates, the inhibitor-based assay detected 25 (59.5%) isolates, while the disk approximation test detected nine (21.4%) isolates.

Conclusion Our findings suggest that inhibitor-based assay using boronic acid can be used for the detection of the isolates that harbor AmpC β-lactamases. This method is cost-effective, simple to perform, and easy to interpret. Thus AmpC detection as a routine in clinical laboratories can help in appropriate therapeutic intervention and improved infection control.

Keywords
- *Enterobacteriaceae*
- AmpC β-lactamases
- inhibitor-based assay
- disk approximation test
- multiplex PCR

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Background

AmpC β-lactamases are cephalosporinases that confer resistance to cephalothin, cefazolin, cefoxitin, penicillin, and β-lactamase inhibitor-β-lactam combinations.[1] Under Ambler classification scheme, AmpC β-lactamases are class C enzymes, which utilize serine for beta-lactam hydrolysis.[2] AmpC β-lactamase resistance mechanisms can be: (1) inducible resistance via chromosomally encoded AmpC genes which is present in *Citrobacter freundii, Enterobacter cloacae,* etc., (2) non-inducible chromosomal resistance due to promoter and/or attenuator mutations seen in *Escherichia coli*, *Shigella* species, and (3) plasmid-mediated resistance in *Klebsiella pneumoniae*, *E. coli*, *Salmonella* species, etc.[3]

Even though the AmpC resistance is reported, but the accurate occurrence of AmpC β-lactamases in *Enterobacteriaceae* members is still unknown.[4] In *Enterobacteriaceae,* cefoxitin resistance is used for screening of AmpC β-lactamase producers. Its resistance may also be due to alterations to outer membrane permeability.[5] Techniques to identify AmpC producers are still evolving but not yet optimized for clinical laboratory.[6] Disk-based assays using cloxacillin and inhibitors (boronic acid [BA] compounds), cefoxitin-cloxacillin double disk synergy, AmpC disk tests, disk approximation tests, etc. have been developed for detection of AmpC-producing β-lactamase isolates in *Enterobacteriaceae*.[5,6] Molecular tests are also available but their use is restricted to research settings.[3] The Clinical and Laboratory Standards Institute (CLSI) guidelines 2019 recommended criteria for AmpC resistance detection do not exist.

However, surveillance and monitoring activity is significantly important in this epoch of multidrug resistance as failure in antimicrobial resistant mechanisms detection may result in the spread of resistant pathogens and ultimately, complicating the clinical outcome.[5] Well-designed studies on diagnostic techniques for the detection of AmpC β-lactamases, that is easy-to-perform and provides reliable results in a short time, and suitable for treatment recommendations for AmpC-producers are needed.[7]

This study aimed to compare the test performance of two different phenotypic methods that is inhibitor-based assay using BA and disk approximation test for AmpC detection in *Enterobacteriaceae* isolates from a tertiary hospital microbiology laboratory.

Material and Methods

Bacterial Isolate Collection and Identification

The study was based on laboratory surveillance from July 2018 to February 2019. The analysis includes 137 nonrepeat *Enterobacteriaceae* strains (60 *E. coli*, 60 *K. pneumoniae*, 10 *Proteus* species, 5 *Citrobacter koseri, 1 Salmonella typhi*, and 1 *Enterobacter* species samples). The clinical isolates were identified by their colony morphology, Gram staining characteristics, and standard biochemical tests.[8] We evaluated antimicrobial susceptibility by Kirby Bauer method as per CLSI (formerly NCCLS) guidelines, 2018.

AmpC β-lactamase Detection

Bacterial isolates that yielded a zone diameter of less than 18 mm for cefoxitin by disk diffusion method were considered potential AmpC producers[9] which was further confirmed by phenotype methods—inhibitor-based assay using BA and disk approximation test.

Inhibitor-based assay: Mueller Hinton agar (MHA) plates were inoculated with the bacterial isolate. Cefoxitin-BA disks were prepared as per Coudron.[10] Cefoxitin and cefoxitin with BA disks were placed on the inoculated MHA plates and incubated overnight at 37°C. An isolate that demonstrated a zone diameter of 5 mm or more in the presence of cefoxitin with BA in comparison with cefoxitin alone was considered an AmpC producer (►**Fig. 1**).

