



Original Research Article

Phenotypic methods for detection of Amp C β lactamases in Gram negative clinical isolates of a tertiary care hospital

Dhanashree P Inamdar^{1,*}, Anuradha B¹

¹Dept. of Microbiology, Mamata Medical College and Hospital, Khammam, Telangana, India



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ABSTRACT

Introduction: ESBLs (Extended spectrum beta lactamases) are detected routinely in most laboratories but Amp c Beta lactamases are neglected to large extent. Pseudosusceptibility seen in Amp C producers leads to resistance to extended-spectrum cephalosporins resulting in inappropriate antimicrobial regimens and therapeutic failure. Thus, there is a need to know an appropriate phenotypic method for easy detection of Amp C β -lactamases as they have clinical relevance.

Aim: To detect and compare different phenotypic methods for Amp C β -lactamases in Gram-negative clinical isolates of Enterobacteriaceae family

Materials and Methods: A prospective study was done at Mamata Medical college, Khammam for period of three months. Phenyl boronic acid method, Cefoxitin Cloxacillin-Double disc synergy test, TRIS EDTA method, Disc approximation test were done on isolates after screening by Cefoxitin disc.

Results: Out of 140 isolates tested, 80(57%) were positive (resistant) for screening test by cefoxitin. Out of them 61(76.2%) were Escherichia coli, 16 (20.1%) isolates were Klebsiella pneumoniae and 3 (3.75%) were Enterobacter sp. Phenotypic confirmatory methods by Cefoxitin Cloxacillin Double disc Synergy (CC-DDS) test showed zone difference of >4 mm in 38(47.5%) isolates, by Phenylboronic acid method (PBA) > 5 mm zone difference was observed in 34(42.5%) isolates, by TRIS EDTA method 32 (40%) showed indentation near EDTA disc and by Disc approximation test 32(40%) were positive for Amp C production. E coli was the commonest isolate showing Amp c production by all four methods.

Conclusion: CC-DDS method has better detection rate compared to other phenotypic confirmatory methods. We suggest CC-DDS method as it is easy to perform.

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1. Introduction

Amp C Beta lactamase was first bacterial enzyme discovered to destroy penicillin which was seen in Escherichia coli. Stepwise-enhanced resistance in beta lactamases due to mutations were termed amp A and amp B.^{1,2} A mutation in an amp A strain causing reduced resistance was called as amp C. In many Enterobacteriaceae, Amp C expression is low but inducible in response to Beta lactam antibiotic exposure. The induction mechanism is very complex.³⁻⁵ The most common cause of Amp C overexpression in most clinical isolates is due to mutation

in amp D leading to Amp C hyperinducibility or constitutive hyperproduction.

Amp C b-lactamases are clinically significant because they confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., cefoxitin and cefotetan) and monobactams. Amp C b-lactamase activity is not affected by the beta lactamase inhibitor like clavulanic acid.⁶

In Gram-negative bacteria, Amp C b-lactamase can be of two types: chromosomal or plasmid mediated. Chromosomal Amp C genes are expressed constitutively at a low level. Few Enterobacteriaceae like Enterobacter species, Citrobacter spp., and Serratia spp., carry an

* Corresponding author.

E-mail address: dhanashreelmmc@gmail.com (D. P. Inamdar).

inducible Amp C gene.⁷ Plasmid-based Amp C genes are expressed constitutively in most cases. All plasmid-carried Amp C genes have clinical significance as plasmids not only transfer Amp C but also ESBL enzymes in the same plasmid. Although reported with increasing frequency in case isolates, the true rate of occurrence of plasmid mediated Amp C b-lactamases in *Klebsiella pneumoniae*, *E. coli*, and *Proteus mirabilis* remains unknown due to difficulties involved in laboratory testing methods.⁸ Also there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for Amp C detection, whereas criteria for ESBL detection is present. Organisms producing Amp C b-lactamase will typically give ESBL screening test positive but increased sensitivity with clavulanic acid is not seen and Amp C producers are inhibited by cloxacillin and phenyl boronic acid when tested.^{7,9} Testing is not considered as necessary in organisms that produce an inducible chromosomal Amp C β -lactamase; i.e. 100% isolates of *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, but, detection of an Amp C β -lactamase in *Escherichia coli*, *Klebsiella* sp., *Citrobacter koseri* is confirmatory for plasmid-mediated Amp C production as these organisms lack a chromosomal Amp C β -lactamases usually.

