IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861. Volume 6, Issue 2 (Mar.- Apr. 2013), PP 74-77
www.iosrjournals.org

Simultaneous detection of Extended-spectrum-β-lactamase, AmpC-β-lactamase and Metallo-β-lactamase in gram negative clinical isolates on a single plate

Rajesh Bareja[1], Shinu Pottathil[2], Rajesh Kumar Shah[3], Prem Singh Grover[4], Varsha Ashok Singh[5]

[1,2,4,5] *(Department of Microbiology, M.M.I.M.S.R, Mullana, Ambala, Haryana, India – 133207)*
[3] *(Department of Microbiology, D.A.V. Dental College, Yamunanagar, Haryana, India – 135001)*

Abstract: *A total of 840 isolates were screened for ESBL, AmpC and MBL by double disk potentiation method. In addition double disk approximation test was also applied for detection of ESBL. The strains, where the synergy was observed between disks of ceftazidime / cefotaxime and clavulanate were considered as ESBL producers. For the detection of AmpC ceftazidime and cefazidime with APB, for the detection of MBL imipenem and imipenem with EDTA disks were used. Isolates showing enlargement of the diameter of growth inhibitory zone ≥ 5mm were considered as AmpC and MBL producers. A total of 259 of isolates were found to be ESBL positive. ESBL was detected in 139 (35%) isolates of Escherichia coli, 79 (28.7%) of Klebsiella species, 25 (22.5%) of Proteus species and 16 (28%) of Citrobactor species. The occurrence of AmpC-β-lactamase was found to be in 129 isolates out of which 73 (18.3%) isolates were Escherichia coli, 37 (13.4%) of Klebsiella species, 10 (9.0%) of Proteus species and 9 (15.7%) of Citrobacter species None of the strain were MBL positive. The results indicate double disk potentiation test to detect ESBL, AmpC and MBL in one plate was easier, reliable and rapid method.*

Keywords: *ESBL; AmpC; MBL; double disk potentiation*

I. Introduction

Extended-spectrum-β-lactamases (ESBL), AmpC-β-lactamases (AmpC) and Metallo-β-lactamases (MBL) enzymes are of increasing clinical concern. ESBLs are most commonly produced by *Escherichia coli* and *Klebsiella spp.* but may also occur in other gram negative bacteria. They are typically plasmid mediated, clavulanate susceptible enzymes that hydrolyze penicillins, extended-spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime and others) and aztreonam [1].

AmpC-β-lactamases are the cephalosporinases that are poorly inhibited by clavulanic acid. They can be differentiated from other ESBLs by their ability to hydrolyze cefamycins as well as othter extended spectrum cephalosporins [1]. AmpC-β-lactamases have been described in pathogens like, *Escherichia coli (E.coli), Klebsiella pneumoniae, Salmonella* species, *Proteus mirabilis, Citrobacter freundii, Acinetobacter* species, *Enterobacter* species, *and Pseudomonas aeruginosa* [1]. Although reported with increasing frequency, the actual rate of occurrence of AmpC-β-lactamases in different organisms, including members of *Enterobacteriaceae*, remains unknown.

An increasing prevalence of carbapenem resistance, mediated by acquired Metallo-β-lactamases (MBLs) is being reported [2]. This has been particularly so in *Pseudomonas aeruginosa* [3]. The resistance may spread rapidly to various species of gram negative bacilli [4,5].

The rapid detection of ESBL, AmpC and MBL positive gram negative bacilli is necessary to aid infection control. A PCR method was simple to use in detecting these enzymes initially but it became more difficult with the increased number of types of these enzymes (ESBL, AmpC and MBL). So in the present study we used a single plate method to detect all the three enzymes simultaneously in gram negative isolates of *Enterobacteriaceae* family (*E.coli, klebsiella, Proteus, Citrobacter*).

