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Original Article

Evaluation of various methods for detection of metallo-β-lactamase (MBL) production in gram negative bacilli

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A R T I C L E I N F O

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A B S T R A C T

Introduction: The emergence of metallo-β-lactamase (MBL) in gram negative bacilli (GNB) is becoming a therapeutic challenge worldwide. Detection of MBL is also a challenge for routine microbiology laboratories, since there are no standardized methods for MBL detection. The aims of this study were to know prevalence of MBL production in various gram negative bacilli, to evaluate different phenotypic methods to detect MBL production and to find out antibiotic sensitivity profile of MBL producing gram negative bacilli. Material and methods: Total 450 clinical isolates of GNB including E. coli, Pseudomonas, Klebsiella, Acinetobacter and Other GNB were subjected to antibiotic susceptibility testing. Imipenem, ertapenam, meropenam and third generation cephalosporins resistant clinical isolates were taken as positive for MBL screening. Four different methods using EDTA as MBL inhibitor were evaluated: (i) Combined disk synergy test with imipenem (CDST-IPM), (ii) Double-disk synergy test with imipenem (DDST-IPM), (iii) CDST with ceftazidime (CDST-CAZ) and (iv) DDST with ceftazidime (DDST-CAZ). Result: Out of 450 clinical isolates of GNB, 27 isolates (6.00%) were resistant to imipenem, ertapenam, meropenam and third generation cephalosporins. These 27 isolates were considered screening positive and further tested for MBL production by four different methods. 26 isolates (96.30%) were MBL positive by CDST-IPM and 22 isolates (81.48%) were MBL positive by DDST-IPM. 23 isolates (85.19%) were MBL positive by CDST-CAZ and 12 isolates (44.44%) were MBL positive by DDST-CAZ. Prevalence of MBL production was highest in Pseudomonas (9.92%), followed by Klebsiella pneumoniae (7.26%), Acinetobacter spp. (7.14%) and E. coli (2.87%). Conclusion: The detection of MBL-producing isolates is of crucial importance in GNB isolates especially in Pseudomonas. CDST-CAZ is the most sensitive method for MBL detection.

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1. Introduction

The introduction of carbapenem into clinical practice represented a great advancement for the treatment of β-lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most β-lactamase, the carbapenems have been the drugs of choice for treatment of infections caused by penicillin or cephalosporin resistant gram negative bacilli [1]. Metallo-β-lactamase was first detected in 1960, in Bacillus cereus which was chromosomal in location. Then, first plasmid mediated MBL isolates was found in Pseudomonas aeruginosa in 1991 in Japan. Since early 1990s, metallo-β-lactamase (MBL) encoding genes have been reported all over the world in clinically important pathogens, such as Pseudomonas spp., Acinetobacter spp., and members of the Enterobacteriaceae family [2]. MBL in gram negative bacilli is becoming a therapeutic challenge, as these enzymes usually possess a broad hydrolysis profile that includes all β-lactam antibiotics including carbapenems [3] .

MBLs spread easily on plasmids and cause nosocomial infections and outbreaks. Such infections mainly concern patients admitted to Intensive Care Units with several co-morbidities and a history of prolonged administration of antibiotics [4]. Moreover, MBL producing isolates are also associated with higher morbidity

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and mortality [5]. Early detection of MBL-producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their interhospital and intrahospital dissemination. [2] Arakawa et al. first described that the specificity and sensitivity of the disk diffusion tests, using a ceftazidime disc and two MBL inhibitors [EDTA and 2-mercaptopropionic acid], were comparable with those of PCR for detection of MBL production [6]. Later Lee et al. described DDST method and Yong et al. described CDST method for easy detection of MBL in routine laboratories [7,8]. Microbiology laboratories must be prepared to screen for MBL-producing isolates by a low cost, convenient and sensitive procedure.

