

COMPARISON OF DIFFERENT PHENOTYPIC METHODS FOR AmpC DETECTION

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ABSTRACT**BACKGROUND**

AmpC β -lactamases are clinically important cephalosporinases which confer resistance to cephamycins, penicillins, aminoglycosides and β -lactam- β -lactamase inhibitor combinations and produced in several Enterobacteriaceae. The aim of this study was to compare various phenotypic detection methods of AmpC β -Lactamases.

MATERIALS AND METHODS

This was a descriptive study. Three hundred multidrug-resistant bacterial isolates were tested for AmpC β -lactamase enzyme production. Cefoxitin (30 μ g) resistance was used as the screening test for AmpC enzyme detection. Phenotypic confirmation was done by conventional disk combination method (Cefoxitin/ cefoxitin + boronic acid), 3D extract method, double disk synergy test (DDST) and AmpC disk test.

RESULTS

All 300 strains were found to be AmpC positive by the screening test. Out of these 300 strains, 100 strains were positive by disk combination method, 107 strains by DDST, 111 strains by 3D extract method and 170 strains by AmpC disk test.

CONCLUSION

Cefoxitin resistance can be used as the screening test for AmpC. AmpC disc test is simple to perform. Many strains can be checked on single petri plate and get best results in comparison to other methods of AmpC detection. So, it can be used as a phenotypic method for detection of AmpC enzyme production in resource-limited settings, where genotypic detection methods are not available.

KEY WORDS

AmpC β -Lactamase, Double Disk Synergy Test, 3D Extract, Boronic Acid, AmpC Disk Test.

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BACKGROUND

AmpC β -lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms where they mediate resistance to cephalothin, ceftazidime, cefoxitin, most penicillins and β -lactamase inhibitor- β -lactam combinations. Microbes acquire these enzymes by horizontal gene transfer of the plasmid DNA. Persistent treatments with standard antibiotics lead to the origin of these enzymes worldwide in diverse species of Enterobacteriaceae such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Salmonella* spp. and *Proteus mirabilis*.⁽¹⁻⁹⁾ Plasmid-mediated AmpC β -lactamase are expressed constitutively in Enterobacteriaceae and are relatively rare.⁽¹⁰⁻¹¹⁾ Several AmpC enzyme producing bacteria are retrieved from hospitalised patients after some days of admission in the hospital. Although, more than two decades have passed since plasmid mediated AmpC- β -lactamases were discovered, still

most clinical laboratories remain unaware of their clinical importance. As a result of which microorganisms producing them remain hidden and are mainly responsible for various nosocomial infections in hospital.

Numerous AmpC detection tests based on clinical microbiology techniques include screening for AmpC β -lactamases producing bacteria; modified three dimensional tests; disc approximation method; cefoxitin agar media (CAM) based assay; extraction of β -lactamase and analysis by isoelectric focusing; detection and characterisation of β -lactamases by PCR amplification and DNA sequencing; detection of AmpR regulatory gene and pulsed-field electrophoresis are available. Till now there is no recognised gold standard test for detection of AmpC β -lactamases.

It is highly desirable that clinical laboratories should undertake such additional tests capable of detecting latent resistance in time, so that they can effectively guide therapeutic as well as infection control strategies. The present study of phenotypic detection of AmpC β -lactamase producing bacteria will prevent unnecessary use of antibiotics and benefit patients by administration of appropriate antibiotics. This will also reduce the duration of hospital stay and treatments cost of the patient and thus help in reducing national health expenditure.

Aims of the Study

To compare various phenotypic detection methods of AmpC β -Lactamases.

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MATERIALS AND METHODS

Study Group

This descriptive study was performed at a tertiary care hospital in Western UP, India. 300 cefoxitin resistant strains of *E. coli* and *Klebsiella* spp. were subjected to various phenotypic methods of AmpC detection.

1. Three-dimensional extract method for AmpC detection⁽¹²⁾

Material Required

90 mm petri plate, MHA plate, McFarland 0.5 standard, sterile cotton tipped swab, tryptic soy broth, scalpel blade, antimicrobial disk- Cefoxitin (30 µg).

