Original Article

Two simple modifications of modified three-dimensional extract test for detection of AmpC β-lactamases among the members of family *Enterobacteriaceae*

Abstract

Aims and Objectives: An AmpC enzyme differs from the Extended-spectrum β-lactamases (ESBL) in their preferential hydrolysis of cephamycins along with other classes of cephalosporins (except fourth generation) and being resistant to inhibition by clavulanic acid. Various phenotypic methods of AmpC detection has been described but they are technically intricate and difficult to interpret. Present study was aimed to evaluate two simple modifications of modified three dimensional enzyme extract test – disk method and well method to detect AmpC enzymes among clinical isolates of *Enterobacteriaceae*. **Materials and Methods:** A total 160 consecutive clinical isolates of *Enterobacteriaceae* from various clinical samples were tested for extended spectrum β-lactamase production using CLSI described phenotypic confirmatory test. AmpC production was determined by using boronic acid disk potentiation test. All the strains were tested with modified three dimensional test and two simple modification of three dimensional extract test – disk method and well method. **Results & discussion:** Among 160 clinical isolates of Enterobacteriaceae 80 were AmpC producers. Modified three dimensional test detected only 70% of AmpC producers. Disk method and well method using enzyme extract showed 100% and 91% sensitivity respectively. Disk method was technically simple, easy to interpret and gave consistent results on repeated testing. Disk method using enzyme extract can be reliably used for AmpC detection in routine clinical microbiological laboratories.

Key words:
AmpC, AmpC disk test, boronic acid, modified three dimensional test, well method

Introduction

AmpC β-lactamases are group C enzymes belonging to Class I of Bush's functional classification. They confer resistance to penicillins, cephalosporins (except advanced spectrum cephalosporins like cefepime, cefpirome, and cefclidin), and monobactams.[1] They can be differentiated from extended-spectrum β-lactamases (ESBLs) by their ability to hydrolyze cephamycins and not inhibited by clavulanic acid (CA). AmpC enzymes may be plasmid mediated or chromosomal. Organisms such as *Enterobacter*, *Citrobacter*, *Shigella*, *Morganella*, *Serratia*, and *Escherichia coli* possess AmpC enzymes on their chromosomes.[2] Chromosomal expression is usually inducible variety, but it can be expressed constitutively when there is a promoter mutation (derepressed strains).[1] Plasmid-mediated AmpC enzymes were first identified in 1980, since then, they have spread among the members of the family *Enterobacteriaceae*.[1] Inappropriate use of cephalosporins in clinical practice led to the emergence of bacteria producing multiple β-lactamases. This leads to therapeutic failure when β-lactam drugs or β-lactam/inhibitor combination are used.[3] These phenotypes not only limit the therapeutic options, but they also pose a challenge to the clinical microbiology laboratories to identify them. Identification of AmpC or combined AmpC

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and ESBL is essential for appropriate infection control and correct management of infection.

Clinical laboratory standard institute (CLSI) described phenotypic confirmation test for ESBL production in routine use.[4] Currently, there is no CLSI-recommended guidelines to detect AmpC β-lactamases. Previously, many AmpC detection methods like three-dimensional test,[5] modified three-dimensional test[6] (M3D), AmpC disk test,[7] cefoxitin agar-based test,[8] and inhibitor-based disk potentiation test[9] are described. Disk potentiation test using boronic acid, cloxacillin, and Ro 48-1220 has been evaluated and used by many centers.[5] Many of these tests are intricate and need careful interpretation. Molecular methods like PCR though remains gold standard cannot be adapted on routine basis and instrument availability limits its use in many laboratories.

Manchand and Singh[6] reported that the M3D extract test reliably detect AmpC enzymes among *Enterobacteriaceae*. M3D test is cheap, reliable, and can be adapted in routine laboratory, but it is technically demanding. The present study was aimed at evaluating two simple modification of M3D test using enzyme extract for detection of AmpC enzymes among the members of the family *Enterobacteriaceae*.