II. Material And Methods

Eight hundred and forty gram negative isolates of Enterobacteriaceae family were subjected to study. These were consecutive, nonrepetitive samples from wound, pleural fluid, pus, endotracheal tube, tracheal aspirates, etc. All the isolates were identified by colony morphology and standard biochemical methods [6]. Clinical and Laboratory Standard Institute (CLSI) reference strains, *Klebsiella pneumoniae* ATCC (American Type Culture Collection) 700603, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginsa* ATCC 27853 were also included as control strains.

Screening for ESBL: All the above strains were initially screened for the production of ESBL by the standard technique as recommended by CLSI guidelines [7]. A double disk approximation method was used

(Fig.1). The inoculum was standardized by using 0.5 McFarland's and swabbed on Muller Hinton Agar (MHA) plates. The disks of cefotaxime (30 µg) and amoxicillin / clavulanate (20 µg /10 µg) were placed, 20 mm apart from each other. After overnight incubation, a clear extension of the edge of the cefotaxime inhibition zone towards the disk containing clavulanate was interpreted as synergy, indicating the presence of an ESBL [8].

All ESBL positive isolates thus identified were further subjected to the rapid detection method, which involved the simultaneous detection of three enzymes on a single plate.

Fig. 1; *Double disk approximation method:* screening of ESBL by using disks of cefotaxime and amoxicillin / clavulanate

Rapid detection in single plate: All ESBL producing isolates were studied for the presence of AmpC enzyme and MBL enzyme by double disk potentiation method. A standardized test isolate (0.5 McFarland) was spread on MHA plate with cotton swab [7]. For detection of ESBL, cefoparazone and combination of cefoparazone and sulbactum, for AmpC, ceftazidime (30 µg) and combination of ceftazidime and APB and for MBL, imipenem and combination of imipenem and EDTA (Ethylenediaminetetraacetic acid) disks were used.

Three hundred micrograms of 3-aminophenyleboronic acid (APB) was added to the commercially available disk containing 30 µg of ceftazidime / cefotaxime. 3-aminophenyleboronic acid (APB) was dissolved in DMSO (dimethylsulfoxide) at a concentration of 100 mg/ml and used for test [9].

In the present study EDTA was used with imipenem because it is simple to perform and highly sensitive in differentiating MBL producing isolates [10]. Dissolving 186.1 gram of disodium EDTA.2H2O in 1000 ml of distilled water and adjusting it to pH 8 by using NaOH prepared a 0.5M EDTA solution. The mixture was sterilized by autoclaving. Imipenem (10 µg) disks were placed on the plate and 10 µl EDTA solution was added to each disk. The imipenem-EDTA disks can be stored at -20°C till used within 16 weeks [10].

These disks were placed on the MHA plate in pairs (plain disk plus plain disk with respective additive) with a center to center distance of 30 mm. Incubate the plate in incubator at 37ºC for 16-18 hours. The diameter of the growth inhibitory zone was compared in each three pairs of disks. Isolates showing enlargement of the diameter of growth inhibitory zone ≥ 5 mm in their respective pair considered positive for that enzyme (Fig. 2). Fig. 2 shows the production of ESBL, AmpC enzymes and non-production of MBL enzyme by a strain of *E.coli*.

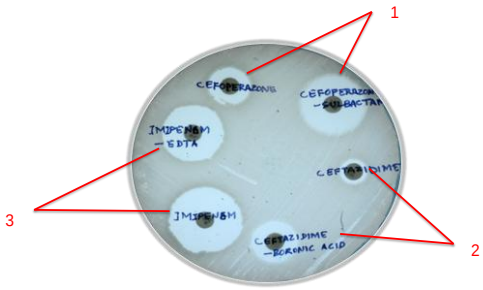

Fig. 2; *Double disk potentiation method:* detection of ESBL by using disks of Cefoperazone and Cefoperazone with Sulbactum (1), AmpC by using disks of Ceftazidime and Ceftazidime with Boronic acid (2) and MBL by using disks of Imipenem and Imipenem with EDTA (3), in a single plate