Detection of AmpC is important not only to improve the clinical management of patients suffering from infections but it would also provide us with sound epidemiological data. Thus, there is a need to know an appropriate phenotypic method for easy detection of Amp C β -lactamases which can help in patient improvement.

Thus the aim of our study was to detect Amp C production by screening test followed by comparison of different phenotypic confirmatory methods in Gram-negative clinical isolates of Enterobacteriaceae family in a tertiary care hospital.

2. Materials and Methods

Ethical committee clearance was first taken. A Prospective study was done at Mamata Medical college & Hospital, Khammam for period of three months. Isolates were first identified by appropriate biochemical reactions. From a total of 140 Enterobacteriaceae clinical isolates tested phenotypic confirmatory tests were conducted on 80 AmpC b-lactamase as they were positive by Cefoxitin screening. Phenyl boronic acid method (PBA), Cefoxitin Cloxacillin-Double disc synergy test (CC-DDS), TRIS EDTA method, Disc approximation test were done on isolates for Amp C confirmation after screening by cefoxitin disc.

2.1. Amp C Screening method:¹⁰

Cefoxitin disk diffusion method : Cefoxitin 30-ug disc was used. Isolates with zone diameters less than 18 mm were considered AmpC producers. Confirmation by various

phenotypic methods was further done on them.

2.2. Phenotypic AmpC confirmation methods

2.2.1. Phenyl boronic acid method-PBA¹¹ (Inhibitor based method)

120 mg of phenyl boronic acid+ 3ml Dimethyl sulfoxide(DMSO) was added to 3ml of distilled water. Then 20 μ l of this liquid was added on each cefoxitin(CX) disc. Disk containing 30 μ g of CX and another disk containing 30 μ g of CX with BA were placed on the agar at a distance of 30 mm. Inoculated plates were incubated overnight at 37°C. An organism demonstrating a zone difference >5 mm was considered as an AmpC producer

2.2.2. Cefoxitin Cloxacillin-Double disc synergy test(CC-DDS):¹²

This test was based on the inhibitory effect of cloxacillin on AmpC production. The isolates were inoculated on Mueller Hinton agar. Cefoxitin/cloxacillin disks (200 μ g/ 30 μ g) and cefoxitin disk (30 μ g) were used. A difference of 4 mm zone between the two discs was an indication of AmpC production.

2.2.3. Amp C TRIS EDTA disc test:¹³

The test is based on the use of Tris-EDTA to permeabilize a bacterial cell and release b-lactamases into the external environment. AmpC disks were prepared by adding 20 μ l of a 1:1 mixture of saline and Tris-EDTA to sterile filter paper disks and then dried, refrigerated. Prior to use, Amp C disks were rehydrated with 20 ml of saline and several colonies of each test organism were applied to a disk. A 30 μ g cefoxitin disk was placed at the inoculated surface of the Mueller-Hinton agar containing ATCC E coli strain. The inoculated Amp C disk was then placed nearly touching the antibiotic disk with the inoculated disk face coming in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C in ambient air. An indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin was considered as positive.

2.2.4. Disk approximation test (Induction based method):¹⁴

A 0.5 McFarland bacterial suspension was prepared, the surface of the MHA plate was inoculated with suspension. A 30 μ g ceftazidime disk was placed at the center of the plate then 10 μ g imipenem, 30 μ g cefoxitin, and 20/10 μ g amoxicillin/clavulanate disks were placed at a distance of 20 mm from the ceftazidime disk. The plate was inverted and incubated overnight at 37°C. After overnight incubation, if there is any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates (imipenem, cefoxitin and amoxicillin/clavulanate disk) it was considered as a positive result for AmpC

production.

