Detection of co-existence of β-lactamases in Gram negative bacteria using disc potentiation tests

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Abstract

Background: The extended spectrum β-lactamases(ESBLs), AmpCβ-lactamases and metalloβ-lactamases (MBLs), have emerged worldwide as a cause of antimicrobial resistance in Gram negative bacteria (GNB). So the present study was conducted to detect all three β-lactamases in GNB using disc potentation tests.

Materials and Methods: A total of 200 Gram negative clinical isolates from a tertiary care centre were identified and antibiotic susceptibility testing was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The screening and phenotypic confirmatory test recommended by CLSI were carried out for ESBL detection. Detection of AmpCβ-lactamase was done by phenyl boronic acid test. MBL were detected using EDTA disc potentation test.

Result: Of a total of 200 Gram negative isolates, pure ESBL were seen in 50(25%) isolates and pure AmpC were seen in 35(17.5%) isolates. ESBL and AmpC co-existed in 38(19%) isolates. AmpC and MBL co-occurred in a single isolate.

Conclusion: we recommend the phenotypic tests using various inhibitors for the detection of β-lactamases.

Keywords: β-lactamases, Extended spectrum β-lactamases (ESBLs), AmpCβ-lactamases, Metalloβ-lactamases (MBLs), Co-existence.

Introduction

The commonest cause of bacterial resistance are β-lactamases, leading to an unexhausted impact on antimicrobial chemotherapy.[1]In Gram negative organisms, β-lactamases namely extended spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo β-lactamases (MBLs) are the major cause of β-lactam resistance.[2] The genes of these enzymes are carried on plasmids, facilitating spread between micro-organisms and often co-expressed in the same isolate.[3] The co-expression of ESBLs and AmpC in a single isolate inactivates β-lactam-β-lactamase inhibitor combinations, while MBLs and AmpC β-lactamases confer carbapenem resistance. Since the susceptibility tests are unreliable, special tests are required to detect the "hidden" resistance mechanisms.[4]

The Clinical and Laboratory Standards (CLSI) has established the ESBL and MBL confirmatory method. But there is no CLSI recommended method to detect AmpC β-lactamases. The phenotypic methods for AmpC detection are labour intensive and lack sensitivity and specificity. Polymerase chain reaction (PCR) has high sensitivity and specificity, but costly and limited to few reference laboratories.[5] Hence the present study was done to detect the different β-lactamases and their co-existence by using different substrates and inhibitors by disc potentiation method in Gram-negative bacteria isolated from various clinical samples.

Materials and Methods

The present study was carried out in the Department of Microbiology for a period of one year from November 2010 to October 2011. A total of 200 consecutive, non-repetitive Gram negative isolates from various clinical samples of a tertiary care hospital were obtained. The institutional ethical committee clearance was obtained to conduct the study. Samples were processed and isolates were identified by standard laboratory methods.[6] Antibiotic susceptibility testing was done according to CLSI guidelines.[7]

Screening and detection of ESBL: All isolates showing reduced susceptibility to ceftazidime (zone diameter of ≤22mm) and cefotaxime (zone diameter of ≤27mm) were selected for ESBL production. The CLSI double-disk diffusion test was performed for the confirmation of ESBL. A 0.5 McFarland of Gram negative isolate was swabbed on Mueller Hinton agar plate and disk of ceftazidime (30µg) and ceftazidime/clavulanic acid (30/10 µg) were placed. After incubation, an enhanced zone of inhibition ≥5mm around ceftazidime/clavunic acid in comparison with ceftazidime disc alone was confirmed as ESBL.[8] *Klebsiellapneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as positive and negative controls respectively.(Fig. 1)
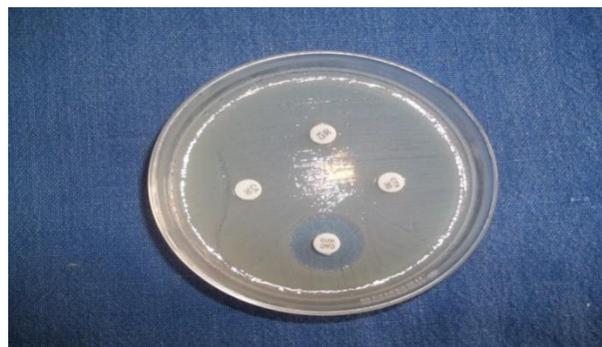

