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Metallo- β - lactamase producing nonfermentative gram-negative bacteria: An increasing clinical threat among hospitalized patients

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ABSTRACT

Objective: To detect and evaluate the various methods for metallo- β -lactamases (MBL) production in *Pseudomonas aeruginosa (P. aeruginosa)* and *Acinetobacter* species. **Methods:** A total of 109 *P. aeruginosa* and 85 *Acinetobacter* species were screened for imipenem resistance by Kirby- Bauer disc diffusion methods. Detection of MBL production was done by imipenem-EDTA combined disc test, double disc synerygy test (DDST) and imipenem-EDTA MBL E test. **Results:** A total of 63 (57.8%) strains of *P. aeruginosa* and 46 (54.1%) strains of *Acinetobacter* spp. were found to be resistant to imipenem. Of the 63 imipenem resistant *P. aeruginosa* tested for MBL production, 44 (69.8%) were found to be positive and among 46 imipenem resistant *Acinetobacter*, 19 (41.3%) were shown to be the MBL producers. **Conclusions:** Imipenem-EDTA combined disc test and MBL E test are equally effective for MBL detection in both *P. aeruginosa* and *Acinetobacter* spp., but given the cost-constraints, combined disc can be used as a convenient screening method in the clinical microbiology laboratory.

1. Introduction

Infections caused by gram negative bacteria are difficult to treat as the majority of isolates exhibit varying degrees of beta-lactamase mediated resistance to most of the beta-lactam antibiotics. The genes coding for β - lactamase enzymes mutate continuously in response to the heavy pressure of antibiotic use lead to development of newer β - lactamases with broad spectrum of activity[1]. Amongst β -lactamases, the carbapenemases especially metallo- β -lactamases (MBL) are a major cause of concern because of their ability to hydrolyze most beta-lactams including the carbapenems, drugs considered reserve for the treatment of gram negative multidrug resistant strains[2]. The worldwide spread of MBLs represents a great fear nowadays not only due to their ability to confer a high level of resistance but also because their genes carried highly mobile elements that facilitates their spread among different bacterial species and genera[3]. Their ability to inactivate many broad spectrum antimicrobial agents, and increase in their prevalence would drastically compromise the ability to effectively treat hospital or community acquired infections caused predominantly by gram negative bacilli[4]. Moreover, MBLs are not susceptible to therapeutic β - lactamase inhibitors and no new inhibitor of these enzymes is yet in the pipeline, hence their continued spread would be a clinical disaster[5]. This situation prompts an early and accurate detection of MBL producing organisms of crucial importance like non-fermenting pathogens[4]. The present study was undertaken to evaluate the accuracy of various phenotypic tests for detection of MBL-producing isolates among *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter* spp.

2. Materials and methods

A prospective study was conducted to 109 consecutive non-duplicate isolates of *P. aeruginosa* and 85 isolates of *Acinetobacter* spp., which were obtained from patients admitted in various wards and ICUs at a tertiary care hospital over a period of 7 months (July 2009-January 2010). These organisms were isolated from various specimens included sputum, bronchoalveolar lavage, tracheal aspirate, pus, pleural fluid, ascitic fluid and were identified by the standard laboratory techniques[6,7].

Antimicrobial sensitivity testing was performed on Mueller Hinton agar (MHA) by Kirby-Bauer disk diffusion method according to the CLSI recommendations[8]. The following antibiotics were tested: imipenem (10 μg), cefoperazone

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(75 μg), cefoperazone/sulbactam (75 μg/30 μg), ciprofloxacin (5 μg), piperacillin (100 μg), piperacillin/tazobactam (100 μg/10 μg), amikacin (30 μg), and polymixin B (300 units) (BD Diagnostics Pvt. Ltd., India).

Screening for MBL production was performed in all imipenem resistant isolates and randomly selected ten imipenem sensitive isolates by different phenotypic methods.