Procedure

50 mL of 0.5 McFarland bacterial suspensions prepared from an overnight blood agar plate was inoculated into 12 mL of tryptic soy broth and incubated for 4 hrs. at 35°C. The cells are concentrated by centrifugation and crude enzyme preparations made by freeze thawing the cell pellets five times. The surface of a Mueller-Hinton agar (MHA) plate was inoculated with *E. coli* strains ATCC 25922 as described for the standard disk diffusion by NCCL guidelines 1997.⁽¹³⁾ A 30 µg cefoxitin disk was placed on the inoculated agar. With a sterile scalpel blade, a slit beginning 5 mm from the edge of the disk was cut in the agar in an inward radial direction. By using a pipette, 25 - 30 µL of enzyme was dispensed into the slit; beginning near the disk and moving inward avoiding overflow. The inoculated media was incubated overnight at 35°C.

Interpretation of Result

Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition or indentation in the zone of inhibition was considered a positive 3D test result and was interpreted as evidence for the presence of AmpC β-lactamase.

2. AmpC Disk Test⁽¹⁴⁾

Material Required

Petri plates 90 mm, MHA plate, Tris-EDTA, normal saline, Cefoxitin disk (30 µg).

Procedure

AmpC disk was prepared in-house by applying 20 µL of a 1:1 mixture of saline and 100X Tris-EDTA to sterile filter paper disks allowing the disks to dry and storing them at 2-8°C. The surface of MHA plate was inoculated with a lawn of *E. coli* ATCC 25922 as for standard disk diffusion method. Immediately prior to use, AmpC disks were rehydrated with 20 µL of saline and several colonies of each test organism was applied to the disk. A 30 µg cefoxitin disk was placed on the inoculated surface of the MHA plate. The inoculated AmpC disk was placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate was incubated at 35°C for 16 - 18 hrs.

Interpretation of Result

An indentation or a flattening of the zone of inhibition indicates enzymatic inactivation of cefoxitin, which is interpreted as an AmpC producer strain. The absence of distortion indicates no significant inactivation of cefoxitin and the test strain is negative for AmpC β-lactamase production.

3. Boronic Acid/ Cefoxitin Disk Combination Test⁽¹⁵⁾

Material Required

Petri plates 90 mm, MHA plate, McFarland 0.5 standard, normal saline, aminophenylboronic acid, Cefoxitin (30 µg).

Preparation of Boronic Acid Disk (400 µg)

120 mg of phenylboronic acid was dissolved in 3 mL of dimethyl sulfoxide. 3 mL of sterile distilled H₂O was added to this solution. Now 20 µL of the stock solution was dispensed onto a disk containing 30 µg of cefoxitin. Disks were allowed to dry for 30 mins and used immediately or stored in air tight container at 4°C and at 70°C.

Procedure

Test strain was inoculated on MHA plate as mentioned in standard disk diffusion method. A disk containing 30 µg of cefoxitin and 400 µg of boronic acid was placed onto the agar plate.

Interpretation of Result

An organism that demonstrated a zone diameter around the disk containing cefoxitin and boronic acid that was 5 mm or greater than the zone diameter around the disk containing cefoxitin was considered an AmpC producer.

4. Double Disk Synergy Test⁽¹⁶⁾

Material Required

90 mm petri plates, MHA plate, McFarland 0.5 standard, sterile cotton tipped swabs, antimicrobial disks- boronic acid disk (400 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg) and Cefoxitin (30 µg).

Procedure

MHA plate was used. Inoculum, incubation condition, incubation times were same as standard disk diffusion. A boronic acid disk was placed in the centre. Disk containing extended-spectrum cephalosporins was placed at a distance of 10 mm (center to center) from boronic acid disk.

Interpretation of Result

Production of an AmpC by the test organism was inferred by the presence of characteristic enhancement of the inhibition zone towards the boronic acid disk.

RESULTS

AmpC β-Lactamase Detection by Screening Method (n=300)

Cefoxitin disk (30 µg) was used for AmpC screening. All the 300 *E. coli* and *Klebsiella* isolates included in the study were tested for cefoxitin susceptibility and not a single isolate was found to be susceptible to cefoxitin 0 (0%), i.e. all the 300 (100%) isolates were resistant (Table 1).

