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Original Article

Microbiology Section

Comparative Evaluation of Four Phenotypic Tests for Detection of Metallo-β-Lactamase and *Carbapenemase* Production in *Acinetobacter baumannii*

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ABSTRACT

Introduction: *Acinetobacter baumannii* is an emerging multi-drug resistant opportunistic pathogen that causes a variety of nosocomial infections. In recent years, carbapenem resistance in *A.baumannii* has increased due to Ambler class B Metallo β-lactamases or class D OXA Carbapenemases.

Objective: The present study was undertaken to detect and compare the various phenotypic methods for MBL production in nosocomial *A.baumannii* isolates.

Materials and Methods: One hundred sixty eight *A.baumannii* isolates were subjected to disc diffusion assay. Imipenem resistant isolates were subjected to 4 different phenotypic tests. MBL screening was done by Imipenem-EDTA double disc synergy test, Imipenem-EDTA combined disc test, Modified Hodge test and MBL E-test.

Results: Out of 168 *A.baumannii* isolates, 85 (50.59%) were imipenem resistant. Among these 85 isolates, 57 (67.05%) were MBL positive by DDST, 69 (81.18%) by CDT, 85 (100%) by MHT and all these 85 isolates were confirmed to be MBL positive by MBL E-test method.

Conclusion: Combined disc test, Modified Hodge test & E-test are equally effective to detect MBL production. However, considering the cost constraints of E-test, simple MHT and CDT can be used. They are easy, economical and can be incorporated into routine testing in laboratories to monitor the emergence of MBLs in MDR *A.baumannii.*

Keywords: MDR *Acinetobacter baumannii* (MDR AB) Combined disc test (CDT), Modified Hodge test (MHT), MBL E-test

INTRODUCTION

Acinetobacter baumannii has emerged as one of the most troublesome pathogens for health-care institutions globally. Over the last 16 years it has developed remarkable ability to acquire resistance determinants. Along with its intrinsic and acquired resistance mechanisms, it has the uncanny ability to survive for prolonged period throughout a hospital environment. Its ability to survive under a wide range of environmental condition, makes it a frequent cause of outbreaks of infection and endemic health-care associated pathogen. This multi-drug resistant and Pan resistant *A.baumannii* is threatening the current antibiotic era.

As reported from reviews dating back to1970s, hospital acquired pneumonia is still the most common infection caused by *Acinetobacter*. In most recent times, infections involving urinary tract, blood stream, CNS, skin, soft tissues, surgical sites and bones have been reported. Significant advances have been made in understanding this multi-drug resistant organism in last two decades [1].

A.baumannii is often resistant to wide variety of antimicrobials, including carbapenems. Carbapenem group of antibiotics play a vital role in the management of nosocomial infections due to gram negative organisms because of their broad spectrum activity and the ability to hydrolysis by most of the β-lactamases including ESBLs [2]. Carbapenem resistance in *A.baumannii* is due to variety of combined mechanisms such as hydrolysis by β-lactamases, alterations in the outer membrane proteins and penicillin-binding proteins and increased activity of efflux pumps [3,4]. In addition to this, acquired resistance is mediated by OXA-type carbapenemases and metallo β-lactamases (MBLs). The MBLs efficiently hydrolyse all the β-lactams, except for Aztreonam, in-vitro. Three groups of MBLs (IMP, VIM & SIM) and three groups of CHDLs (OXA group) have been reported in *A.baumannii* [5,6]. Therefore detection of MBL-producing multi-drug resistant *A.baumannii* (MDR AB) is crucial for the optimal and modified therapy and also to initiate effective control of the dissemination of resistance.

Few Indian studies have documented the presence of MBLs in *A.baumanni*. No extensive study has been conducted in Karnataka, especially in and around Mangalore,India regarding the detection of MBL and Carbapenemase producing *Acinetobacter sps*. Hence the aim of this study was to detect the MBL producing strains among MDR AB, isolated from clinical specimens in this geographical area by various phenotypic tests and their comparative evaluation.

MATERIALS AND METHODS

This study was conducted in coastal Karnataka, Southern India for a duration of one year. A total of 168 strains of *A.baumannii* were collected from various clinical samples such as blood, pus, urine, respiratory secretions and other body fluids such as ascitic fluid and synovial fluid, including the patients from ICUs. The multi-drug resistant (MDR) isolates which were resistant to Imipenem were selected and tested for MBL and Carbapenemase production by the following phenotypic tests:

Imipenem-EDTA Double Disc Synergy Test (DDST)

The IMP-EDTA double disc synergy test was performed to detect MBL production [7]. The overnight broth cultures of test isolates along with standard control strains (opacity adjusted to 0.5 McFarland opacity standard) were inoculated onto Mueller-Hinton agar plates as lawn culture according to the CLSI recommendations. After drying, a 10 µg Imipenem disc was placed on the lawn culture with a distance of 15 mm centre to centre from a blank disc. 10 µl of 0.5 M EDTA was added to the blank disc and incubated overnight.

Presence of an enlarged zone of inhibition towards EDTA disc was interpreted as positive for MBL production [Table/Fig-1].