2.1. Imipenem (IMP)-EDTA combined disc test[9]

A 0.5 McFarland suspension of the test organism was inoculated on a MHA plate as per CLSI guidelines[8]. Two 10 μg imipenem disks were placed on the plate. An appropriate amount of 10 μL of 0.5 M EDTA solution was added to one of the disc to obtain the desired concentration. Plates were incubated at 37 ℃ for 18-24 hours. The zone of inhibition of the imipenem and imipenem-EDTA disks was compared for the detection of MBLs. For *P. aeruginosa*, if the increase in the inhibition zone with the imipenem-EDTA disk was ⩾7 mm than imipenem disk alone it was taken as MBL positive. However for *Acinetobacter* spp. zone of inhibition of imipenem-EDTA disk was measured which was ⩾ 17 mm for MBL positive and ⩽ 14 mm were taken as MBL negative (Figure 1).

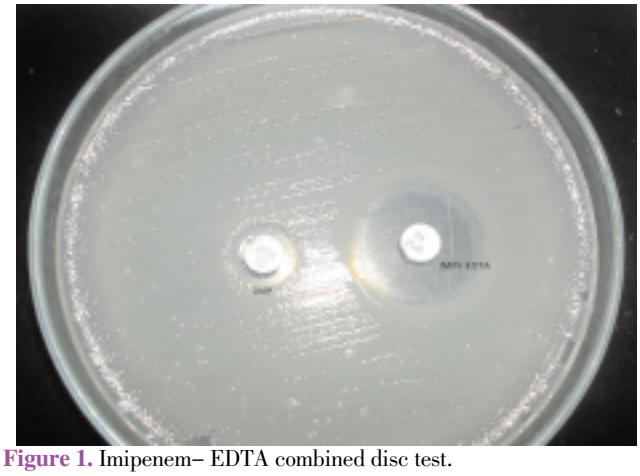

Figure 1. Imipenem- EDTA combined disc test.
Increase in the diameter of zone of inhibition around imipenem+ EDTA disc in comparison to imipenem disc alone indicate MBL positive.

2.2. Double disc synergy tests (DDST)[10]

An overnight liquid culture of the test isolate was adjusted to turbidity 0.5 McFarland standards and inoculated on the surface of MHA as recommended by CLSI[10]. An imipenem disk (10 μg) was aligned around blank filter paper disk at a distance of 20 mm from centre to centre on MHA plate. To the blank disk either 10 μL of 0.5 M EDTA or 3 μL of concentrated 2-mercaptopropionic acid (MPA) solution was added directly. After overnight incubation 37 ℃, an enhancement of the zone of inhibition between imipenem and the inhibitor disk was indicative of MBL production.

2.3. MBL E-test

The E-test MBL strip (AB Biodisk, Solna, Sweden) containing double sided seven dilution range of imipenem (4-256 μg/mL) and imipenem (1 to 64 μg/mL)in combination with a fixed concentration of EDTA. MIC ratio of IP/IPI of ⩾8 or ⩾3 $\log_2$ dilutions indicated MBL production[11]. Appearance of a phantom zone or deformation of the ellipse is also taken as positive for MBL regardless of the IP/IPI ratio (Figure 2).

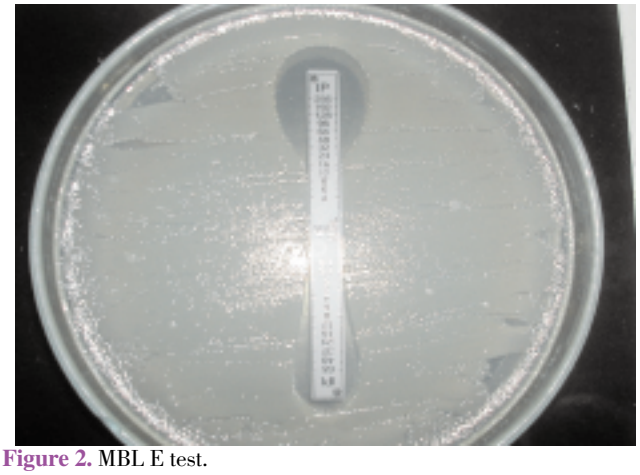

Figure 2. MBL E test.
MIC ratio of IP (imipenem)/IPI (imipenem-EDTA) of >8 or >3 $\log_2$ dilutions indicates MBL positive.

