

DETECTION OF AMPC BETA LACTAMASES IN CEFOXITIN-RESISTANT GRAM-NEGATIVE CLINICAL ISOLATES USING PHENYLBORONIC ACID

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ABSTRACT

BACKGROUND

AmpC β -lactamases are Ambler class C enzymes that confer resistance to extended spectrum cephalosporins and are not inhibited by β -lactamase inhibitors. These enzyme-producing organisms produce infections that are associated with significant morbidity and mortality. Resistance to third-generation cephalosporins develop in these organisms after exposure to these agents. This complicates treatment options and carbapenems are considered optimal.

MATERIALS AND METHODS

In this cross-sectional study, AmpC β -lactamase production was determined in Gram-negative clinical isolates from various clinical samples. Isolates resistant to cefoxitin and third generation cephalosporin (3GC) antibiotics were tested for the production of AmpC β -lactamases by using an inhibitor-based method (IBM) with phenylboronic acid.

RESULTS

It was observed that, among the 100 Gram-negative isolates, 48 (48%) were resistant to cefoxitin. Using IBM, the occurrence of AmpC β -lactamases was found in 24 (24%) of these 48 isolates. ESBL/AmpC co-carriage was found in 13 (13%) of these isolates by E-Test. Among the 24 AmpC positive isolates, 10 (41.6%) were *E. coli*, 5 (20.8%) were *Enterobacter cloacae*, 5 (20.8%) were *Klebsiella pneumoniae*, 2 (8.3%) were *Acinetobacter baumannii* and 2 (8.3%) were *Pseudomonas aeruginosa*.

CONCLUSION

AmpC production can be determined in routine clinical microbiology laboratory using IBM as it is a simple, rapid and technically easy procedure. Thus, their accurate detection and characterisation plays an important role in their epidemiological survey, infection control and treatment.

KEYWORDS

Gram-negative Bacteria, AmpC β -Lactamase, Phenylboronic Acid, Cefepime.

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BACKGROUND

Production of β -lactamases by Gram-negative bacteria is the predominant mechanism for their resistance to β -lactam antibiotics. ESBLs and AmpC β -lactamases are the most commonly produced.¹ They have become a major cause of hospital-acquired infection, particularly in the intensive care unit (ICU). Such bacteria are associated with severe infections such as bacteraemia, intra-abdominal infection, urinary tract infections and respiratory tract infections. AmpC β -lactamases are mostly chromosomally mediated cephalosporinases produced by Gram-negative bacteria that make them resistant to a wide range of beta lactam drugs thereby leading to serious problem in therapy.

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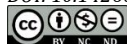
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In the Ambler structural classification of β -lactamases, AmpC enzymes belong to class C, while in the functional classification scheme of Bush et al,² they belong to group 1. Their clinical importance lies in they being resistant to both narrow and broad spectrum cephalosporins, β -lactam/ β -lactamase inhibitor combinations and aztreonam.³ The lack of inhibition by cephamycins and β lactam/ β -lactamase inhibitor combination helps in differentiation between AmpC β -lactamase producer and ESBL producers. They are usually sensitive to the carbapenems and 4th generation cephalosporins.

There are two types of AmpC β -lactamases – chromosomal and plasmid-mediated. They have been found around the world in nosocomial and non-nosocomial isolates. AmpC production in Gram-negative bacilli is normally repressed. AmpC β -lactamase production returns to low levels after antibiotic exposure is discontinued, unless spontaneous mutations occur in the AmpD locus of the gene, leading to permanent hyperproduction (derepression). Chromosomal AmpC enzymes are seen in *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Serratia marcescens* and are inducible by antibiotics like cefoxitin and imipenem. The plasmid mediated AmpC β -lactamases are

derived from chromosomally encoded genes and hydrolyse all β -lactam antibiotics except cefepime and carbapenems.

