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COMPARISON OF PHENOTYPIC METHODS FOR AMPC DETECTION IN GRAM NEGATIVE ISOLATES FROM A TERTIARY HEALTH CARE HOSPITAL

Microbiology

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ABSTRACT

The purpose of the study was to compare different phenotypic methods;M3DT,AmpC disc test with and without EDTA, boronic acid inhibitor test for AmpC detection and screen for ESBL and AmpC production among gram negative isolates from a tertiary health care teaching hospital. Out of 149 cefoxitin resistant isolates, boronic acid method detected 53.02% of AmpC producers. While ESBL was detected in 46.97% of isolates, coproduction of ESBL and AmpC was seen in 30%. Boronic acid inhibitor method was found to be the most sensitive, simple and easy method to perform on a routine basis in the laboratory.

KEYWORDS

AmpC betalactamases, Boronic acid, ESBL

Introduction

The increasing prevalence of gram negative bacterial infections pose a therapeutic problem not only in the hosital setting but also in the community due to the acquisition of resistance to multiple antibiotics.ESBL production, AmpC production, efflux mechanism and porin deficiency include the different mechanisms of drug resistance in gram negative bacteria. Among them, ESBLs and AmpC betalactamases are more common. AmpC betalactamases , chromosomally or plasmid mediated have been described in Enterobacteriaceae and non fermenters.

These enzymes confer resistance to penicillins, first, third generation cephalosporins, cephamycins and monobactams such as aztreonam(1-2). These enzymes are poorly inhibited by the commercially available β-lactamase inhibitors such as clavulanic acid, sulbactam etc. but are inhibited by cloxacillin and phenylboronic acid (3-4).There are no recommended guidelines for detection of this resistance mechanism by CLSI. Moreover detection of AmpC is equally important as ESBLs since both may coexist and mask each other. Even though PCR is a gold standard method to detect AmpC beta lactamase genes many clinical laboratories show interests in performing phenotypic tests as these are cost effective. Therefore, it is important to come up with a practical and simple method to detect this type of resistance.The aim of the study was to detect the presence of AmpC betalactamases by different methods and to evaluate and compare boronic acid inhibitor test (5) with other phenotypic tests like M3DT (6), AmpC disk test with and without EDTA(7) , and detect the occurrence of both AmpC and ESBLs in clinical isolates of gram negative bacteria.

Materials and methods

The study was conducted in the clinical Microbiology laboratory of a tertiary health care referral centre for a period of six months.A total of 149 gram negative isolates showing resistance to one or more extended spectrum cephalosporins but sensitive to carbapenems were isolated from different clinical non repetative specimens like urine, blood, tracheal aspirate, pus and csf .After identifying the organisms using standard biochemical tests, antibiotic susceptibility testing was performed by kirby bauer method on MHA as per CLSI protocols. Isolates which showed cefoxitin zone diameter <18mm were considered to be screen positives for AmpC betalactamase production (8) and were then subjected to following phenotypic tests:

Modified three dimensional test (M3DT)

An overnight growth on Mueller Hinton Agar (MHA) plate was transferred to a preweighed sterile micro centrifuge tube to obtain 10-15 mg of bacterial wet weight. The bacterial mass was suspended in peptone water and pelleted by centrifugation at 3000 rpm at 4°C for 15 minutes. Crude enzyme extract was prepared by repeated freezing and thawing of the bacterial pellet. Lawn culture of *E .coli* ATCC 25922 was prepared on MHA plates and cefoxitin disc of 30µg was placed in the centre of the plates. Linear slits of 3cm were cut using a sterile surgical blade, up to 3mm away from cefoxitin disc. At the other end of the slit, a small circular well was made which was loaded with 30-40µl of the enzyme extract. The plates were kept upright for 5-10 minutes until the liquid dried and incubated overnight at 37°C. Extension of growth into the zone of inhibition due to neutralization of cefoxitin by the enzyme at the point where the slit inserted the zone of inhibition of cefoxitin was considered a positive M3D test and interpreted as evidence for the presence of AmpC beta-lactamases.

AmpC Disc Test

A lawn culture of *E.coli* ATCC 25922 was prepared on MHA plate. A sterile disc of 6 mm moistened with 20µl of sterile saline was kept and several colonies of test organism were inoculated on this disc. A cefoxitin disc was placed next to this disc (almost touching) on the inoculated plate. The plates were incubated overnight at 37°C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disc was considered a positive test.

AmpC disc test with Tris-EDTA

The AmpC discs were prepared by applying an aliquot of 20µl of a 1:1 mixture of normal saline and 100x Tris- EDTA to each of sterile filter paper discs, which were allowed to dry. The discs were stored at 2 to 8°C. The surface of a MHA plate was inoculated with a lawn culture of *E.coli* ATCC 25922. A 30µg cefoxitin disc was placed on the inoculated MHA plate. An AmpC disc was rehydrated with 20µl of saline and growth of several colonies of the test organism was applied on the disc using a sterile inoculating loop. The inoculated AmpC disc was placed with its inoculated surface in touch with the agar surface and in close proximity (almost touching) to the cefoxitin disc. The plate was incubated overnight at 37°C. An indentation or a flattening in the zone of inhibition of cefoxitin disc indicated a positive result.