AmpC β-Lactamase Detection by Disk Combination Method

All the isolates of *E. coli* and *Klebsiella*, which were screened by using cefoxitin disk for detection of AmpC (Resistant) were tested with cefoxitin (Cn) Cefoxitin + Boronic acid (BA) disk combination methods for AmpC detection. Out of 300 isolates, this method detected 100 (33.33%) isolates of *E. coli* and *Klebsiella* for AmpC production (Fig. 1; Table 2).

AmpC β-Lactamase Detection by 3D Extract Method

All the 300 isolates were tested for AmpC detection by 3D extract method and this method detected 111 (37.00%) *E.coli* and *Klebsiella* isolates for AmpC production. Out of this, *E. coli* and *Klebsiella* were 70 (37.63%) and 41 (35.96%) respectively (Fig. 2; Table 3).

AmpC β-Lactamase Detection by DDST Method

Various DDST combination ceftazidime/ boronic acid, cefotaxime/ boronic acid, ceftriaxone/ boronic acid were used for AmpC detection. Among these combinations ceftriaxone/ boronic acid detected maximum 107 (35.67%) isolates followed by ceftazidime/ boronic acid 106 (35.33%) and cefotaxime/ boronic acid 105 (35.00%) from 300 isolates of *E. coli* and *Klebsiella* (Fig. 3; Table 4).

AmpC β-Lactamase Detection by AmpC Disk Test

AmpC disk test detected a total of 170 (56.67%) isolates of *E. coli* and *Klebsiella* as AmpC β-lactamase producer out of a total of 300 isolates. By this method, 110 (59.19%) isolates of *E. coli* and 60 (52.63%) isolates of *Klebsiella* were found to be AmpC producer (Fig. 4; Table 5).

Comparison of Various Methods of AmpC Detection (n=300)

Table 6 shows comparison of different methods of AmpC β-lactamase detection. AmpC disk test detected maximum number 170 (56.67%) followed by 3D extract method 111 (37.00%), DDST [(Ca/BA), (Ce/BA)] 108 (36.00%) and Disk combination method 100 (33.33%) out of 300 3rd generation cephalosporin resistant *E. coli* and *Klebsiella* isolates.

Screening Method	E. coli No. (%)	Klebsiella No. (%)	Total	E. coli No. (%)	Klebsiella No. (%)	Total
	Sensitive	Sensitive		Resistant	Resistant	
Cefoxitin disk diffusion method	0(0.00)	0(0.00)	0	186	114	300 (100)

Table 1. AmpC β-Lactamase detection by Screening Method

Organism No.	Cn/ Cn + Boronic Acid (BA) No (%)
E. coli 186	65
Klebsiella 114	35
Total 300	100 (33.33)

Table 2. AmpC detection by Disk Combination Method

Organism No.	3D Extract Method No. (%)
E. coli 186	70 (37.63)
Klebsiella 114	41 (35.96)
Total 300	111 (37.00%)

Table 3. AmpC β-Lactamase detection by 3D Extract Method

Organism	DDST No. (%)		
	Ca/ BA	Ce/ BA	Ci/ BA
E. coli 186	69 (37.10)	66 (35.48)	68 (36.56)
Klebsiella 114	37 (32.46)	39 (34.21)	39 (34.21)
Total 300	106 (35.33)	105 (35.00)	107 (35.67)

Table 4. AmpC β-Lactamase detection by DDST Method

Organism No.	AmpC Disk Test No. (%)
E. coli 186	110 (59.14)
Klebsiella 114	60 (52.63)
Total 300	170 (56.67)

Table 5. AmpC β-Lactamase detection by AmpC Disk Test

Methods for AmpC Detection	E. coli 186	Klebsiella 114	Total No. (%)	P value
AmpC disk test	110	60	170 (56.67)	>0.05
Disk combination method (Cn/Cn+BA)	65	35	100 (33.33)	>0.05
DDST	69 (Ca/BA)	39 (Ce/BA)	108 (36.00)	>0.05
3D extract method	70	41	111 (37.00)	>0.05