3. Results

A total of 63 (57.8%) strains of *P. aeruginosa* and 46 (54.1%) strains of *Acinetobacter* spp. were found to be resistant to imipenem.

Of the 63 imipenem resistant *P. aeruginosa* tested for MBL production, 44 (69.8%) exhibited a ⩾7 mm zone size enhancement in imipenem-EDTA combined disk test indicated MBL producers. 39 (57.1%) isolates gave positive result by DDST using imipenem and EDTA. 33 (52.3%) were found to be positive on DDST using imipenem and MPA. MBL E-test was done in 15 isolates (12 of which were both combined disk test and DDST test positive and 3 isolates were randomly tested from those strains which gave positive combined disk test and negative DDST). All the 15 isolates were shown to be MBL producers by E-test method. Phantom zone between IP/IPI was noted in 3 isolates.

Among the 46 imipenem resistant *Acinetobacter* spp., 19 (41.3%) were found to be MBL producers by combined disc test, 12 (26.0%) gave positive results by EDTA disk synergy test and 9 (19.5%) were positive on MPA disk synergy test. E-test was performed in 12 isolates (MBL producers by combined disk test) and was found to be positive in all these isolates.

No MBL production was seen in 10 randomly selected imipenem sensitive strains screened by various phenotypic methods.

The antimicrobial resistance patterns of the isolates are depicted in Table 1. *P. aeruginosa* showed maximum sensitivity (100.0%) to polymyxin B followed by imipenem (42.2%), piperacillin-tazobactam (36.7%), cefoperazone-sulbactam (34.9%) and amikacin (34.9%). All *Acinetobacter* spp. were also found to be sensitive to polymyxin B and showed moderate sensitivity to imipenem (45.9%), piperacillin-tazobactam(38.8%), cefoperazone-sulbactam (36.5%) and ciprofloxacin (25.9%). Poor susceptibility was seen with the rest of the drugs as shown in Table 1.

Table 1
Antimicrobial resistance pattern of various isolates.

Antimicrobial agent	*P. aeruginosa*(*n*=109)	*Acinetobacter* spp (*n*=85)
Ciprofloxacin	79.8%	74.1%
Amikacin	65.1%	81.2%
Piperacillin	90.8%	95.3%
Cefoperazone	96.3%	95.3%
Piperacillin - tazobactam	63.3%	61.1%
Cefoperazone-sulbactam	65.1%	63.5%
Imipenem	57.8%	54.1%
Polymyxin B	0.0%	0.0%

4. Discussion

Carbapenems are β - lactam antibiotics, considered as the most potent agents of treatment of multidrug resistant gram negative bacterial infections due to the stability of these agents against the majority of β - lactamases and their high rate of permeation through bacterial outer membranes. However, in the last decade there is an alarming increase in reports of resistance to these life saving antimicrobials and poses a significant clinical challenge[12]. Production of MBLs has emerged as the most important mechanism of carbapenem resistance among non-fermentative gram negative bacterial isolates due to the facilitation of rapid spread by the transfer of integrons containing gene cassettes for resistance to multiple antibiotics. For many years, these MBL producing isolates were restricted to several countries but now it has disseminated worldwide[1,13,14].

The present study reports high level of imipenem resistance (57.8% and 54.1%) among *P. aeruginosa* and *Acinetobacter* spp. respectively. Previous studies from India also showed rising trend (14.0%-36.4%) in the carbapenem resistance among the non-fermenters[1,15,16]. In *P. aeruginosa*, resistance has gone up to even 60% across the world[17,18]. Surveillance in Brooklyn, New York revealed that approximately 2 of every 3 isolates of *Acinetobacter* were resistant to carbapenem[19]. In another study, 64% of resistance to meropenem was reported in *Acinetobacter* spp[20]. High antibiotic pressure due to greater empirical or indiscriminate use of antibiotics could have result the carbapenem resistance in the hospital setting. We also found that 69.8% of the imipenem resistance among *P. aeruginosa* was attributable to production of MBLs which is comparable to the another study reporting a similar pattern of resistance (61.3%)[12]. In India, MBL producing *P. aeruginosa* was first reported in 2002 and been seen to vary from as low as 7.5% to 75%.[17,21,22] The prevalence of MBL positive strains among imipenem non susceptible *Acinetobacter* was lower than in *P. aeruginosa*. Among the imipenem resistant *Acinetobacter*, 41.3% were MBL producers which was relatively less compared to the other Indian studies[1,21] according to which MBL may not play a major role in resistance among the *Acinetobacter*. But our findings clearly show a rising trend in the carbapenem resistance due to MBLs among the *Acinetobacter* spp. especially in hospitalized patients.