3. Results

Out of 140 isolates tested, 80(57%) were positive (resistant) for screening test by cefoxitin (Table 1)(Figure 1). Out of them 61(76.2%) were *Escherichia coli*, 16 (20.1%) isolates were *Klebsiella pneumoniae* and 3 (3.75%) were *Enterobacter sp* (Table 2). Phenotypic confirmatory methods by Cefoxitin Cloxacillin Double disc Synergy (CC-DDS) test showed zone difference of >4mm in 38(47.5%) isolates (Figure 2), by Disc approximation test 32(40%) were positive for Amp C production(Figure 3), by TRIS EDTA method 32 (40%) showed indentation near EDTA disc (Figure 4) and by Phenylboronic acid method > 5mm zone difference was observed in 34(42.5%) isolates (Figure 5)(Table 3). As per Table 4 *Escherichia coli* was the commonest isolate showing Amp c production by all four methods followed by *Klebsiella pneumoniae* and *Enterobacter spp*.

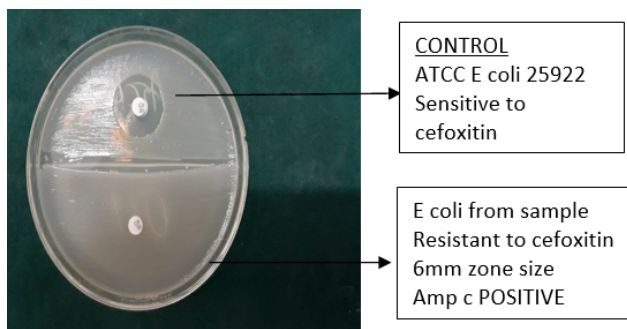


Fig. 1: Cefoxitin screening test

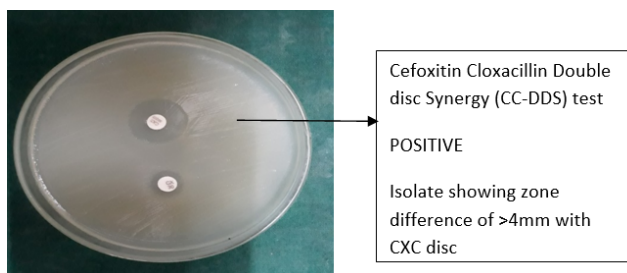


Fig. 2: CC-DDS test

Table 1: Screening test (Total Isolates tested: 140)

	Resistant to Cefoxitin(CX)	Sensitive to Cefoxitin(CX)
N=140	80 (57%)	60 (43%)