They are seen in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Unlike chromosome-mediated AmpC, plasmid-encoded AmpC enzymes are almost always expressed constitutively.⁴ Detection of an AmpC β -lactamase in *Klebsiella* spp., *Citrobacter koseri* or *Proteus mirabilis* is confirmatory for plasmid-mediated AmpC production because these organisms lack a chromosomal AmpC β -lactamases.⁵

AmpC enzyme producing Gram-negative bacteria are isolated from hospitalised patients who have been exposed to β -lactam antibiotics. Cefepime is an effective drug in treating infections due to AmpC producers. It is a poor inducer of AmpC, penetrates through the outer cell membrane of bacteria and is little hydrolysed by the enzyme. Detection of AmpC production in Gram-negative clinical isolates helps to improve the clinical management of patients suffering from these infections. At present, there are no CLSI guidelines for detection of AmpC-mediated resistance in Gram-negative bacteria. A problem in therapy can arise due to misleading results, in phenotypic tests. Cefoxitin resistance is suggestive of AmpC production, but it is not specific as resistance to cefoxitin can also be mediated by certain class A β -lactamases, carbapenemases and decreased production of outer membrane porins.⁶ Boronic acid (BA) derivatives have been considered to be reversible inhibitors of AmpC enzymes.⁷ Many studies have validated the use of BA to detect AmpC β -lactamases among Gram-negative bacteria.^{8,9}

Aim of the Study

To detect the production of AmpC enzymes in Gram-negative clinical isolates resistant to cefoxitin and third generation cephalosporin (3GC) by inhibitor-based method using boronic acid.

MATERIALS AND METHODS

Study Setting

The study was conducted in the Department of Microbiology, Government Medical College, Kozhikode, during March 2011-February 2012 for a period of one year.

Study Design

Cross-sectional study.

Study Group

Patients admitted in Govt. Medical College, Kozhikode.

Specimen

Specimens such as pus aspirate, blood, cerebrospinal fluid, sputum, urine, pleural fluid, corneal scrapings and ascitic fluid received in Microbiology laboratory for culture and sensitivity.

Sample Size

A total of 100 randomly chosen non-repetitive isolates obtained from cultures of above specimens.

Inclusion Criteria

1. Specimens from patients admitted in Government Medical College, Kozhikode.
2. Gram-negative bacteria which were resistant to 1st, 2nd and 3rd generation cephalosporins.

Ethical Committee Approval

Ethical clearance to perform the study was obtained from the Institutional Ethical Committee.

Detection of AmpC

Identification of microorganisms was performed according to standard procedures.¹⁰ Antibiotic sensitivity testing was done by Kirby-Bauer's method as per CLSI guidelines (2012). The following antibiotic discs were tested: Ceftazidime (30 μ g), Cefotaxime (30 μ g), Ceftriaxone (30 μ g), Cefepime (30 μ g), Cefoxitin (30 μ g), Ampicillin (10 μ g), Gentamicin (10 μ g), Amikacin (30 μ g), Ciprofloxacin (5 μ g) and Meropenem (10 μ g) [HiMedia laboratories, Mumbai, India]. The isolates found resistant to cefoxitin (zone size ≤ 14 mm) were considered as potential AmpC β -lactamase producers and were further tested with combined disc diffusion test.¹¹

Phenotypic Detection Method for Detection of AmpC Enzymes

Combined disc diffusion test using Phenyl boronic acid (PB): The differences in inhibition zones for cefoxitin (30 μ g) discs alone and in combination with 400 μ g of phenylboronic acid (PB) (120 mg dissolved in 3 mL dimethyl sulfonic acid + 3 mL distilled water) [HiMedia laboratories, Mumbai, India] were determined. An increase of >5 mm in zone diameter in the presence of phenylboronic acid compared with cefoxitin tested alone was considered to be positive for the presence of AmpC β -lactamase.¹¹ (Figure 1).

Control Strains used in all Methods

Negative control ATCC E.coli 25922 and positive control *Klebsiella pneumoniae* 700603.¹²

ESBL Detection was done on the AmpC Positive Isolates by E-test Strips

E-Test for ESBLs: E-test strips (bioMerieux) were applied on to MHA with the MIC scale facing upwards. One end of strip contains a gradient of Ceftazidime (TZ) (MIC test range of 0.5 to 32 μ g/mL) and the other end with a gradient of Ceftazidime plus a constant concentration of clavulanate (TZL) (4 μ g/mL). The presence of ESBL is confirmed by the appearance of a phantom zone or deformation of the TZ ellipse or when either the MIC of TZ is reduced by ≥ 3 log₂ dilutions in the presence of clavulanic acid (Figure 2, 3).