Materials and Methods

A total of 160 consecutive, nonrepetitive isolates of *Enterobacteriaceae* isolated from different clinical samples like exudates (*n*=91) (wound swab, fluids, etc), urine (*n*=32), and sputum (*n*=37) between March 2010 and July 2010 were included in the study. Samples were processed and isolates were identified by standard laboratory methods.[10] The antibiotic susceptibility testing was done by Kirby Bauer disk diffusion method according to CLSI recommendations.[11]

ESBL production was detected by using CLSI described phenotypic confirmatory test using cefotaxime (CTX) and ceftazidime (CTZ) alone and in combination with CA. A ≥5-mm increase in zone was considered as confirmation of ESBL production. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 (HiMedia laboratories, Mumbai) was used as positive and negative controls, respectively.

AmpC production was detected by 3-aminophenyl boronic acid (APB; Sigma Aldrich, India) disk potentiation test, as described previously.[12] Briefly, 5 µl of APB stock solution (240 mg APB in 3 ml of Dimethyl sulfoxide (DMSO)) was added to CTX, CTZ, and cefoxitin (FOX) disks. The final concentration of APB on each disk was 400 µg. A ≥5-mm increase in zone of CTX and/or CTZ and/or FOX disk alone and in combination with APB was considered as AmpC production.

All the 160 isolates were taken for M3D and two simple modifications of M3D using enzyme extract. Briefly, 10 to 15 mg of fresh overnight growth of test organism from Mueller-Hinton agar (MHA) was suspended in peptone water and was pelleted by centrifugation at 3000 rpm for 15 minutes. Crude enzyme extract was prepared by repeated freezing and thawing of pellet for 10 times in the freezer portion of the ordinary refrigerator. The enzyme extract obtained was used for the following tests

M3D: A lawn of *E. coli* ATCC 25922 was made on MHA plate, 30 µg FOX (HiMedia laboratories, Mumbai) disk was placed in the center. Linear slits (3 cm) was made radially, 3 mm away from the edge of FOX disk. A well was cut 5 mm inside the outer end of the linear slit using pasture pipette. 30 to 40 µl of enzyme extract was put into the well and liquid was allowed to absorb for 15 minutes. The plates were incubated at 37^{0}C for 18 to 24 hours. Three types of results were recorded. Isolate showing clear distortion was taken as AmpC producer, isolate showing no distortion was taken as AmpC nonproducer, and isolate showing minimal distortion was considered indeterminate [Figure 1a].

AmpC Disk method: A lawn of *E. coli* ATCC 25922 was made on MHA plate and a 30-µg FOX disk was placed. A sterile filter paper disk (AmpC disk) was placed adjacent to FOX

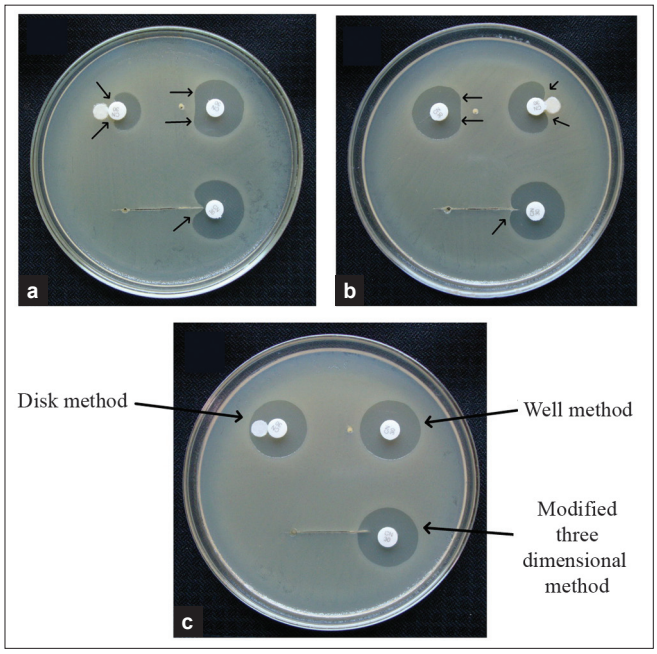

Figure 1: Comparison of modified three-dimensional test and two modifications disk and well method using enzyme extract. (a) AmpC-positive isolate showing clear distortion (arrow) of zone of FOX in all the three tests; (b) AmpC-positive isolate showing indeterminate result on M3D test but showing clear distortion in disk and well method; (c) AmpC-negative isolate showing no distortion in all the three tests

