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Research Article

PREVALENCE OF VARIOUS β-LACTAMASE (ESBL, AMPC AND MBL) PRODUCING MULTIDRUG RESISTANT CLINICAL ISOLATES OF *ACINETOBACTER* SPP.IN A TERTIARY CARE HOSPITAL.

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ABSTRACT

Background: Extensive use of β- lactam group of antibiotics to treat the gram negative bacterial infections the antibiotic resistance are increasing and *Acinetobacter* species are budding as multidrug resistant (MDR) gram negative bacteria showing various β-lactamases mediated resistance.
Materials and Methods: The presence of β-lactamases in the multi drug resistant clinical isolates of *Acinetobacter* species were screened by β-lactamase assay method. The presence of ESBL was detected by phenotypic confirmatory test recommended by Clinical and Laboratory Standards Institute (CLSI), AmpC was detected by CX/BA inhibitor method and carbapenem (IMP or MER)/EDTA combined disc method and Modified Hodge tests were performed to detect MBL mediated resistance mechanism.
Results: We found high level of beta lactamase mediated resistance mechanism as part of multidrug resistance. Among 180 MDR isolates, 147 isolates were shown positive for beta lactamase assay method and these 147 isolates were further subjected to confirm presence of beta lactamase such as AmpC 105 (71.42%) followed by ESBL 84 (57.14%), MBL 43 (29.25%). Co-existence of ESBL and MBL also identified in 13 (8.84%) isolates.
Conclusion: β-lactamase mediated resistance mechanisms are accounting very high in the multidrug resistant isolates of *Acinetobacter* species. Therefore early detection is necessary to screen and confirm beta lactamase mediated resistant strains to avoid treatment failure and prevent the spread of MDR.

Keywords: *Acinetobacter* species, Multi drug resistance, β-lactamases.

INTRODUCTION

Acinetobacter species are emerging multidrug resistant pathogens among non fermentative gram negative bacilli[1.] *Acinetobacter* species are recognized as multidrug resistant bacteria in the listed six top priority dangerous drug resistant microbes, released by Infectious Disease Society of America[2]. The β- lactam group of antibiotics are widely used to treat the infections caused by the pathogenic bacteria[3-4]. Due to the extensive use of these antibiotics, β- lactamase mediated resistance has raised as major clinical crisis[5]. The newer β-lactamases, including extended-spectrum β-lactamases (ESBLs), ampC β-lactamases (AmpC), and metallo-β-lactamases (MBLs) have emerged Worldwide as a cause of antimicrobial β- lactamase mediated resistance in gram negative bacteria (GNB) [6].

In India there have been few studies of beta lactamase mediated resistance mechanisms especially in *Acinetobacter* spp. Therefore, the objective of this study was to determine the β- lactamase mediated resistance mechanisms in MDR clinical isolates of *Acinetobacter* spp.

Materials and Methods

Bacterial isolates

A total of 180 multi drug resistant isolates of *Acinetobacter* spp. were obtained over a period of 12 months (January2012-December2012) at a tertiary care hospital in South India. All isolates were non duplicate and were identified as *Acinetobacter* species [7]. These organisms were taken as MDR, if it was resistant to more than three of the following eight antimicrobial agents such as ampicillin/sulbactam, aztreonam, ceftazidime, ciprofloxacin, gentamicin, imipenem, piperacillin and trimethoprim/ sulfamethoxazole[8].

β-lactamase assay

The presence of beta lactamase was screened and assayed by performing beta lactamase assay[9-10]. A loopful culture of the test organism was spot inoculated on Mueller Hinton agar containing 1% starch and penicillin (10,000 Units). After incubation of plates at 37^{0}C for 18-24 hrs, the plates were flooded with freshly prepared phosphate buffered saline (PBS) containing potassium iodide, iodine. The appearance of clear colourless zone around the bacterial growth is an indication of beta lactamase production (Fig 1). The beta lactamase converts penicillin to penicilloic acid, which reduces iodine to iodide monitored via decolourisation of the starch iodine complex. All MDR clinical isolates of *Acinetobacter* spp. were tested for the production of beta lactamases.