RESULTS

As per the criteria, 100 Gram-negative bacteria which were resistant to cefotaxime, ceftazidime and ceftriaxone obtained from various clinical specimens were tested for the presence of AmpC. E-test for the detection of ESBL was done on AmpC positive isolates. Majority of patients were male. Females constituted 46% and males 54%. Patients in the age group of 1 month to 90 years were included in this study. Most of the cases were in the elderly age group of >50 years (47%).

Twenty five percent belonged to age group of 13-49 years (Table 1).

The antimicrobial susceptibility pattern of 100 Gram-negative isolates is summarised in Table 2. Among the 100 study isolates, 48 (48%) were found to be ceftaxitin resistant (Graph 1). Of the 48 ceftaxitin resistant isolates, 24 (24%) were found as AmpC producers (Graph 1). Among the 24 AmpC positive isolates, majority 8 (33%) were isolated from blood. AmpC-positive organisms were also isolated from pus, urine, sputum, CSF and ascitic fluid (table 3). Among the 24 AmpC-positive isolates, *E. coli* comprises the majority 10 (41.6%). The percentage distribution of AmpC production in different Gram-negative isolates is given in Graph 2.

Of the 24 AmpC positive isolates, 13 (54%) were ESBL positive by E-test. *E. coli*, *Enterobacter spp.* and *Klebsiella spp.* were isolated, of which *E. coli* (54%) were in majority. The remaining 11 were pure AmpC producers (Graph 3). Among the 13 ESBL/AmpC co-carriage isolates, 12 (92.3%) were resistant to ceftaxime. Nine of 11 (81.8%) pure AmpC isolates were also ceftaxime resistant. Among the 24 AmpC positive isolates, only 7 (29%) isolates showed resistance to meropenem.

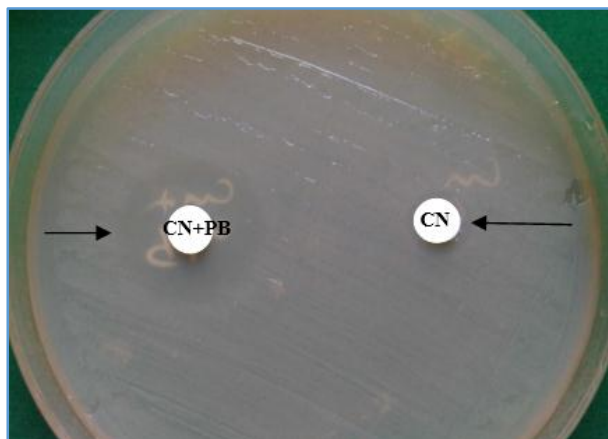


Figure 1. Combined Disc Diffusion for AmpC Detection. Test Organism showing >5 mm Increase in Zone Diameter in the Presence of Phenylboronic Acid (CN-Ceftaxitin: 6 mm, CN+PB-Ceftaxitin+ Phenylboronic Acid: 12 mm).



Figure 2. E-Test for the Detection of ESBL: MIC of TZ is Reduced by ≥ 3 log₂ dilutions in the Presence of Clavulanic Acid (MIC of TZ-Ceftazidime: 32, MIC of TZL Ceftazidime+Clavulanate: 0.25)

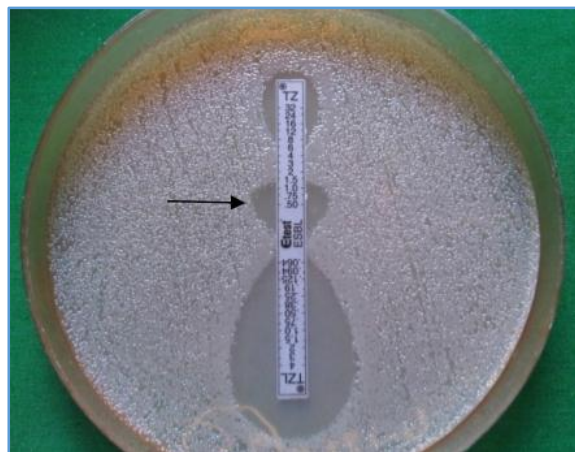


Figure 3. E-Test - Phantom Zone Indicative of ESBL

Age	Number	Percentage
<1 yr.	18	18
1-12 yrs.	10	10
13-49 yrs.	25	25
>50 yrs.	47	47