Fig.1: CLSI phenotypic confirmatory test for ESBL

Screening and detection of AmpC β-lactamases: Isolates resistant to cefoxitin (30μg) or showing no increase in zone diameter with addition of inhibitor were suspected to be AmpC producers. These isolates were subjected to phenyl boronic acid (PBA) disc enhancement method. In this method, two cefoxitin discs (30μg) were placed on Mueller Hinton agar plate lawn inoculated with a 0.5 McFarland turbidity adjusted suspension of the test strain. To one of the discs, 400 µg phenyl boronic acid (Sigma-Aldrich) was added. After overnight incubation at 37°C, the zones of inhibition were measured. Enhancement of zone by 5mm around a cefoxitin disc with PBA, in comparison with a disc with cefoxitin alone, was taken as a positive result for AmpC production.[9] (Fig.2)

when the isolate was resistant to imipenem. Screening and confirmation for the detection of MBL was done by disc potentiation test with EDTA- impregnated imipenem discs. Test organism was inoculated onto plates of Mueller-Hinton agar plate (opacity adjusted to 0.5 McFarland opacity standards). A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA IN 1000ml of distilled water and adjusting it to Ph 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10 µg imipenem discs were placed on the plate; 5 µl of EDTA solution was added to one of the disc. The inhibition zones of imipenem-EDTA discs were compared after 16-18 hours incubation at 35°C. An increase in the zone size of atleast 7mm around the imipenem-EDTA disc compared to plain imipenem disc was recorded as an MBL- positive strain.[10] (Fig. 3)

Fig.2: Phenyl boronic acid test for AMP

Fig.3: EDTA disc potentiation test for MBL

Screening and detection of metallo- β-lactamases: MBL-producing Gram negative isolates were suspected

Results

Of a total of 200 Gram negative clinical isolates, 126(63%) were screening positive for ESBL and ESBL was confirmed in 88 (44%) isolates. Among 200 Gram negative isolates, 87(43.5%) were cefoxitin resistant andAmpC β-lactamases were detected in 74 (37%) isolates. Out of 200 organisms 4(2%) isolates were imipenem resistant, but MBL was found in only 1 (0.5%) isolate.ESBL and AmpC co-existed in 38 (19%) isolates. AmpC and MBL co-occurred in a single organismthe rest 74 (37%) organisms did not harbor any enzymes and 2 (1%) isolates had porin channel loss.Table 1 shows the organism-wise distribution of different beta lactamases and their co-production. Table 2 shows the antibiotic resistance patterns of β-lactamase producers and non-β-lactamase producers.

Table 1: Shows the organism-wise distribution of different beta lactamases and their co-production

Organism	Pure ESBL	Pure AmpC	ESBL+AmpC	AmpC+MBL	No mechanism	Total
Escherichia coli	31 (15.5%)	18 (9%)	23 (11.5%)	0	39 (19.5%)	111 (55.5%)
Klebsiellapneumoniae	6 (3%)	4(2%)	7(3.5%)	1(0.5%)	10(5%)	28(14%)
Klebsiellaoxytoca	5(2.5%)	2(1%)	0	0	6(3%)	13(6.5%)
Pseudomonas aeruginosa	2(1%)	2(1%)	3(1.5%)	0	11(5.5%)	18 (9%)
Acinetobacter spp.	1(0.5%)	4(2%)	3(1.5%)	0	1(0.5%)	9(4.5%)
Atypical Escherichia coli	2(1%)	1(0.5%)	0	0	2(1%)	5(2.5%)
Citrobacterspp	0	3(1.5%)	0	0	2(1%)	5(2.5%)

Enterobacter spp.	2(1%)	0	0	0	2(1%)	4(2%)
Shigellaflexneri	1(0.5%)	0	0	0	2(1%)	3(1.5%)
Proteus mirabilis	0	0	1(0.5%)	0	1(0.5%)	2(1%)
Proteus vulgaris	0	0	1(0.5%)	0	0	1(0.5%)
Serratiamarcescens	0	1(0.5%)	0	0	0	1(0.5%)
Total	50(25%)	35(17.5%)	38(19%)	1(0.5%)	76(38%)	200(100%)

Table 2: Shows the antibiotic resistance patterns of β-lactamase producers and non-β-lactamase producers