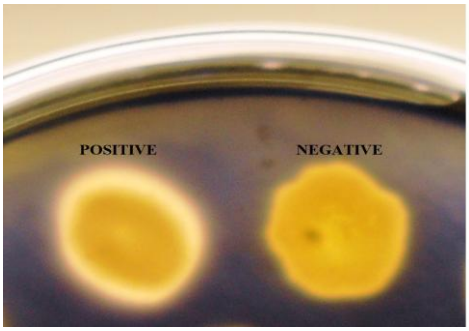


Fig 1: β-lactamase assay

Inhibition zone around bacterial culture (left) indicates positive and no inhibition zone (right) indicates negative.

Confirmatory tests for beta lactamases production

Extended spectrum beta lactamase

The CLSI recommended phenotypic confirmatory method[11] was performed to confirm the presence of ESBL production **(Fig 2).** *K. pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) were used as quality control.

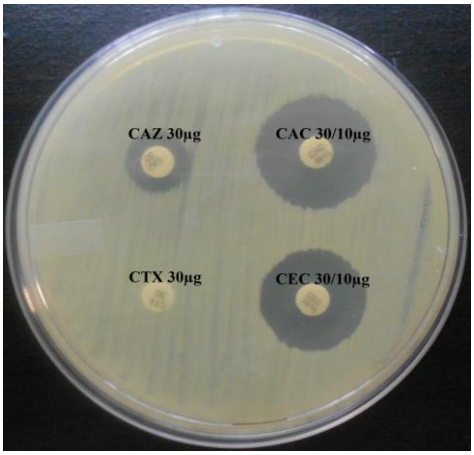


Fig 2: ESBL detection by CLSI method.

CAZ: ceftazidime; CAC: ceftazidime/clavulanic acid; CTX: cefotaxime; CEC: cefotaxime/clavulanic acid.

Moreover, the detection of ESBL in *Acinetobacter* spp. is masked by intrinsic and acquired AmpC[12]. As the boronic acid is an AmpC inhibitor, [13] we added boronic acid to commercially available discs ceftazidime and ceftazidime/clavulanic acid as well as cefotaxime and cefotaxime/clavulanic acid. The test culture (0.5 McFarland suspension) was swabbed on the Muller Hinton agar (Hi-media, Mumbai) plates. The discs were placed at a distance of 20 mm each other, ceftazidime/boronic acid (CAZ/BA), ceftazidime/clavulanic acid/boronic acid (CAC/BA) and cefotaxime/boronic acid (CTX/BA) cefotaxime/clavulanic acid/boronic acid (CEC/BA) and incubated at 37^{0}C for 16-18hrs in ambient air. The results were interpreted as ESBL positive if the inhibition zone is $\geq$ 5mm for CAC/BA then CAZ/BA and $\geq$ 3 mm for CEC/BA then CTX/BA[14].

AmpC detection

The inhibition zone size $\leq$18 mm around the cefoxitin disc was used as a screening test for the presence of AmpC β- lactamase production[15]. The cefoxitin (30µg) and cefoxitin/ boronic acid (30/400µg) was placed on the Muller Hinton agar (Hi-media, Mumbai) containing 0.5 McFarland turbid test culture. After the incubation period of 16-18hours at 37^{0}C, the inhibition zone $\geq$ 5mm of CX/BA than CX alone was considered as AmpC producers (Fig 3)[12]. The boronic acid was prepared by dissolving the 120 mg of phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Bangalore, India) in 3 ml of dimethyl sulfoxide (DMSO) and 3 ml of sterile distilled water was added to this solution. Finally 20 µl of stock solution was dispensed on to cefoxitin disc (30mcg). Discs were allowed to dry for 45-60 minutes and used immediately or stored in airtight vials with desiccant at 4^{0}C[12].