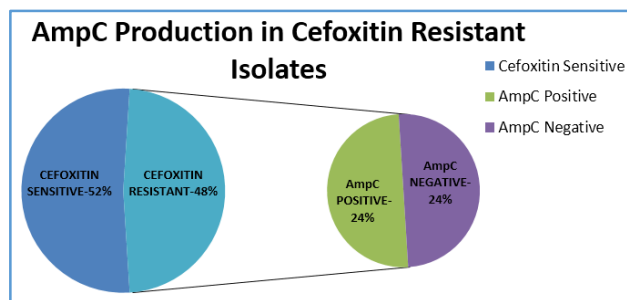
Table 1. Distribution of Cases According to Age

Antimicrobial Agent	Total No. Resistant	Percentage
Ampicillin (10 µg)	100	100
Ceftaxitin (30 µg)	48	48
Ceftriaxone (30 µg)	100	100
Cefotaxime (30 µg)	100	100
Ceftazidime (30 µg)	100	100
Ceftaxime (30 µg)	91	91
Gentamicin (10 µg)	68	68
Amikacin (30 µg)	29	29
Ciprofloxacin (5 µg)	56	56
Meropenem (10 µg)	24	24

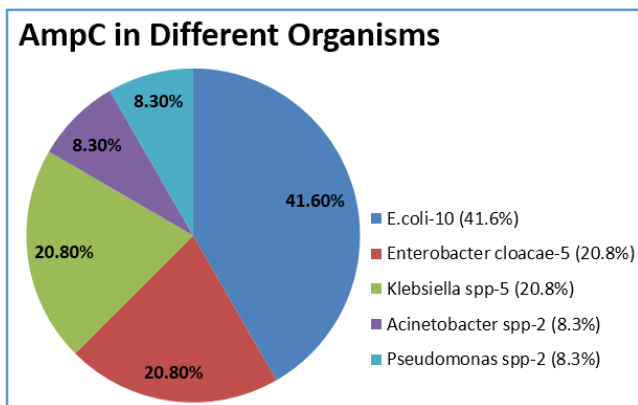
Table 2. Antibiotic Susceptibility Pattern of 100 Gram-negative Isolates

Specimen	Total Number	AmpC Positive (%)
Blood	39	8 (33%)
Pus	16	6 (25%)
Urine	22	4 (16%)
Sputum	15	3 (12%)
CSF	5	2 (8%)
Ascitic fluid	1	1 (4%)
Corneal scraping	1	0
Pleural fluid	1	0
Total	100	24 (24%)

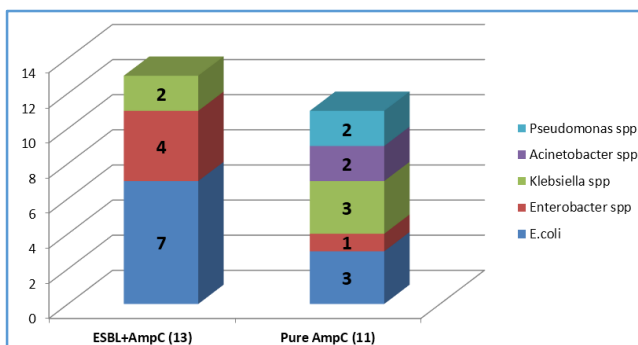
Table 3. AmpC Production in Isolates from Various Clinical Specimens



Graph 1. AmpC Production in Cefoxitin-Resistant Isolates



Graph 2. AmpC Detection in Different Organisms



Graph 3. ESBL Positives in AmpC Producers

DISCUSSION

Cephalosporins are the first-line drugs used in the treatment of infections caused by members of family *Enterobacteriaceae*. Their extensive use has led to increased prevalence of plasmid-mediated AmpC among these organisms. AmpC β -lactamase production is determined by genes commonly found on the chromosomes of members of the family *Enterobacteriaceae* like *Enterobacter spp.*, *Shigella spp.*, *Providencia spp.*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens* and *Escherichia coli*. Plasmid-mediated AmpC β -lactamases are acquired by the transfer of chromosomal genes for the inducible AmpC β -lactamase and are seen in isolates of *E. coli*, *Klebsiella pneumoniae*, *Salmonella spp.*, *Citrobacter freundii*, *Enterobacter aerogenes* and *Proteus mirabilis*.¹³