With the global increase in the types of MBLs, early detection is crucial. The CLSI does not have performance standards documented so far, various screening methods have been employed for screening of clinical isolates for MBL production. Though MIC detection is gold standard, combined disc test and DDST are comparable with the former and at the same time are simple, reliable, less cumbersome and cheap, as per previous reports[17,23]. In this study, we have used four different methods of screening of MBL production. With the imipenem - EDTA combined disk test, the positive and negative results were more clearly discriminated and were found to be more superior to DDST and detected highest number of MBL producing isolates in both *P. aeruginosa* and *Acinetobacter* spp. The reason for the difference in the performance of these two tests is exactly not clear but interpretation of the combined disk assay results is more objective than that of DDST results because the DDST depends upon the expertise in discriminating true synergism from intersection of inhibition zones. One major disadvantage of DDST was the subjective interpretation of result in some instances as both the EDTA and MPA discs alone frequently produced undesirably large inhibition zones. Our findings are supported by other published studies which have found the combined disc method to be the more sensitive technique for detecting MBLs[17,24,25]. Among the DDST, the performance of IMP-EDTA was compared with IMP-MPA and the former provided the better results over MPA in both of *P. aeruginosa* and *Acinetobacter* isolates .

In present study, the MBL E test is found to be very sensitive for detection of MBLs in *P. aeruginosa* as well as in *Acinetobacter* spp. All isolates positive by combined disc test were also found to be positive with E test and other few which were positive by combined disc test and negative by DDST were also detected as MBL producers by E test. The MBL E test strip has the ability to detect both chromosomally and plasmid mediated MBLs, is simple to perform with ease of application as well as interpretation. However, it has a disadvantage of being unable to provide conclusive results in strains with low MICs to be detected by the test strip and of utility only in differentiating MBL producers in imipenem resistant strains. Moreover, given the cost constraints of E test, other screening methods like combined disc test and DDST have been reported to be simple, inexpensive phenotypic resources for the detection of MBL that could be easily incorporated into the routine testing of any microbiology laboratory[11,17].

The unique problem with MBLs is their broad spectrum resistance profile. Multidrug resistance was observed with a higher frequency among the MBL producers in this study. Apart from imipenem, MBLs were found to be resistant to other important groups of antibiotics tested including cephalosporins, aminoglycosides and fluoroquinolones. All the isolates were seen to be susceptible to polymyxin B. Since these organisms carry other drug-resistant

genes, limited treatment options are available and the only therapeutic alternative remains the potentially toxic polymyxin B and colistin[6]. So,early identification of the infections due to these organisms is necessary as the appropriate treatment might reduce the spread of these resistant strains as well as reduce the mortality among hospitalized patients.

The limitations in the present study are relatively small sample size and were restricted mainly to the detection of metallo- beta- lactamases among non fermentative bacteria. However use of additional features including large study sample and clinical usefulness of detection of MBLs would have enhanced the value of study and may have provided greater insight into the possible link.

To conclude, MBL production is an important mechanism of carbapenem resistance among non-fermentative gram negative bacilli. Detection of MBLs by simple, inexpensive and reproducible methods should be routinely performed for all imipenem-resistant isolates. We found imipenem-EDTA combined disc test and MBL E test to be equally effective for MBL detection, however given the cost factor, combined disk test could be used as convenient screening method in the clinical microbiology laboratories. But at the same time, standardization of phenotypic methods is of crucial importance. Moreover, a strict antibiotic policy, timely implementation of infection control practices and antibiotic resistance surveillance programs should be carried out from time to time.

Conflict of interest statement

We declare that we have no conflict of interest.

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