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COMPARISON OF PHENOTYPIC METHODS TO STUDY THE PREVALENCE OF AmpC β -LACTAMASES

Vandana Upadhyay, Deepika Verma, Atul Garg, ME Siddique, GC Upadhyay
Department of Microbiology, Rohilkhand Medical College and Hospital, Bareilly, India

Abstract

Introduction: Amp C β -lactamases are often misidentified as extended spectrum β -lactamases (ESBL's). With the wide occurrence of AmpC strains all over the world it is important to know their prevalence and formulate guidelines to detect them.

Aim: To study the prevalence of AmpC β -lactamases in isolates of family Enterobacteriaceae and compare methods of detection in a tertiary care hospital in Lucknow.

Material and Method: A total of 200 Gram negative isolates were screened by Modified Double Disc Approximation Method (MDDM). Those isolates which gave a positive screening test were then subjected to modified Three Dimensional Enzyme Extract Test (TDET) and AmpC Disk Test (ADT) to confirm the presence of AmpC β -lactamase enzymes.

Results: 50 isolates were screened positive and hence suspected to be AmpC producers. These were then subjected to modified TDET and ADT to confirm the presence of AmpC enzyme. By TDET, 12 isolates were tested positive. By ADT, 14 isolates gave positive result.

Conclusions: Amp C β -lactamase is prevalent in our setting. The two confirmatory tests carried out to detect the presence of enzyme AmpC β -lactamase ie. TDET and ADT gave concordant results. However, the AmpC disk test is found to be a better option to be done routinely in laboratories as it more sensitive and easy to carry out.

Key words: AmpC β -lactamases, MDDM, TDET, ADT

Author for correspondence: Dr. Vandana Upadhyay, Department of Microbiology, Rohilkhand Medical College and Hospital, Bareilly, India E mail: dr.vandana027@gmail.com

Introduction:

Decades after their discovery, β -lactams continue to remain key components of modern antimicrobial armamentarium for the treatment of infectious diseases. Nevertheless, resistance to these antibiotics is increasing alarmingly all over the world.¹ The most important mechanism of microbial resistance to β -lactam antibiotics (penicillins, cephalosporins, monobactams, and

carbapenems) is hydrolysis of their β -lactam ring by enzyme β -lactamase. Genes coding for β lactamase enzymes mutate continuously in response to the heavy pressure of antibiotic, leading to emergence of newer β -lactamases having a broad spectrum of activity.² Beginning about 20 years ago, new agents were introduced (cephamycins, cephalosporins with an oxyimino side chain, carbapenems, and the monobactam aztreonam) to overcome the early

resistance of the then common β -lactamases. Bacteria responded with a plethora of “new” β -lactamases — including extended- spectrum β -lactamases (ESBLs), plasmid-mediated AmpC enzymes and carbapenem hydrolyzing β -lactamases (carbapenemases) — that, with variable success, could confer resistance to the latest β -lactam antibiotics.³

AmpC β -lactamases belong to the “new” plethora of β -lactamases classified under Class C of Ambler i.e. molecular classification and Class 1 of the Bush, Jacoby and Medeiros i.e. functional classification systems.⁴ Besides being active on penicillins they are even more active on cephalosporins and can hydrolyze cephamycins such as cefoxitin and cefotetan; oxyiminocephalosporins such as ceftazidime, cefotaxime, and ceftriaxone; and monobactams such as aztreonam.⁵

AmpC β -lactamases are chromosomally or plasmid mediated. They have been described in pathogens eg. *Klebsiella pneumonia*, *Escherichia coli*, *Salmonella spp.*, *Proteus mirabilis*, *Citrobacter freundii*, *Acinetobacter*, *Enterobacter spp.* and *Pseudomonas aeruginosa*.⁶

Therapeutic and infection control considerations argue that the recognition of plasmid-mediated AmpC enzymes is necessary even for an average laboratory. Although AmpC β -lactamase producing isolates may appear to be susceptible in vitro to some cephalosporins and aztreonam yet fail to respond if those agents in clinical practice.⁷ They also have the potential for developing resistance to carbapenems. Furthermore, plasmid mediation of AmpC carries the threat of spread to other organisms within a hospital or geographic region.³

With the wide occurrence of AmpC strains all over the world it necessary to know their prevalence in a hospital so as to formulate an empirical antibiotic policy in any hospital.

Material and methods:

Gram negative isolates obtained from different clinical specimens (urine, pus, wound swab, catheter tips, blood, sputum, throat swab, cerebrospinal fluid, high vaginal swab and other body fluids) were collected over a period of one year. These were then characterized by standard biochemical tests : Indole, Methyl red (MR), Voges Proskeur (VP), Citrate, Urease, Triple sugar iron (TSI). A total of 200 non-repeat Gram-negative organisms isolated from patients in and around Lucknow were included in the study after approval from the ethical committee.