Aims and Objectives

- To know prevalence of MBL production in various gram negative bacilli
- To evaluate the accuracy of four different phenotypic methods to detect MBL production in gram negative bacilli
- To find out antibiotic sensitivity profile of MBL producing gram negative bacilli

2. Material and Methods

From August 2010 to July 2011, total 1400 various clinical specimens were processed. Out of these 1400 specimens, 450 clinical isolates of gram negative bacilli including *E. coli, Pseudomonas, Klebsiella, Acinetobacter* and other gram negative bacilli were tested for MBL production. The isolates were identified by conventional methods[9]. All isolates were non-duplicate.

2.1.MBL screening method

Gram negative bacilli isolates were subjected to antibiotic susceptibility testing by Kirby Bauer disk diffusion method as per the CLSI guidelines [10]. Isolates resistant to imipenem, ertapenam, meropenam and third generation cephalosporins were considered screening positive.

2.2.MBL confirmation tests

A 0.5 M EDTA (Hi-Media, India) solution was prepared by dissolving 18.61 g. of EDTA in 100 ml of distilled water and adjusting it pH 8.0 by using NaOH. [7] The test organisms were inoculated on to Mueller Hinton agar plates.

2.2.1. Imipenem - EDTA Combined disk synergy test (CDST-IPM) [7]

Two imipenem (10ug) discs were placed on the surface of an agar plate and 10 µl EDTA solution was added to one of them to obtain a desired concentration of 750 ug. Plates were incubated for 16 to 18 hours at 35°C. If zone of inhibition of imipenem- EDTA disc was ≥ 7 mm more than that of imipenem disc alone, it was considered MBL positive.

2.2.2. Imipenem - EDTA Double disc synergy test (DDST-IPM) [8]

An imipenem (10ug) disc was placed 20 mm center to center from a blank disc containing 10 µl of 0.5 M EDTA (750 ug). Plates were incubated for 16 to 18 hours at 35°C. If there is enhancement of zone of inhibition between imipenem and EDTA disc, it was considered MBL positive.

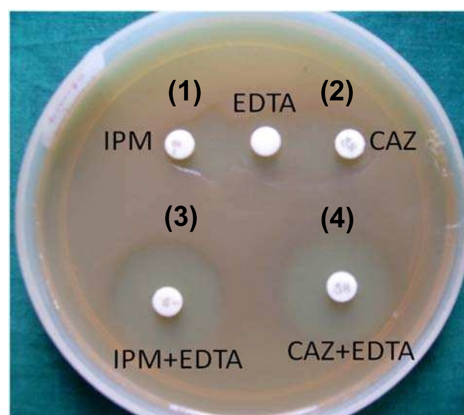
2.3.3. Ceftazidime - EDTA Combined disk synergy test (CDST-CAZ) [3]

Method and interpretation is same as CDST-IPM method except using ceftazidime (30ug) discs in place of imipenem.

2.4.4. Ceftazidime - EDTA Double disc synergy test (DDST-CAZ) [8]

Method and interpretation is same as DDST-IPM method except using ceftazidime (30ug) discs in place of imipenem.

Figure 1. MBL positive isolate by all methods



IPM – Imipenem, CAZ – Ceftazidime

(1) DDST-IPM: Enhancement of zone of IPM towards EDTA
(2) DDST-CAZ: Enhancement of zone of CAZ towards EDTA
(3) CDST-IPM: Zone of inhibition of IPM+EDTA disc is ≥7 mm than that of IPM disc alone
(4) CDST-CAZ: Zone of inhibition of CAZ+EDTA disc is ≥7 mm than that of CAZ disc alone

3.Result

Out of 450 clinical isolates of GNB, 27 (6.00%) isolates were resistant to imipenem, ertapenam, meropenam and third generation cephalosporins.

Prevalence of MBL production was highest in Pseudomonas (9.92%), followed by Klebsiella pneumoniae (7.26%), Acinetobacter spp. (7.14%) and E. coli (2.87%), as shown in table 1.