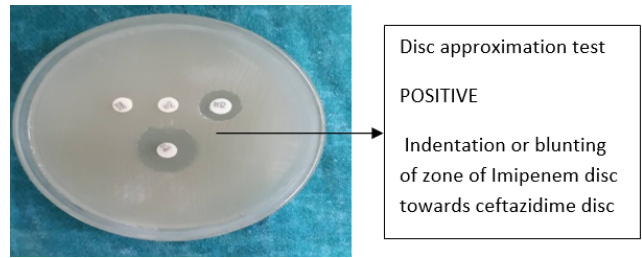


Fig. 3: Disc approximation test

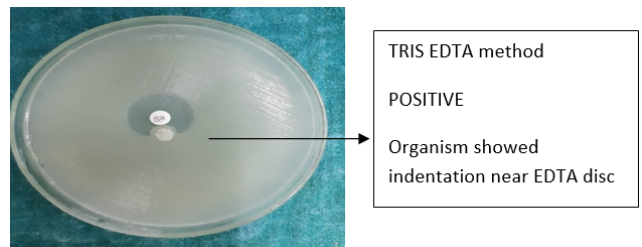


Fig. 4: TRIS-EDTA method

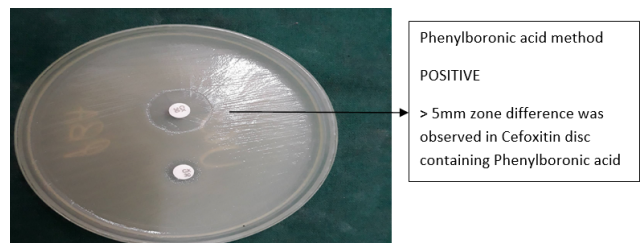


Fig. 5: Phenylboronic acid method

Table 2: Distribution of isolates (Amp c: POSITIVE)

N=80	Isolates
<i>Escherichia coli</i>	61 (76.2%)
<i>Klebsiella pneumoniae</i>	16 (20.1%)
<i>Enterobacter sp</i>	3 (3.75%)

Table 3: Phenotypic methods

Methods	n=80 (Confirmatory tests positive)
CC-DDS (Cefoxitin Cloxacillin -double disc synergy test)	38 (47.5%)
Phenyl boronic acid method	34 (42.5%)
Disc approximation test	32 (40%)
TRIS EDTA method	32 (40%)

Table 4: Distribution of organisms based on various phenotypic methods

	Phenyl boronic acid (n=34)	CC- DDS (n=38)	Disc Approximation test (n=32)	TRIS EDTA method (n=32)
Escherichia coli	24 (70.5%)	24 (63.15%)	20 (62.5%)	23 (71.8%)
Klebsiella pneumoniae	8 (23.5%)	12 (31.5%)	12 (37.5%)	8 (25%)
Enterobacter sp	2 (5.8%)	2 (5.26%)	—	1 (3.2%)

4. Discussion

In our study out of 140 isolates tested, 80(57%) isolates showed resistance to Cefoxitin which is similar to study done by Handa, et al.¹⁵ where they had 59% isolates tested positive by screening. Escherichia coli was the commonest isolate in our study followed by Klebsiella pneumoniae as most of our clinical isolates were from urine sample followed by pus. This study is similar to study done by Polfuss et al.⁸ in 2011 where Escherichia coli was the commonest isolate. Our study is in contrast to study done by Soha et al.¹⁴ in 2015 where Klebsiella pneumoniae were common isolates.

Phenotypic confirmatory tests showed maximum detection by Cefoxitin Cloxacillin Double disc Synergy (CC-DDS) test in 38(47.5%) isolates. In one study done by Polfuss et al.⁸ in 2011, the phenotypic detection and characterization of AmpC beta-lactamases showed high specificity of the cefoxitin-cloxacillin CC-DDS confirmation test, which is similar to present study. A study done in 2013 by Tanushree Baru¹⁶ showed that inhibitor based detection method (Phenyl boronic acid method) was 100% sensitive and 96% specific in detecting AmpC producers hence they recommended this test as it is easier to perform and sensitive. But in our study only 34(42.5%) isolates were positive by Phenylboronic acid method which is in contrast to present study. By TRIS EDTA method 32 (40%) showed indentation near EDTA disc in our study which is in contrast to study done by Ingram et al.¹⁷ in 2011 where they suggested this method for confirmation as it would detect AmpC with a sensitivity of 95% and a specificity of 98%. By all these methods the commonest isolate showing Amp C production was Escherichia coli as it was most common isolate and most of the samples were from urine.

5. Limitations

Only phenotypic methods for AmpC detection could be done in our study as genotypic methods which are considered gold standard were not available in our setup and not cost effective also. Clinical correlation and follow up of patients after Amp C detection was done in some cases, so this objective could not be included in our study.

6. Conclusion

CC-DDS method had better detection rate compared to other phenotypic confirmatory methods. We suggest CC-

DDS method for routine Amp C detection when needed as it is easy to perform.

7. Source of Funding

None.

8. Conflict of Interest

None.

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Author biography

Dhanashree P Inamdar Associate Professor

Anuradha B Professor and HOD

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