Modified Double Disc Approximation Method (MDDM)

Organisms once characterized were then screened for AmpC β -lactamase presence by this method. A 0.5 McFarland suspension of the test organism was swabbed on a Mueller Hinton Agar (MHA) plate. A disc of cefotaxime (30 μ g) and ceftazidime (30 μ g) were placed adjacent to cefoxitin (30 μ g) disc at a distance of 20 mm (centre to centre) from each other. The plate was incubated at 37°C overnight.

Blunting of zone of inhibition between cefotaxime or ceftazidime and cefoxitin disc or reduced susceptibility to ceftazidime or cefotaxime and cefoxitin suggested production of AmpC β -lactamase.

Modified Three Dimensional Test

Fresh overnight bacterial growths were taken from subculture plates (Mueller Hinton Agar) and a growth of 10-15 mg of bacterial wet weight was suspended in 1 mL peptone water in sterile microcentrifuge tubes. The bacterial mass was then pelleted by centrifugation at 3000 rpm for 15 minutes. Crude enzyme extract was prepared by repeated freezing (-200°C) and thawing (37°C) cycles of bacterial pellet (approx 10 cycles).

Lawn culture of standard *Escherichia coli* ATCC 25922 strain was made on MHA plates and a cefoxitin disc (30 μ g) placed in the centre. Linear slits were cut using sterile surgical blade, 3mm away from the cefoxitin disc. At the other end of the slit a small circular well were made such that ~30-40 μ L of enzyme extract could be loaded. The plates were kept upright for 5–10 min until the solution dried and then incubated at 37°C overnight. Indentation of growth of the sensitive *E. coli* strain inwards along the linear slit was regarded as a positive test.

Amp C Disc Test/ Double disc test

This method was performed by using EDTA discs. Plain 6 mm discs were made from Whatmann No.3 paper and sterilized in hot air oven. These discs were then impregnated with 20 μ L of a 1:1 mixture of saline and 100x Tris EDTA (1.0 M Tris-HCl, pH 8.0, containing 0.1 M EDTA, and filter sterilized). The discs were stored at 2 – 8°C.

To perform the test lawn culture of *E. coli* ATCC 25922 was inoculated on MHA plates and cefoxitin (30 μ g) discs placed on it. Sterile Triss EDTA discs were moistened with sterile saline and inoculated with several colonies of test organism and placed just adjacent to the cefoxitin disc such that the inoculated side was in contact with the agar surface. The plates were incubated overnight at 35°C. An indentation or a flattening of the zone of inhibition of cefoxitin disc in vicinity of test disc was regarded as positive. A negative test had an undistorted zone.

Quality Control

Standard strain of *Escherichia coli* (ATCC 25922) was used. It was tested each time before the commencement of tests using various antibiotic discs on Muller Hinton agar.

Results:

The 200 Gram negative isolates included 99 of *E.coli*, 48 of *Klebsiella spp*, 15 of *Acinetobacter spp.*, 5 of *Citrobacter spp.*, 3 of *Pr.mirabilis*, 1 of *Enterobacter spp* and 29 of the isolates were of *Pseudomonas spp.*(as shown in Table 1)

Table 1: Distribution of isolates

| Samples | <i>E.coli</i> | <i>Klebsiella spp</i> | <i>Acinetobacter spp</i> | <i>Citrobacter spp</i> | <i>Proteus spp.</i> | Enterobacter spp | <i>Pseudomonas spp</i> | Total |
|-------------------|---------------|-----------------------|--------------------------|------------------------|---------------------|------------------|------------------------|------------|
| Urine | 81 | 16 | 3 | 3 | 2 | - | 4 | 109 |
| Pus | 8 | 12 | 5 | 1 | 1 | - | 9 | 36 |
| Wound swab | 3 | 5 | 1 | - | - | 1 | 4 | 14 |
| Catheter tip | 1 | 1 | 1 | - | - | - | 2 | 5 |
| Blood | 1 | 2 | 2 | 1 | - | - | 1 | 7 |
| Sputum | 1 | 2 | 1 | - | - | - | 6 | 10 |
| Throat swab | 0 | 4 | - | - | - | - | 2 | 6 |
| HVS | 2 | - | - | - | - | - | - | 2 |
| Other body fluids | 2 | 6 | 2 | - | - | - | 1 | 11 |
| Total | 99 | 48 | 15 | 5 | 3 | 1 | 29 | 200 |

These isolates were subjected to MDDM, 50 isolates gave positive result. These included –14/99 *E.coli* isolates, 8/48 *Klebsiella* isolates, 9/15 *Acinetobacter* isolates, 2/5 *Citrobacter* isolates, 1/3 *Proteus* isolates and 16/29 *Pseudomonas* isolates screened positive and hence were suspected to AmpC producers. However, the *Enterobacter* isolate gave negative result. These were suspected to AmpC producers and were subjected to modified TDET and ADT to confirm the presence of AmpC enzyme. By TDET, Amp C β -lactamase production was confirmed in 2 *E.coli* isolates, 2 *Klebsiella* isolates, 5 *Acinetobacter* isolates, 1 *Citrobacter* isolate and 2 *Pseudomonas* isolates. By ADT, 2 *E.coli*, 3 *Klebsiella spp*, 5 *Acinetobacter spp.* and 3 *Pseudomonas spp.* were confirmed to be AmpC producers. (as shown in Table 2)