Inhibitor (boronic acid) based detection method

Screened positive isolates were also checked by this method for the presence of AmpC β-lactamases. Boronic acid was prepared by dissolving 120mg of phenyl boronic acid in 3ml DMSO, to which 3ml of sterile distilled water was added. Discs were prepared by dispensing 20µl of the stock solution on discs containing 30 µg of cefotetan. This test was performed by inoculating MHA plate by the standard disc diffusion method , by placing a disc containing 30µg of cefotetan along with a disc containing 30µg of cefotetan and 400µg of boronic acid. The inoculated plates were incubated overnight at 37ºC. Organism showing an increase of 5mm, of zone diameter around the disc containing cefotetan and boronic acid as compared to that of zone diameter around the disc of cefotetan alone was considered as AmpC producer.

Detection of ESBL

Isolates showing reduced susceptibility to third generation cephalosporins were tested for ESBL production by CLSI double disc synergy method(9).

Statistical analysis was done using SPSS version 16.

Results

A total of 149 cefoxitin resistant isolates were included in the study. These isolates with cefoxitin zone diameter <18mm were identified as

presumptive AmpC producers. Among them, E.coli constituted 34.2% (51/149), klebsiella 26.84% (40/149), Acinetobacter 28.85% (43/149), Pseudomonas 4.69% (7/149) and other coliforms forming the rest 5.36% (8/149). (Table1)

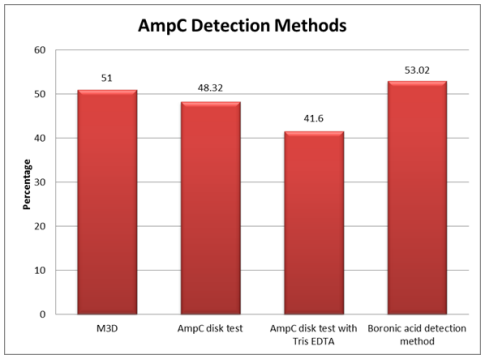
Table 1.Distribution of various gram negative isolates in the study

ISOLATES	NUMBER
Ecoli spp	51 (34.2%)
Klebsiella spp	40 (26.84%)
Acinetobacter spp	43(28.85%)
Pseudomonas spp	7(4.69%)
Other coliforms	8(5.36%)
Total	149

The isolates were obtained from specimens like urine 58(38.93%), pus 37(24.83%), tracheal aspirate 33(22.15%), blood18 (12.08%), and csf 3 (2.01%). All the isolates were recovered from inpatients of our hospital only.

These 149 screen positive isolates were subjected to M3D test for AmpC production.Out of these 76(51%) showed presence of AmpC betalactamase production. The AmpC disk test detected 72(48.32%) isolates as Amp C producers. By boronic acid method AmpC production was observed in 79(53.02%) isolates. By AmpC disk test with EDTA only 62(41.6%) of isolates were found to be AmpC producers (fig1)

Fig 1. Comparison of four phenotypic methods for AmpC detection



Ecoli formed the major group of isolates (34.2%) followed by klebsiella among enterobacteriaceae. AmpC was detected in 43.13% and 37.25% of clinical isolates of E.coli using boronic acid inhibitor method and M3D methods respectively(table 2).Among klebsiella isolates M3D method and inhibitor based test identified 47.5% and 50% of Amp C isolates respectively.

Acinetobacter formed the major isolate among the nonfermenter group (28.85%) followed by pseudomonas (4.69%). Inhibitor based test with boronic acid identified 69.7% and 42.8% of Acinetobacter and pseudomonas respectively as AmpC producers

Table 2 Results of AmpC phenotypic tests among screen positive isolates

Organism	Phenotypic tests			
	M3D	Amp C disk test	AmpC disk test with Tris EDTA	Boronic acid based detection test
E.coli(n=51)	19(37.25%)	19(37.25%)	12(23.5%)	22(43.13%)
Klebsiella sp(n=40)	19(47.5%)	20(50%)	19(47.5%)	20(50%)
Acinetobacter sp(n=43)	29(67.4%)	26(60.4%)	20(46.5%)	30(69.7%)
Pseudomonas sp(n=7)	7(100%)	2(28.5%)	7(100%)	3(42.8%)
Others	2(25%)	5(62.5%)	4(50%)	4(50%)
Total	76(51%)	72(48.32%)	62(41.6%)	79(53.02%)

Prevalence of AmpC production was highest in Acinetobacter spp followed by klebsiella spp and other coliforms (Table 3)