Table 1. Prevalence of MBL in gram negative bacilli

	E. coli	*Klebsiella pneumoniae*	*Pseudo-monas*	*Acineto-bacter*	Other-GNB	*Total*
Isolates	174	124	121	14	17	450
MBL	5	9	12	1	0	27
%	2.87	7.26	9.92	7.14	00	6.00

Using imipenem as a substrate, 26 (96.30%) isolates were MBL positive by CDST and 22 (81.48%) isolates were MBL positive by DDST. Using ceftazidime as a substrate, 23 (85.19%) isolates were MBL positive by CDST and 12 (44.44%) isolates were MBL positive by DDST, as shown in table 2. One isolate that was negative with CDST-IPM was positive with DDST-IPM & CDST-CAZ. The zone

diameters were similar and reproducible when the procedure was repeated.

Table 2. Comparison of MBL detection methods

Screening positive	CDST- IPM	DDST-IPM	CDST-CAZ	DDST-CAZ
27	26	22	23	12
%	96.30	81.48	85.19	44.44

Antibiotic sensitivity pattern of MBL producing gram negative bacilli isolates is as shown in table 3. Polymyxin B is found to be most sensitive drug. Tetracycline and gentamicin are the least sensitive drugs.

Table 2. Antibiotic sensitivity profile of MBL producing isolates

Antibiotic Polymyxin B	Sensitive isolates 23 (85.19%)
Piperacillin-Tazobactum	4 (14.81%)
Chloramphenicol	4 (14.81%)
Ampicillin-Sulbactum	3 (11.11%)
Cotrimoxazole	2 (7.41%)
Gatifloxacin	2 (7.41%)
Tetracycline	1 (3.70%)
Gentamicin	1 (3.70%)

4.Discussion

Since there are no standard guidelines for detection of MBL, different studies have reported the use of different methods. PCR analysis is the gold standard method for the detection of MBL production, but it is not feasible in routine microbiology laboratory. In present study, out of 450 gram negative bacilli isolates, 27 (6.00%) isolates were screening test positive for MBL production. Out of four methods used for confirmation of MBL production, CDST-IPM was found to be the most sensitive (96.30%) method, followed by CDST-CAZ (85.19%) and DDST-IPM (81.48%). DDST-CAZ was found to be the least sensitive (20.83%) method.

In other studies, CDST-CAZ and DDST-IPM are most sensitive method for detection of MBL producing GNB, contrasting to present study. However, all studies found DDST-CAZ as the least sensitive method similar to present study, as shown in table 4. Interpretation of DDST results is more subjective as it depends upon the technician's expertise to discriminate true synergism from the intersection of inhibition zones.

Table 4. Comparison of present study with other published studies

Study	CDST-IPM (%)	DDST-IPM (%)	CDST-CAZ (%)	DDST-CAZ (%)
Galani et al.[3]	94.7	100	100	77.9
Picao et al.[2]	80	82.6	83	45.7
Franklin et al.[11]	100	79	-	-
Present study	96.30	81.48	85.19	44.44

Prevalence of MBL production, as per CDST-IPM method, was found to be highest in Pseudomonas (9.92%). MBL production was also detected in Klebsiella pneumoniae, Acinetobacter and E. coli, but not in other gram negative bacilli.

MBL positive isolates usually shows resistance to all β-lactam antibiotics, aminoglycosides, tetracycline, and fluoroquinolones. However they remain sensitive to polymyxin B [12]. In present study 23 out of 27 isolates (85.19%) were sensitive to polymyxin B.

5. Conclusion

The early detection of MBL-producing isolates would be important for the reduction of mortality rates for patients infected with MBL producing isolates and also to avoid the intra hospital dissemination of such strains.

'Imipenem-EDTA combined disk test' (CDST-IPM) is the most sensitive method for detection of MBL production in gram negative bacilli. There is variation in subjective interpretation of result in DDST. Positive and negative results are more clearly seen in CDST than DDST. CDST-IPM is the method that can be used as a convenient screening method for detection of MBL production in gram negative bacilli in routine microbiology laboratory.

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