Antibiotic	β-lactamase producers(n=124)	Non β-lactamase producers (n=74)
Cotrimoxazole	103 (82.93%)	54 (72.97%)
Gentamycin	63 (50.41%)	35 (47.30%)
Amikacin	39 (30.89%)	27 (36.49%)
Netilmycin	34 (27.41%)	12 (16.22%)
Amoxyclav	119 (95.97%)	70 (94.56%)
Cefoperazonesulbactum	87 (70.16%)	48 (64.87%)
Piperacillintazobactum	93 (75%)	41 (55.41%)
Aztreonam	113 (91.13%)	46 (62.16%)
Cefepime	105 (84.68%)	42 (56.76%)
Ciprofloxacin	80 (64.52%)	30 (40.54%)
Ceftazidime	108 (87.1%)	37 (50%)
Ceftazidimeclavulanic acid	59 (47.58%)	40 (54.05%)
Cefotaxime	109 (87.91%)	45 (60.81%)
Cefoxitin	79 (63.71%)	18 (24.32%)
Imipenem	02 (1.61%)	0 (0%)

Discussion

The present study showed a high prevalence rate of beta lactamases among various Gram negative bacteria isolated in our Hospital. The rise in resistance mechanisms pose a serious threat to effective antimicrobial therapy in the near future.[11]This emphasizes the need for the detection of isolates that co-produce β-lactamases and prevent therapeutic failures and nosocomial outbreaks.[12]

In the present study, 50(25%) were pure ESBL producers, 35(17.5%) were pure AmpC producers. Co-existence of ESBL and AmpC were in 38(19%) organisms. MBL and AmpC co-occurred in a single isolate. This was close to the study from Karnataka.[13] Other studies have shown ESBL prevalence rates ranging from 27.33% to 98.51%, AmpC occurrence varying from 14.8% - 52.1% and the presence of multiple enzymes varied from 1.33%-42.75%.[3,5,11,14-17] This difference could be due to the factors like antibiotic usage pattern causing gene mutation leading to β-lactamase production. The additional factor could be variations in the normal flora due to cultural, nutritional and ethnic difference in various populations. Different phenotypic methods in various studies could also be another reason.[18] Even within the same Hospital, the frequency of occurrence of various β-lactamases varied in differents ward and was more in intensive care units (ICUs). The high prevalence of ESBLs and AmpC co-production indicates the inappropriate use of extended spectrum cephalosporins.[13]

Antibiotic resistance was high among β-lactamase producers when compared to β-lactamase non producers. This may be due to plasmids simultaneously carrying β-lactamase enzymes and genes coding for resistance to other antibiotics.[13]Imipenem was the most effective antibiotic, showing a maximum susceptibility of 98%, which is in agreement with earlier studies.[5,14,16]

In the present study, two isolates (*Citrobacter* spp. and *Pseudomonas aeruginosa*) were resistant to all antibiotics and produced no beta lactamases. This may be due to the activity of efflux pump or lack of outer membrane proteins (OMPs).[18]

Various boronic acid derivatives were used to detect AmpC enzymes but 3-aminophenyl boronic acid gave most convincing results.[19] So we adapted the disk potentiation test using phenylboronic acid as it was cheaper and convenient to procure. The cefoxitin-boronic acid method is reliable for AmpC detection but may give false negative results when other mechanisms for cefoxitin resistance are at play.[13]

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. Tazobactum and sulbactum 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.[20] This result has also been observed in the present study, which showed only 5(2.5%) isolates were sensitive to cefepime, out of 35(17.5%) pure AmpC producers. The remaining 30(15%) isolates resistant to cefepime co-produced ESBL, which was missed by the CLSI confirmatory test. So the total ESBL producers were 118(59%), in which 50(25%) isolates were pure ESBL producers, the rest 68(34%) organisms co-produced ESBL and AmpC. This finding reinforces the stability of cefepime in the presence of AmpC enzyme.

The limitation of the study was molecular analysis and characterization of β-lactamases were not done due to financial constrains.

Conclusion

The phenotypic tests using inhibitors to detect β-lactamases were very simple, cost effective, faster and easy to adapt on a routine basis by a clinical microbiology laboratory. The test results were reproducible when carried out in duplicate. Hence we suggest to incorporate the inhibitor based tests in diagnostic laboratoriesto monitor the development of antimicrobial resistance and for implementation of proper hospital infection control measures.

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