In the present study, among 100 Gram-negative isolates, 48 (48%) were resistant to cefoxitin by disc diffusion method. Of these 48 Cefoxitin-resistant isolates, 24 (24%) were AmpC producers by combined disc diffusion test using phenyl boronic acid as inhibitor (IBM). Cefoxitin resistance in the remaining 24 isolates that did not show any enhancement with the addition of BA may be due to mechanisms other than AmpC such as porin channel mutation.¹⁴ The Cefoxitin-BA method cannot differentiate between plasmid-mediated AmpC, iAmpC or derepressed AmpC enzymes.

The prevalence of plasmid-mediated AmpC varies widely in different parts of the world ranging from 2% to 46%.⁶ In India, the prevalence ranges from 8% to 47%.¹⁵ In the present study, the occurrence of AmpC β -lactamases was found in 24% of the isolates. Other studies have shown AmpC occurrence varying from 14.8% - 52.1%.¹⁶

In the present study, among the 24 AmpC positive isolates, ESBL and AmpC co-existed in 13 (13%) isolates. The remaining 11 were pure AmpC producers. Similar results

(19% ESBL AmpC co-carriage) were obtained by Vijay Shivanna and Achut Rao.¹⁶ ESBL detection was done by an inhibitor-based method (Ceftazidime and Ceftazidime+Clavulanate) using E-test in our study. The inhibitor-based confirmatory tests are most appropriate for isolates not co-producing an inhibitor-resistant β -lactamase like AmpC, since high level production of AmpC may prevent the detection of ESBL. Moreover, in these organisms, clavulanic acid may act as an inducer of high level AmpC production resulting in false negative result in ESBL confirmatory test. Tazobactam and sulbactam are much less likely to induce AmpC β -lactamases and are therefore, preferable inhibitors for ESBL detection tests in AmpC co-producers. Another approach is to include cefepime as an indicator drug. High level AmpC production has a minimal effect on the activity of cefepime, making this drug a more reliable detection agent for ESBLs in the presence of AmpC.¹⁷

In this study, of the 24 AmpC positive isolates, 10 (41.6%) were *E. coli*, 5 (20.8%) were *Enterobacter cloacae*, 5 (20.8%) were *Klebsiella pneumoniae*, 2 (8.3%) were *Acinetobacter baumannii* and 2 (8.3%) were *Pseudomonas aeruginosa*. Although reported with increasing frequency, the true rate of occurrence of AmpC β -lactamases in different organisms including members of Enterobacteriaceae remains unknown. In India, AmpC prevalence has been reported in *Klebsiella spp.* (24.1%) and *E. coli* (37.5%). In a study by Hemalatha et al, they have reported 8% detection of AmpC β -lactamases production in *E. coli* and *Klebsiella spp.* by inhibitor-based method.¹⁵

AmpC enzymes primarily confer resistance to penicillins, oxyimino- β -lactams and cephamycins with reduced affinity for cefepime and ceftiofime.¹⁸ Contrary to the above statement, in this study, a higher rate of cefepime resistance in both AmpC isolates and ESBL/AmpC co-carriage isolates were observed. Among the 11 pure AmpC producers, 9 (81.8%) were resistant to cefepime. In ESBL/AmpC co-carriage isolates, 12 of 13 (92.3%) were also cefepime resistant. Cefepime resistance has been reported in a study by Jose M. Rodríguez-Martínez¹⁹ in a clinical isolate of *Enterobacter aerogenes*. In recent years, rare AmpC-type β -lactamases that confer resistance to cefepime and ceftiofime have been recovered from *E. coli*, *Serratia marcescens* and *Enterobacter cloacae*.^{20,21,22}

CONCLUSION

The boronic acid disk test is a practical and efficient method based on the current CLSI methodology to detect plasmid-mediated AmpC β -lactamases in organisms that usually do not harbour genes for these enzymes. Studies have shown that patients with bloodstream infections with these organisms and treated with expanded-spectrum cephalosporins had poor clinical outcome. AmpC β -lactamase production is frequently accompanied by multidrug resistance. Meropenem is superior to other antibiotics for the treatment of serious infections due to AmpC β -lactamase-producing Gram-negative bacteria.

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