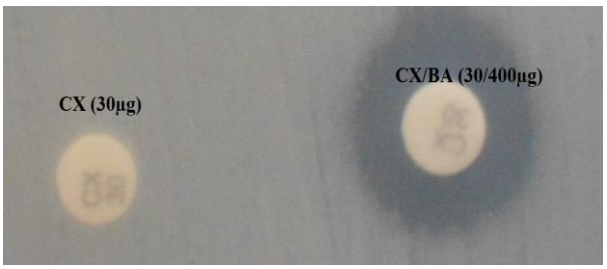


Fig 3: AmpC detection by CX/Boronic acid method

CX: cefoxitin (30 µg); CX/BA: cefoxitin/Boronic acid (30/400 µg)

MBL detection

Double disk diffusion test

The test culture was made on the Muller- Hinton agar (Hi-media, Mumbai) recommended by the CLSI guidelines[11]. Commercially available imipenem (10µg) and imipenem/EDTA (10+750 µg) (Hi-media, Mumbai) discs were placed on the Mueller Hinton agar at a distance of 20mm each other. After the incubation period of 16-18 hours at 37^{o}C, the increase in inhibition zone size ≥ 7mm around the IMP/EDTA disc than the IMP alone was considered as MBL positive (Fig 4)[16]. This test was also performed by using meropenem and EDTA.

A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA · $2H_2O$ in 1,000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving[17] 10 µL of EDTA solution was added to one of them to obtain the desired concentration (750 µg).

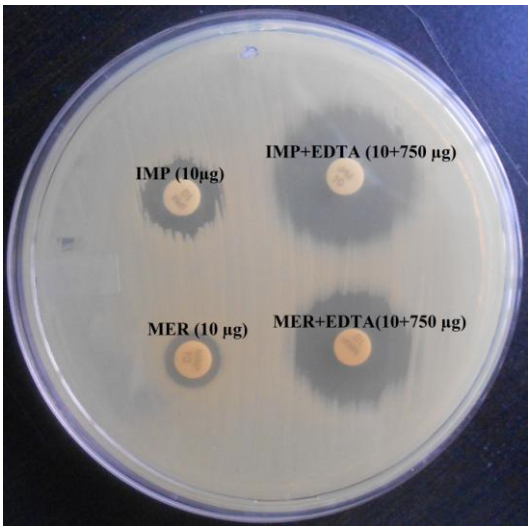
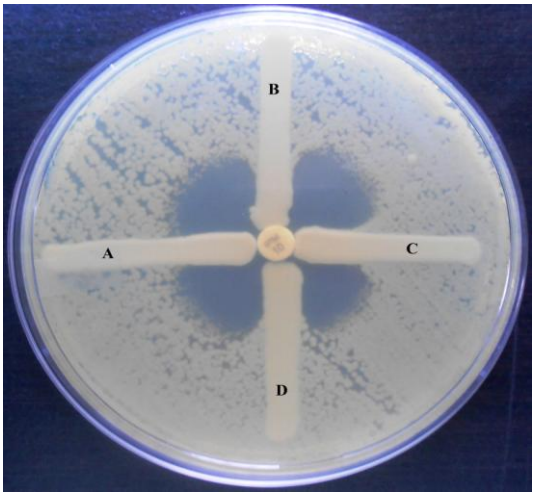


Fig 4: MBL detection by Modified Hodge test.

IMP: Imipenem (10µg); IMP+EDTA: Imipenem+ Ethylenediaminetetraacetic acid (10+750 µg); MER: Meropenem (10µg); MER+EDTA: Imipenem+ Ethylenediaminetetraacetic acid (10+750µg).