Disk approximation test: MHA plates were inoculated with the study isolate. A ceftazidime disk (30 µg) was placed at the center of the inoculated plate. Imipenem (10 µg), cefoxitin (30 µg), and amoxicillin/clavulanate (20/10 µg) disks were then placed at a distance of 20 mm from the ceftazidime disk. The inoculated plates were incubated overnight at 37°C. After overnight incubation, if the isolate demonstrates an obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates (imipenem, cefoxitin, and amoxicillin/clavulanate disk) then the isolate was considered as an AmpC producer[11] (►**Fig. 2**).

Molecular characterization of AmpC β-lactamase: Multiplex polymerase chain reaction (PCR) was used to detect the most common plasmid-mediated AmpC genes: ACC, FOX, MOX, DHA, CIT, and EBC.[11]

The protocol for multiplex was as follows: for the detection of MOX gene, 5GCTGCTCAAGGAGCACAGGAT-3 was used

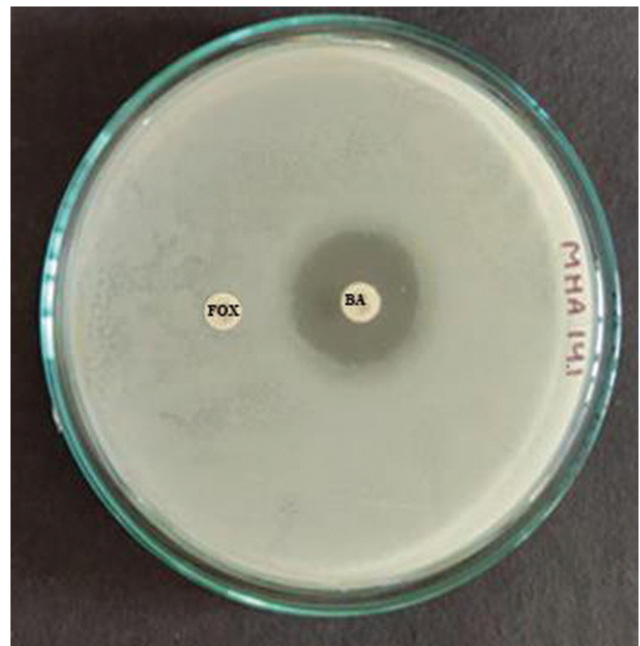

Fig. 1 Representation of inhibitor-based assay. A pure AmpC-producing isolate showing cefoxitin (FOX) zone enhancement (≥5 mm) with the addition of BA. BA, boronic acid.

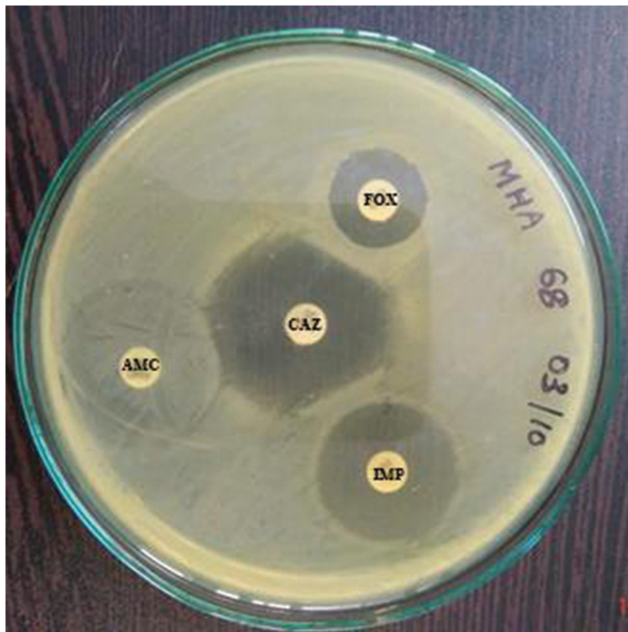

Fig. 2 Representation of disk approximation test. Flattening of the zone of ceftazidime toward imipenem disk and cefoxitin disk showing AmpC producer. IMP, imipenem (10 µg); FOX, cefoxitin (30 µg); CAZ, ceftazidime (10 µg); AMC, amoxicillin-clavulanate (20/10 µg).