Table 6. Comparison of various methods of AmpC Detection



Figure 1. Disc Combination Method for detection of AmpC using Boronic Acid

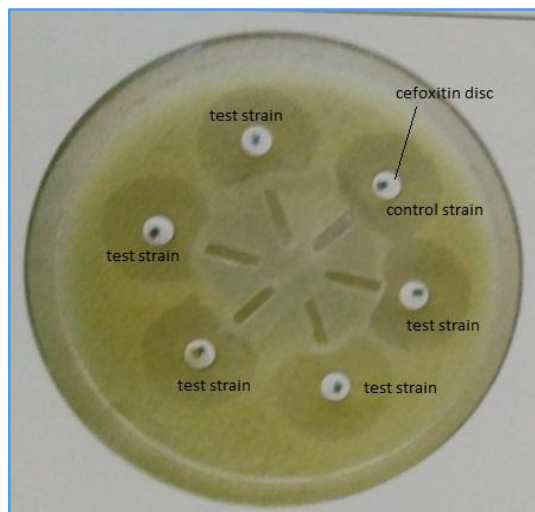


Figure 2. 3D Extract Method for detection of AmpC β-Lactamase

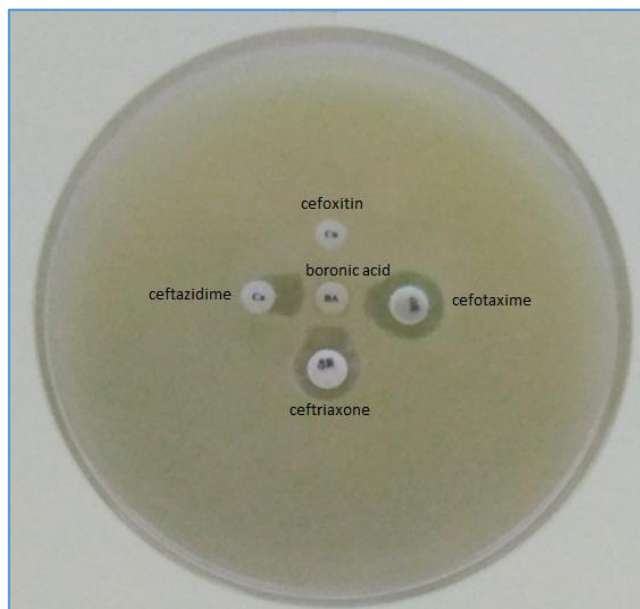


Figure 3. DDST for detection of AmpC by Boronic Acid



Figure 4. AmpC Disc Test for detection of AmpC β -Lactamase

DISCUSSION

A total of 300 resistant *E. coli* and *Klebsiella* isolates were screened for AmpC production with cefoxitin and it was found that all of them were resistant to cefoxitin. This may be due to AmpC production, porin loss⁽¹⁷⁻¹⁸⁾ and/ or not all strains with AmpC enzymes meet NCCLS criteria for resistance to cephamycins and oxyimino-cephalosporins.⁽⁷⁾ Some investigators have reported that cefoxitin resistance in general was a non-specific indicator of AmpC production.⁽¹⁹⁾

All the cefoxitin screened isolates were tested with boronic acid disk combination method (Reversible AmpC inhibitor).⁽¹⁵⁾ This method confirmed AmpC production in 33.33% of isolates out of 300 cefoxitin screened positive isolates of *E. coli* and *Klebsiella*. Study carried by Coudron⁽¹⁵⁾ showed that out of total 271 positive screened clinical isolates, 55 isolates showed AmpC production as was confirmed by PCR. Later these were subjected to boronic acid disk test, which detected presence of AmpC in 54 isolates.

Hemlatha et al⁽²⁰⁾ worked on ceftazidime/ boronic acid disk combination and showed that 47.3% of their resistant *E. coli* and *Klebsiella* isolates harbour AmpC enzyme.