Modified Hodge Test

The Modified Hodge test[18] was performed to detect metallo β-lactamases. An overnight culture was *E.coli* ATCC 25922 was adjusted to 0.5 McFarland standard and made on Muller Hinton agar (Hi-media, Mumbai). The 10 µg of imipenem was placed on the centre of the plate and test culture was streaked from the edge of the disc to the periphery of the plate. After the incubation period of 16-18 hours at 37^{0}C, the presence of a cloverleaf zone or distortion of inhibition around the imipenem disc was screened for the presence of the carbapenemases (Fig 5).



MBL detection by Modified Hodge Method

A: Negative; B, C and D: Positive

RESULTS

Among 180 MDR *Acinetobacter* spp. 147 were positive for beta lactamase assay method. Only 147 beta lactamase positive isolates were subjected to confirm beta lactamase mediated resistance mechanisms and we found ESBL 84 (57.14%), AmpC 105 (71.42%) and MBL 43 (29.25%) were confirmed by the phenotypic methods. We found the dual β-lactamase presence such as ESBL plus MBL 13 (8.84%).

DISCUSSION

The prevalence of multidrug resistance strains expressing ESBL, AmpC and MBL are increasing all around the World[19]. Our study also revealed high level of beta lactamase mediated resistance mechanisms in 147 (81.66%) out of 180 MDR *Acinetobacter* spp. The presence of β-lactamase was screened by β-lactamse assay method and this method was simple and easier to perform but it was difficult to distinguish specific type of β-lactamase such as ESBL, AmpC, and MBL. Distinguishing the beta lactamases into ESBL, AmpC and MBL production has epidemiological significance and has therapeutic importance as well. Therefore, we performed further confirmatory tests to distinguish different beta lactamase mediated resistance mechanisms for 147 screened beta lactamase positive isolates by confirmatory tests, the performed phenotypic methods are minimal cost and easier to perform in all diagnostic laboratories.

The ESBLs ability to hydrolyze third-generation cephalosporins and aztreonam and efficiently inhibited by clavulanic acid. We identified 84 (57.14%) of *Acinetobacter* spp. as ESBL producers and our reports are extremely high when compared with previous study by Sinha *et al*.[20] and low with another study by Kansal *et al* [21] in India.

AmpC beta-lactamases are cephalosporinases and can be differentiated from other extended-spectrum β-lactamases by their ability to hydrolyse cephamycins as well as other extended-spectrum cephalosporins that are poorly inhibited by clavulanic acid. They can be differentiated from other ESBLs by their ability to hydrolyze cephamycins (cefoxitin, cefotetan), as well as other extended-spectrum cephalosporins[22]. AmpC- b-lactamases demonstrated or presumed to be chromosomally or plasmid mediated, have been described in various gram negative bacteria including *Acinetobacter* spp[23]. Although the current CLSI guidelines do not describe any method for detection of isolates producing AmpC beta-lactamases, we followed simple and easily applicable CX/BA method to detect this enzyme. Out of 147 isolates, 105 (71.42%) isolates have shown AmpC beta lactamase production and our report is very high when compared with other studies[24-25]. The CX/BA inhibitor method is applicable simultaneously to detect dual/multi beta lactamase mechanisms are present in bacterial isolates in a single petri plate.

The MBLs hydrolyses all beta lactum groups of antibiotics except for aztreonam *in vitro* [26]. The detection of MBL producing *Acinetobacter* spp. is essential to optimal treatment of patients and to control the spread of resistance[27]. We found 18.55% of clinical isolates of *Acinetobacter* spp. were MBL producers and this was low when compared with a study from Karachi, Pakistan[28]. We observed all MBL producing strains were susceptible to potentially toxic antibiotics such as colistin, tegecyline and polymyxin-B similarly with other studies[29-33]. These drugs may act as alternative choice to treat multidrug resistant isolates of *Acinetobacter* spp. infections based on previous reports[29-33].

In Conclusion, MDR isolates of *Acinetobacter* spp. showed very high level of beta lactamase mediated resistance mechanisms as part of multidrug resistance, so necessity is there to find the beta lactamase producers among MDR *Acinetobacter* spp. to avoid treatment failure and spread.

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