as forward primer and 5-CACATTGACATAGGTGTGGTGC-3 was used as the reverse primer, expected *amplicon* size 520 bp. For the detection of CIT gene, 5-TGGCCAGAACTGACAGGCAAA-3 was used as forward primer and 5-TTTCTCCTGAACGTGGCTGGC-3 was used as the reverse primer, expected *amplicon* size 462 bp. For the detection of DHA gene, 5-AACTTTCACAGGTGTGCTGGGT-3 was used as forward primer and 5-CCGTACGCATACTGGCTTTGC-3 was used as the reverse primer, expected *amplicon* size 405 bp. For the detection of ACC gene, 5-AACAGCCTCAGCAGCCGGTTA-3 was used as forward primer and 5-TTCGCCGCAATCATCCCTAGC-3 was used as the reverse primer, expected *amplicon* size 346 bp. For the detection of EBC gene, 5-TCGGTAAAGCCGATGTTGCGG-3 was used as forward primer and 5-CTTCCACTGCGGCTGCCAGTT-3 was used as the reverse primer, expected *amplicon* size 302 bp. For the detection of FOX gene, 5-AACATGGGGTATCAGGGAGATG-3 was used as forward primer and 5-CAAAGCGCGTAACCGGATTGG-3 was used as the reverse primer, expected *amplicon* size 190 bp.

DNA extraction was by Modified Proteinase K method.[12] For PCR assays, 2-µL cDNA was added to 23-µL master mixture of PCR reagents. The reaction was programmed with initial denaturation step at 94°C for 3 minutes; followed by 25 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, primer extension at 72°C for 1 minute; and a final extension step at 72°C for 7 minutes[6] (Department of Molecular Biology and Immunology, MMNGH Institute of Dental Sciences and Research Centre, Belgaum, for Multiplex PCR). Amplified products were subjected to electrophoresis through 3% Agarose gel. 16 µL of each amplified product was loaded into each well. The gel was visualized under UV light illuminator and analyzed using Gel Documentation System (Major Science, California, United States). Negative control used was PCR mix with distilled water and a 100 base-pair DNA ladder was used as the size reference (►**Fig. 3**).

Statistical analysis: Statistical Package for the Social Science version 20 (IBM, Armonk, New York, United States) was employed to obtain descriptive data.

Results

Antimicrobial susceptibility testing: The clinical isolates showed resistance to multiple antimicrobial drugs. All our isolates showed complete resistance to ampicillin (100%). This was found to coexist with resistance to two or more antimicrobials that is cotrimoxazole (47%), norfloxacin (30.6%), ciprofloxacin (23.1%), and gentamicin (15.5%). Least resistance was observed with amikacin (1.1%) and piperacillin/tazobactam (2.2%).

Phenotypic detection of AmpC β-lactamases: Among 58 (42.3%) cefoxitin-resistant isolates, 53.4 and 18.9% of the isolates were positive by inhibitor-based assay and disk approximation test (►**Table 1**).

PCR detection of AmpC genes: Overall, of 137 *Enterobacteriaceae* members, 42 (30.6%) isolates were positive for AmpC gene subtypes (►**Table 2**).

Comparison of phenotypic test results with PCR detection of AmpC genes: Of the 42 isolates with Amp genes detected by multiplex PCR, inhibitor base assay detected 25 (59.5%) isolates, while disk approximation test detected 9 (21.4%) isolates. ►**Table 3** shows the statistical analysis of inhibitor-based assay and disk approximation test in comparison with gold standard PCR assay.