disk and 10 µl of enzyme extract was added to it and plate was incubated for 18 to 24 hours at 37^{0}C in ambient air. Disk method showed two types of results; indentation of FOX-sensitive zone near AmpC disk was considered as AmpC production and no distortion was considered AmpC nonproduction [Figure 1b].

Well method: A lawn of *E. coli* ATCC 25922 was made on MHA plate and a 30-µg FOX disk was placed. A well was cut 12 mm away from the edge of the disk. 20 µl of enzyme extract was added to the well and incubated for 18 to 24 hours at 37^{0}C in ambient air. Well method showed two results; flattening of FOX zone toward well was considered AmpC production and no distortion was considered AmpC nonproduction [Figure 1c].

Parallel tests were carried out by using cefoxitin added with 400 µg of APB; inhibition of distortion was confirmative of AmpC production. All the tests were done in duplicate.

Results and Discussion

Major group of Gram-negative organisms isolated from clinical specimens belongs to family *Enterobacteriaceae*. β-lactamase production is an important mechanism of drug resistance among these organisms. Inappropriate use of cephalosporins in clinical practice has lead to the increased prevalence of ESBL and AmpC enzymes among Gram-negative bacteria.[12] CLSI described ESBL confirmation method is in routine use.[4] Phenotypic confirmatory test cannot detect ESBL in the presence of AmpC, as the later mask the synergy arising from the inhibition of ESBL by CA. Currently, there are no guidelines for the detection of AmpC or multiple β-lactamases.

Of the 160 *Enterobacteriaceae* isolates, 66 were *Klebsiella spp*, 46 (24.2%) were *E. coli*, 17 *Enterobacter spp*, 16 *Proteus spp*, and 15 *Citrobacter spp*. Among 160 isolates, 41 (25.6%) and 12 (7.5%) were pure ESBL producers (only ESBL production) and pure AmpC producers (only AmpC

Table 1: ESBL and AmpC production among *Enterobacteriaceae*

Organism	Result (n)					Total
	Pure ESBL	Pure AmpC	Combined ESBL and AmpC	iAmpC	Negative	
Klebsiella spp	15	6	24	0	21	66
E. coli	15	1	18	5	7	46
Enterobacter spp	2	1	5	5	4	17
Proteus spp	7	0	4	0	5	16
Citrobacter spp	2	4	3	4	2	15
Total (n)	41 (25.6)	12 (7.5)	54 (33,7)	14 (8.7)	39 (24.3)	160

iAmpC – Inducible AmpC

Table 2: Occurrence of cefoxitin resistance and efficacy of FOX-APB disk test for detection of AmpC among *Enterobacteriaceae*

Result	FOX resistance		FOX-BA disk test for AmpC (%)		
	S (%)	R (%)	≥5-mm enhancement	FOX-R No enhancement	FOX-S No enhancement
ESBL (41)	35	6	00	6	35
AmpC (12)	00	12	8	4	00
ESBL + AmpC (54)	00	54	48	6	00
iAmpC (14)	00	14	12	2	00
None (39)	39	00	00	0	39
Total (160)	74 (46.2)	86 (53.7)	68 (42.5)	18 (11.2)	74 (46.2)

ESBL+AmpC – ESBL and AmpC co-producers; iAmpC – Inducible AmpC; FOX – Cefoxitin; BA – Boronic acid; R – Resistant; S - Sensitive

Table 3: Comparison of modified three-dimensional test, AmpC disk, and Well method using enzyme extract