AmpC β -lactamase production in ceftazidime resistant *E. coli* and *Klebsiella* was confirmed by three-dimensional extract methods. In our study, this method detected 37% *E. coli* and *Klebsiella* as AmpC producer. A different study carried out by Singhal et al⁽²¹⁾ showed 36% AmpC positive isolates out of 61 positive screen isolates by the three dimensional test. Study conducted by Ratna et al⁽²²⁾ showed that out of 520 isolates, 28 were resistant to ceftazidime and among these 16 isolates were positive by three dimensional extract method. In the study of Subha et al,⁽²³⁾ 84 *Klebsiella* isolates and 20 *E. coli* isolates that were found resistant (Zone diameter < 18 mm) were subjected to three dimensional extract test. Out of 84 *Klebsiella* isolates, 20 (20.8%) isolates of *Klebsiella pneumoniae* and 8 (40%) isolates of *Klebsiella oxytoca* were positive AmpC producer by this method. And out of 20, 12 (37.5%) isolates of *E. coli* resistant to ceftazidime showed the production of AmpC β -lactamase by three-dimensional extract tests.

In the present study, DDST using boronic acid (reversible AmpC inhibitor) was used for the detection of AmpC. All the cefoxitin screened positive isolates of *E. coli* and *Klebsiella* were subjected to DDST. Three DDST combinations were used i.e. ceftazidime and boronic acid, cefotaxime and boronic acid and ceftriaxone and boronic acid. Out of these combinations, ceftriaxone and boronic acid detected highest number of AmpC producer isolates (35.67%) followed by ceftazidime and boronic acid (35.33%) and least number by cefotaxime and boronic acid (35.00%). So almost all the third generation cephalosporins used in this test detected almost same number of AmpC producer, but slightly higher for ceftriaxone followed by ceftazidime and least number for cefotaxime. Power et al⁽²⁴⁾ first described the potentiating effect of boronic acid to the antimicrobial activity of ceftazidime and Liebana et al⁽²⁵⁾ used this synergism test for confirmation of the presence of an AmpC like enzyme.

AmpC disk test was also used for the detection of AmpC in cefoxitin screen positive isolates of *E. coli* and *Klebsiella*. Overall, this test detected 56.67% of screened positive isolates as AmpC producer. According to Jennifer et al this test was found to be highly sensitive, specific and convenient means for detection of plasmid mediated AmpC β -lactamases. Singhal et al⁽²¹⁾ also reported in their study that this method was sensitive and specific for the detection of AmpC.

In the present study different methods of AmpC detection was used, i.e. AmpC disk test, inhibitor (boronic acid) disk combination method, DDST by using boronic acid and 3D extract method. All the methods detected more *E. coli* as AmpC producer than *Klebsiella*. So, it was obvious that AmpC production was more prevalent in *E. coli* than *Klebsiella*. Maximum number of AmpC production was detected by AmpC disk test (56.67%) followed by 3D extract method (37.00%), DDST method using boronic acid showed 36.00% and least number by ceftazidime and boronic acid disk combination (33.33%). Except AmpC disk test all the other methods of AmpC detection used in the present study detected almost same number of AmpC producer. So, among the entire AmpC detection test used in this study, AmpC disk test was the best test for the detection of AmpC in clinical strains of *E. coli* and *Klebsiella*. Singhal et al⁽²⁰⁾ in their study

compared AmpC disk test with 3D extract method for AmpC detection. In their study 22 isolates (8%) out of the 272 isolates or 36% of the 61 AmpC screen positive isolates, 22 isolates which were positive by 3D extraction method also showed positive results by AmpC disk test. Indentation indicating strong AmpC producer was observed in 15 isolates, whereas flattening (weak AmpC) was observed in 7 isolates. Three indeterminate isolates (3D tests) also showed flattening in AmpC disk test indicating weak AmpC enzyme. For detection of AmpC class of β -lactamases, no satisfactory technique has been established till date, although various researchers have tried the 3D test with different modifications. This technique first described by Thomson et al,⁽²⁶⁾ has its own limitations, despite being increasingly sensitive. In their study they used an AmpC disk test which is an easier, reliable and rapid method of detection of isolates that harbour AmpC β -lactamases. This suggests that AmpC disk test can be used for routine screening of the AmpC enzymes in clinical laboratory.^(21,14)

CONCLUSION

From our study, it can be concluded that cefoxitin resistance can be used as the screening test for AmpC. AmpC disc test is easy to perform. Many strains can be checked on single petri plate and get best results followed by 3D extract method, DDST and disc combination method. Thus, AmpC disc test can be used above all the other available methods for phenotypic detection of AmpC enzyme production in resource-limited conditions where genotypic detection methods are not available.

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