Discussion

Plasmid-mediated AmpC resistance pose a big challenge to infection control as the AmpC genes are expressed in higher amounts and are highly transmissible to other

Table 1 Detection of AmpC producers by phenotypic methods

Isolates	Cefoxitin screening	Inhibitor-based assay	Disk approximation test
Escherichia coli (*n* = 60)	28 (46.6%)	14 (23.3%)	5 (8.3%)
Klebsiella pneumoniae (*n* = 60)	23 (38.3%)	13 (21.6%)	5 (8.3%)
Proteus species(*n* = 10)	4 (40%)	3 (30%)	1 (10%)
Citrobacter koseri (*n* = 5)	2 (40%)	1 (20%)	0 (0%)
Salmonella typhi (*n* = 1)	0 (0%)	0 (0%)	0 (0%)
Enterobacter species (*n* = 1)	1 (100%)	0 (0%)	0 (0%)
Total (*n* = 137)	58 (42.3%)	31 (22.6%)	11 (8%)

bacterial species.[13] Worldwide prevalence of AmpC resistance is unknown, due to the limited number of surveillance studies and lack of laboratory diagnostic techniques in accurately detecting this resistance mechanism.[14] Detection of AmpC β-lactamases is clinically important so as to avoid therapeutic failures and nosocomial outbreaks.[4] Therapeutic options for AmpC producers are limited due to resistance to most of the β-lactam drugs except for cefepime and carbapenem.[15]

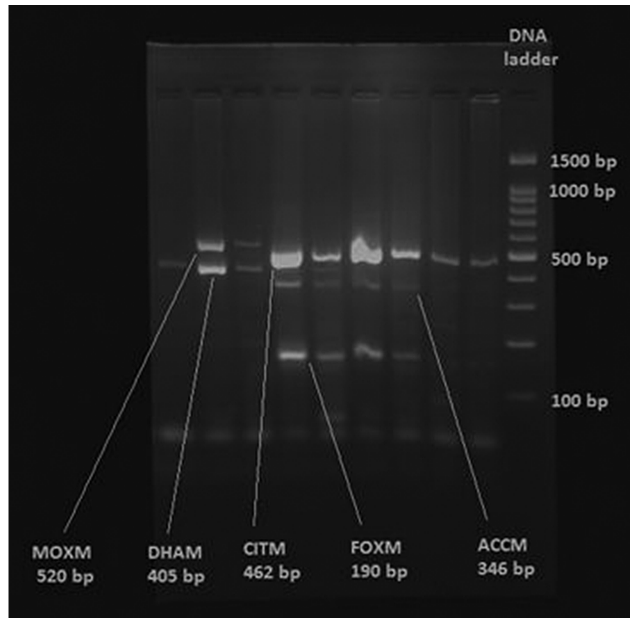

Fig. 3 AmpC genes by multiplex PCR. PCR, polymerase chain reaction.

Table 2 Distribution of AmpC genes among Enterobacteriaceae isolates

Isolates	AmpC genotype positive
Escherichia coli (*n* = 60)	17
Klebsiella pneumoniae (*n* = 60)	19
Proteus species (*n* = 10)	3
Citrobacter koseri (*n* = 5)	2
Enterobacter species (*n* = 1)	1
Total *n* = 137	42

In the present study, the clinical isolates showed complete resistance to ampicillin (100%) followed by cotrimoxazole (47%), norfloxacin (30.6%), ciprofloxacin (23.1%), and gentamicin (15.5%). Least resistance was observed with amikacin (1.1%) and piperacillin/tazobactum (2.2%). Similar antimicrobial resistance patterns have been observed among Indian *Enterobacteriaceae* clinical isolates,[16,17] whereas a study from Romania has reported high antimicrobial resistance against cotrimoxazole (74%), fluoroquinolones (49%), and penicillin (44%). Another study from Iraq has reported high MDR rate with β-lactams, aminoglycosides, and fluoroquinolones among *Enterobacteriaceae*.[18] Studies in different parts of the world have shown different patterns of antimicrobial resistance. This may be due to inappropriate ignorance and overuse of antibiotics, inappropriate infection control, and lack of awareness of the clinical outcome of multidrug-resistant bacterial infections.[16]