Table 3 Prevalence of AmpC in various isolates

Isolates	Ecoli	Klebsiella	Acinetobacter	Pseudomonas	Other coliforms	Total
Number	51	40	43	7	8	149
AmpC prevalence	22 (43.13%)	20 (50%)	30 (69.7%)	3 (42.8%)	4 (50%)	79 (53.02%)

Prevalence of AmpC was highest in isolates obtained from pus samples (table 4)

Table 4 Prevalence of AmpC in clinical samples

Sample	n	Ampc
Urine	58	32(55.1%)
Blood	18	4 (22.2%)
Tracheal aspirate	33	11(33.3%)
Pus	37	30(81.08)
Csf	3	2(66.6%)

By CLSI phenotypic test ESBL was detected in 70(46.97%) of isolates. Coproduction of both ESBL and AmpC were seen in 45(30%) of study isolates (table 5). The study isolates were found to be 100% sensitive to meropenem (table 6)

Table 5 ESBL and AmpC production in the isolates

Isolates	Number	ESBL +VE	ESBL and Amp C +VE
E.coli	51(34.2%)	23(15.4%)	17(11.4%)
Klebsiella	40(26.84%)	19(12.75%)	10 (6.7%)
Acinetobacter	43(28.85%)	19(12.75%)	13(8.7%)
Pseudomonas	7(4.69%)	4(2.68%)	2(1.34%)
Others	8(5.36%)	5(3.3%)	3(2%)
Total	149	70(46.97%)	45(30%)

Table 6 Overall resistance pattern in study isolates

Antibiotics tested	Isolates with resistance(n)	Percentage of resistance(%)
Amikacin	37	24.8
Cefaperazone sulbactum	24	16.1
Cefipime	49	32.8
Ceftriaxone	122	81.8
Meropenem	0	0
Ciprofloxacin	80	53.6%
Cotrimoxazole	62	42.9

Discussion

Ampc betalactamases are clinically significant as they confer resistance to cephalosporins in the oxyiminogroup(cefotaxime, ceftriaxone, ceftazidime) and are not affected by available betalactamase inhibitors (clavulanate, sulbactum) (10). Plasmid mediated AmpC betalactamases differ from chromosomal Amp C in being uninducible and are typically associated with broad multidrug resistantance. Therapeutic optons for infections caused by such isolates are limited to cefipime and carbapenems. This emphasizes the need for detecting AmpC betalactamases harbouring isolates to avoid treatment failures.

In the present study, 149 cefoxitin resistant gram negative isolates obtained from urine, blood, sputum,aspirates and csf were selected to detect Amp C production by different phenotypic methods. There is no CLSI recommended test unlike ESBL detection for testing AmpC production. However cefoxitin or cefotetan resistance along with oxyiminobetalactamases resistance identifies the isolate as presumptive AmpC producers. Another study conducted in our institute (11) showed that cefoxitin is a better screening drug than cefotetan. Hence cefoxitin was used for screening presumptive AmpC isolates. . In the present study out of all four phenotypic test, boronic acid inhibitor test was found to be the most sensitive method(53.02%) in detecting AmpC producers and Ampc disk test with tris EDTA, least sensitive (41.6%) method. We found that IBM method was technically less demanding and less time consuming to perform routinely in a diagnostic lab.Data from various studies in India showed that percentage of AmpC producing bacteria detected by boronic acid inhibitor method varied from 36.9% to 47.3% (12,13,14) which is comparable to our results(53.02%). In a study conducted by Coudron et al., boronic acid disk test detected 54 isolates out of 55 Amp C PCR positive isolates.(15)

Screen positive isolates were also tested for ESBL production by CLSI phenotypic confirmatory method. It has also been reported that AmpC when present along with ESBLs can mask the phenotype of the latter. Out of 149 isolates, ESBL production was seen in 46.97% study isolates and 30% of isolates were seen to be positive for both ESBL and AmpC production. This comparitively high occurrence may be attributed to the fact that our hospital is a tertiary referral centre. A similar frequency (29%) of ESBL and AmpC coproduction was reported in North India. Lower prevalence rates(9.77%) were also reported from other parts of the country.

In spite of many phenotypic tests,a reference laboratory for beta-lactamase isoelectric focusing and gene localization by genotype characterization based on multiplex PCR are considered as the gold standard.(16) This will help us to know the actual prevalence of these enzymes and characterize them for epidemiological purpose. Detection of AmpC by PCR usually gives reliable and satisfactory results; however, because of the cost, it is of limited practical use for routine diagnostic microbiology laboratories.

CONCLUSION

Boronic acid inhibitor test was the most sensitive method for AmpC detection in gram negative bacteria. The method is simple to perform, and the materials used are cheap, nontoxic, and easily accessible, making it highly applicable to routine clinical laboratories. Our study showed an increased rate of prevalence of AmpC producers among gram negative bacteria. It therefore, calls for a serious and concerted effort to rationalize the use of extended spectrum cephalosporins in order to contain this trend.

Limitation

Molecular methods were not used to confirm AmpC production in our study.

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