Enzyme type	M3D			AMPC-disk		Well	
	Positive	Negative	Indeterminate	Positive	Negative	Positive	Negative
ESBL (41)	0	41	0	0	41	0	41
AmpC (12)	10	1	1	12	0	10	2
ESBL + AmpC (54)	39	4	11	54	0	51	3
iAmpC (14)	7	0	7	14	0	12	2
Negative (39)	0	39	0	0	39	0	39
Total	56 (70%)	85	19	80 (100%)	80	73 (91.2%)	87

ESBL+AmpC – ESBL and AmpC co-producers; iAmpC – Inducible AmpC; FOX – Cefoxitin; BA – Boronic acid; R – Resistant; S - Sensitive

production), respectively; 54 (33.8%) were combined ESBL and AmpC producers; and 14 (8.8%) showed inducible AmpC (iAmpC). 39 (24.4%) of isolates did not harbor any enzyme [Table 1].

Cefoxitin resistance was seen in 86/160 (53.75%) isolates [Table 2]. All the 80/80 (100%) AmpC-producing isolates were resistant to FOX, but only 68/80 (85%) showed ≥5-mm zone enhancement with the addition of APB to FOX. Fifteen percent (12/80) of AmpC-positive isolates and 7.5% (6/80) of AmpC-negative and which showed resistance to FOX did not show enhancement of zone with addition of APB. In all such isolates, FOX resistance may be due to mechanism other than AmpC production. None of the FOX-sensitive isolates were AmpC producers. Coudron[12] reported that sensitivity of FOX-APB method was found to be 97% for AmpC detection. But the present study showed a sensitivity of 85% for AmpC detection using FOX-APB disk potentiation method.

Thomson and Sanders first reported three-dimensional test,[13] since then, many modifications have been made. The original method was intricate as it needs customized low-speed rotator and careful loading of slit without spilling the extract. Coudron *et al*.[5] modified original method; they used linear slits instead of circular slits. The method could overcome the need for rotator, but filling these slits without spillage was not solved. Subsequently, Manchand and Singh[6] modified the procedure; they created a well at the outer edge of the slit and the enzyme extract was put into the well. The method overcomes all the problems of three-dimensional test. But the M3D test is laborious, technically demanding requiring careful cutting of slit and well, time consuming, and needs experience. AmpC disk test[7] and spot inoculation test[14] with direct bacterial colonies were introduced to overcome these problems. But these tests were less sensitive than M3D test.

We adapted two simple modifications of M3D test. The results of AmpC detection using M3D test, disk test, and well method are shown in Table 3. M3D test could detect only 56/80 (70%) of AmpC producers. 5/80 (6.25%) AmpC producers were detected as negative and 19/80 (23.75%) AmpC producers showed indeterminate results with M3D test. All the negatives were correctly identified. Among the 19 indeterminate results in M3D test, 11 were combined ESBL and AmpC producers, 7 were iAmpC producers, and one was pure AmpC producer. AmpC disk test using enzyme extract detected all the AmpC producers and AmpC nonproducers correctly (P=0.000). The method also detected iAmpC correctly. Well method could identify 73/80 (91.25%) AmpC enzymes correctly, while it failed to detect 7 (9.75%). All the three tests were 100% specific. With the addition of APB to FOX disks in parallel set, all the three tests showed inhibition of zone distortion, confirming AmpC production.

Isolates which show indeterminate results in M3D test are considered as weak enzyme producers.[6] Such isolates showed clear distortion of zone with AmpC disk test [Figure 1b]. The isolates which showed clear distortion of zone in M3D test showed enhanced distortion of zone around the FOX disk indicating high amount of AmpC production. Disk method detected all the iAmpC enzymes correctly.

We also compared AmpC disk test described by Shahid *et al*.[14] using direct colony on sterile filter paper disks kept adjacent to cefoxitin disks. But the method was least sensitive (data not shown) and test organism would grow in the zone of inhibition around the FOX disk causing false interpretation of results.

To conclude AmpC disk test using enzyme extract was simple to perform, easy to interpret, and gave consistent results on repeated testing. The test is 100% sensitive and specific for AmpC detection. The method avoids the cutting of slits and wells, making it simpler to adapt on routine basis.

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