The present study demonstrated that among 58 cefoxitin-resistant isolates, 42 (72.4%) were found to possess AmpC genes by PCR. Similar results were reported by Yilmaz et al[19] and Helmy and Wasfi.[20] Not all cefoxitin-resistant isolates were AmpC producers. Cefoxitin resistance can be due to the presence of other antimicrobial-resistant mechanisms like extended spectrum beta lactamases, metallo beta lactamases, and mutation of porin channels. Cefoxitin also acts as a substrate to active efflux pumps in clinical strains.[20]

Phenotypic methods are unable to differentiate chromosomal and plasmid AmpC β-lactamases. Plasmid AmpC genes are detected by PCR analysis. But the molecular test is expensive and not available for routine use in all the clinical laboratories.[1] Hence there is a practical need for a simple and cost effective assay to detect plasmid AmpC β-lactamases. Our study compared two phenotypic methods and observed 53.4 and 18.9% of the cefoxitin-resistant isolates positive by inhibitor-based assay and disk approximation test. Plasmid AmpC genes were detected in 25 (59.5%) of the 31 inhibitor-based assay positive isolates and 9 (21.4%) of the 11 disk approximation test positive isolates by molecular analysis. Based on these findings, the inhibitor-based assay exhibited 58% sensitivity and 60% specificity and disk approximation test exhibited 21% sensitivity and 87% specificity.

A study from the United States has noted 58% of the boronic acid disk test positive isolates gave positive result using multiplex PCR.[21] In contrast, Yilmaz et al reported the presence of

Table 3 Statistical analysis of phenotypic AmpC detection tests

Statistic	Inhibitor-based assay		Disk approximation test	
	Value	95% CI	Value	95% CI
Sensitivity	58.14%	42.13–72.99%	21.43%	10.30–36.81%
Specificity	60.00%	32.29–83.66%	87.50%	61.65–98.45%
Positive likelihood ratio	1.45	0.74–2.84	1.71	0.41–7.09
Negative likelihood ratio	0.70	0.41–1.20	0.90	0.70–1.15
Positive predictive value	80.65%	68.08–89.06%	81.82%	52.10–94.90%
Negative predictive value	33.33%	22.51–46.25%	29.79%	24.96–35.11%
Accuracy	58.62%	44.93–71.40%	39.66%	27.05–53.36%

Abbreviation: CI, confidence interval.

AmpC genes in 22% of the boronic acid positive isolates and the inhibitor-based assay revealed 100% sensitivity and 66% specificity.[19] A study from Egypt reported 100% positivity among 50/148 (33.8%) AmpC isolates, by disk approximation test which was inconsistent with our observation.[11]

False positive phenotypic test results encountered in our study may be due to the presence of unknown AmpC genes or the inability of the phenotypic methods to differentiate chromosomal and plasmid AmpC genes. On the other hand, the reason for the false negative results is the ineffective phenotypic AmpC gene expression.[20] The difference between the phenotype methods and the molecular test results can be explained by the presence of chromosomal AmpCs or porin mutations.[19]

Conclusion

Our findings suggest that inhibitor-based assay using BA is a practical and efficient method for the identification of AmpC producers. This method is cost effective, simple to perform, and easy to interpret. Therefore, inhibitor-based assay using BA can be used for the detection of the isolates that harbor AmpC enzymes in the clinical laboratory where multiplex PCR is not affordable and thus help in therapeutic intervention, improved infection control and prevent the dissemination of antimicrobial resistance.

Availability of data and materials: All data generated or analyzed during this study are included in this article.

Financial Disclosure

None.

Ethical Approval

This research is approved by Annamalai Institutional Review Board.

Authors' Contribution

R.M.P. contributed towards concept, design, literature search, experimental studies, data acquisition, data analysis, statistical analysis, and manuscript preparation. G.S. did the data analysis, manuscript editing, and manuscript review. K.M.B. contributed towards data analysis, manuscript editing, and manuscript review.

Conflict of Interest

None declared.

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