Table 2: Results of phenotypic tests conducted

| ORGANISMS | Total isolates subjected to confirmatory test | No. of isolates giving positive mod. 3D enzyme extract test | No. of isolates giving positive ADT |
|--------------------------|---|---|-------------------------------------|
| <i>E.coli</i> | 14 | 2 | 2 |
| <i>Klebsiella spp</i> | 8 | 2 | 3 |
| <i>Acinetobacter spp</i> | 9 | 5 | 5 |
| <i>Citrobacter spp</i> | 2 | 1 | 1 |
| <i>Proteus spp.</i> | 1 | - | - |
| <i>Enterobacter spp</i> | - | - | - |
| <i>Pseudomonas spp</i> | 16 | 2 | 3 |
| TOTAL | 50 | 12 | 14 |

Discussion:

AmpC β -lactamase is often misidentified as ESBL. Detecting AmpC producing isolates is clinically important, not only because of their broader cephalosporin resistance, but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced porin expression.^{8,9} Organisms which express AmpC β -lactamases are a major clinical concern because these are usually resistant to all beta lactam drugs, except for cefepime, ceftiprome and carbapenems.^{10,11}

In contrast to ESBLs, they hydrolyse cephamycins and are not inhibited by β -lactamase inhibitors.¹² The importance of detecting AmpC producing isolates is highlighted by data showing high clinical failure rates when AmpC producing strains of *K. pneumoniae* are treated with cephalosporin agents or the subsequent development of antibiotic resistance in such strains.^{13,14} Detection of any type of AmpC β -lactamase is a challenge to clinical microbiologists. This is because presently there are no guidelines given by CLSI for detection of this

resistance mechanism and yet, there is as much need for clinical laboratories to address this issue as there is for the detection of ESBLs.¹²

The accurate detection of plasmid mediated AmpC is important to improve the clinical management of infection and to provide sound epidemiological data. There is a paucity of data from Indian laboratories on the coexistence of multiple β -lactamases in individual isolates.¹²

In the present study, an assessment was made of the prevalence of AmpC β -lactamase in 200 Gram negative isolates obtained from the various clinical specimens obtained from different clinical settings in our hospital. Screening was done by MDDM. By this method 25% of isolates gave positive result. These were then further subjected to modified TDET and ADT. Prevalence of AmpC enzyme was found to be 11%. Maximum incidence of AmpC production was found in *E.coli* isolates with 36.36% followed by *Pseudomonas spp.* with 27.27%, *Klebsiella spp.* with 22.72%, *Acinetobacter spp* with 9.09% and lastly by *Citrobacter spp* with 4.54%.

A study reported AmpC prevalence in 11% of isolates of *K.pneumoniae*¹⁵. Another study employing an inhibitor based test to detect AmpC β -lactamases in *E. coli* and *K.pneumoniae* reported AmpC β -lactamase production in 9.2% isolates.¹⁶ A similar study reported 21.9% of isolates to harbor pAmpCs using boronic acid (BA) method.¹⁷ However as stated by author the higher percentage of pAmpC in their study was because they had used highly sensitive methods using boronic acid (BA) which gave false positive results in 15% of clinical isolates.

A study detected AmpC enzymes in 8.5% *E. coli* and 5.6% of *K. pneumoniae* isolates respectively using disk based method and agar dilution method.¹⁸ In a recent study, the occurrence of AmpC β -lactamases was found in 10.5% of Gram negative isolates.¹⁹ In another study, the

prevalence of plasmid-mediated AmpC β -lactamases in *E. coli* and *K. pneumoniae* from a tertiary care in Bangalore reported the occurrence of AmpC to be 7.7%.²⁰

The findings of our study suggest that AmpC β -lactamase enzyme is prevalent in our setting. The organism most commonly associated with the enzyme production is *K.pneumoniae*. Majority of the isolates have been obtained from the specimen of patients admitted in the hospital indicating that there is association between the occurrence of enzyme and patient admission to the hospital. The two confirmatory tests which carried out to detect the presence of enzyme AmpC β -lactamase i.e. modified TDET and the ADT gave concordant results. In comparison to the modified TDET, the ADT was found to be much easier to perform. So, the AmpC disk test is a better option to be done routinely in laboratories.

Conclusion:

The occurrence of β -lactamases in institutions can be curbed by determining whether there is a high rate of cephalosporin use, especially third generation cephalosporins. To keep a check on the usage of antibiotics an antibiotic policy should be framed in every institution. There is a need to form an infection control committee and to conduct educational programs for medical staff to increase awareness.

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