III. Results

Eight hundred and forty isolates were screened for ESBL, AmpC and MBL enzyme production by double disk potentiation method. Of these, 259 (30.8%) isolates were found to be ESBL positive. Out of total isolates, 35% of *E.coli*, 28.7% of *Klebsiella* species, 22.5% of *Proteus* species and 28% of *citrobacter* species

were found to be ESBL positive. AmpC-β-lactamase production was confirmed in 129 (15.3%) isolates. Out of total isolates 18.3% of *E.coli*, 15.7% of *Citrobacter* species, 13.4% 0f *Klebsiella* species and 9% of *Proteus* species were found to be AmpC positive. None of the strains were MBL positive as shown in Table 1. Fig. 3 shows the ward wise distribution of isolates.

Table 1; prevalence of ESBL and AmpC in patients

Organisms (n=840)	ESBL positive (%)	Amp C positive (%)
Escherichia coli	139 / 397 (35.0%)	73 / 397 (18.3%)
Klebsiella species	79 / 275 (28.7%)	37 / 275 (13.4%)
Proteus species	25 / 111 (22.5%)	10 / 111 (9.0%)
Citrobacter species	16 / 57 (28.0%)	9 / 57 (15.7%)
Total	259	129

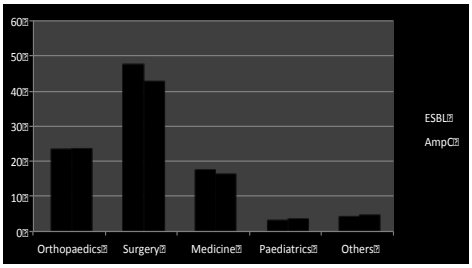

Fig. 3; ward wise distribution of ESBL and Amp C

IV. Discussion

Since the discovery of ESBLs, AmpC-β-lactamases and MBLs, a decade ago, there remains a low level of awareness of their importance. Many clinical laboratories have problem in detecting these enzymes. Confusion exists about the importance of these resistance mechanism, optimal test methods and appropriate reporting conventions. Failure to detect these enzymes has contributed to their uncontrolled spread and some time to therapeutic failure.

The objective of this work was to obtain some experimentally based prediction on the possible emergence of extended spectrum-β-lactamase, AmpC-β-lactamases and MBLs in tertiary care hospital. Detection and reporting of isolates producing AmpC-β-lactamases are more difficult issues than those associated with ESBLs. *E.coli, Klebsiella pneumoniae, Proteus mirabilis* are the species in family *Enterobacteriaceae* most commonly isolated in clinical laboratory [11]. However few studies have assessed the occurrence of AmpC-β-lactamases among these species. The CLSI documents do not indicate the screening and confirmatory test that should be used for detection of AmpC-β-lactamases and MBL in *E.coli* and *Klebsiella pneumoniae*. There is hardly any study involving three parameters in one test.

With the double disk potentiation test, 259 ESBLs producers and 129 AmpC producers were identified. It demonstrated the co-existence phenotype of both ESBL and AmpC in the same isolates. This could be because plasmid mediated AmpC enzymes have also been shown to disseminate among *Enterobacteriaceae*, some time in combination with ESBLs.

In the present study, ESBL and AmpC-β-lactamases producing *E.coli* and *Klebsiella* species were isolated from inpatients units. Surgery and orthopedics patients have the highest burden. It has been reported that at present in India AmpC harbouring isolates are largely restricted to the indoor patients only.

V. Conclusions

The double disk potentiation test to detect all three, ESBL, AmpC-β-lactamase and MBL in one plate was an easier, reliable and rapid method. The present study shows that the single plate method can be used for routine screening of ESBL, AmpC-β-lactamase and MBL enzyme in a clinical laboratory.

Acknowledgement

We are thankful to Dr. Chiranjay Mukhopadhyay, Professor, Department of Microbiology, Kasturba Medical College, Manipal for providing tactical support.

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