Imipenem-EDTA Combined Disc Test (CDT)

The test isolates along with standard control strains (opacity adjusted to 0.5 McFarland opacity standard) were lawn cultured on Mueller-Hinton agar plate as recommended by CLSI. After drying, two 10 µg Imipenem discs were placed on the lawn culture with 20 mm distance from centre to centre of the discs. 10 µl of 0.5 M EDTA was added to one of the imipenem discs and incubated overnight. Isolates showing ≥7 mm increase in the inhibition zone size of Imipenem-EDTA disc than the Imipenem disc alone were considered as MBL producers [8] [Table/Fig-2].

Modified Hodge Test (MHT)

All the isolates were subjected to Modified Hodge test, a screening test which helps in detection of Carbapenemases [3]. *Escherichia coli* ATCC 25922 (an indicator organism sensitive to carbapenems) was cultured in peptone water to achieve 0.5 McFarland opacity standard and was lawn cultured onto a Mueller-Hinton agar plate using sterile cotton swab. After drying, 10 µg Imipenem disc was placed at the centre of the plate on the lawn culture and an overnight growth of test strain was heavily streaked from the edge of the Imipenem disc outwards, to the periphery of the plate in four different directions. The plates were incubated at 37°C overnight. The presence of a distorted zone (Clover-leaf shaped zone of inhibition) was considered as positive test. The test was repeated with Imipenem + 10 µl 0.5 M EDTA. To determine the effect of Zinc ions on the test and to overcome the equivocal or false-negative results, the test was again repeated with 10 µl of 50 mM ZnSO4 solution (Prepared by dissolving 1438 mg of ZnSO4.7H2O in 100 ml deionized water and sterilized by autoclaving) which is added to Imipenem-EDTA disc [Table/Fig-3].

MBL E-Test

All the Imipenem resistant isolates were subjected to E-test [9] to detect minimum inhibitory concentration (MIC) ratio and to confirm MBL production. The E-test MBL strip (bioMerieux SA, France) containing a double sided seven-dilution range of Imipenem (IP) (4 to 256 µg/ml) and Imipenem (1 to 64 µg/ml) in combination with a fixed concentration of EDTA (IPI) was used for MBL detection. The test was done according to manufacturer's instructions (E-test technical manual, bioMerieux SA, France). MIC ratio of ≥8 for the 2 reagent sides or a phantom zone between IP/IPI or deformation of either ellipse was indicative MBL production [Table/Fig-4].

RESULTS

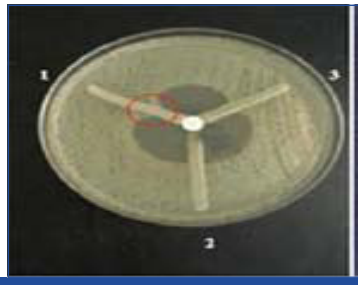
Among 168 *Acinetobacter baumannii* isolates, 40 (23.80%) were isolated from pus, 33 (19.64%) from blood, 30 (17.86%) from urine, 63 (37.50%) from respiratory secretions and 2 (1.19%) from other body fluids. 83 (49.40%) isolates among the 168 A. baumannii were Imipenem sensitive and 85 (50.59%) Imipenem resistant. All the 168 isolates were multi-drug resistant and showed 100% sensitivity to Colistin and Tigecycline.

The results of comparative evaluation of MBL production by four Phenotypic tests are shown in [Table/Fig-5]. In Modified Hodge test, addition of 0.5M EDTA to Imipenem disc improved the test performance whereas addition of 50 mM ZnSO4 to Imipenem-EDTA disc did not show any difference in the test performance.

DISCUSSION

Multi-drug resistant *A.baumannii* (MDR *AB*) is a significant pathogen in health care settings where it causes a multitude of infections that include septicaemia, pneumonia, meningitis, urinary tract infection and wound infections. Less frequently it also causes infections of skin and soft tissues, abdominal and CNS infections. The infection tend to occur in immunosuppressed patients, in patients with serious underlying diseases and in those subjected to invasive procedures and treated with broad spectrum antibiotics [1] especially in ICUs. Nosocomially acquired MDR AB pose a real challenge to the clinician.

Carbapenems are generally the last resort in the treatment of life threatening infections caused by MDR *AB*. However, emergence of Carbapenem hydrolysing β-lactmases of Ambler class B (MBLs) and class D (Oxacillinases/CHDLs), which are proved to be the most important mechanism of carbapenem resistance, have caused serious problem in treatment of MDR AB. These organisms are resistant to carbapenems, fluoroquinolones and aminoglycosides



[Table/Fig-1]: Showing Imipenem-EDTA Double Disc Synergy Test (DDST) **[Table/Fig-2]:** Showing Imipenem-EDTA Combined Disc Test (CDT)
[Table/Fig-3]: Showing Modified Hodge Test (MHT)

[Table/Fig-4]: Showing MBL E-test

Sl.no	Phenotypic tests	Positive Number (n)	Percentage (%)	Negative Number (n)	Percentage (%)
1.	Double Disc Synergy Test	57	67.05	28	32.94
2.	IMP-EDTA Combined Disc Test	69	81.18	16	18.82
3.	Modified Hodge Test	85	100	00	-
4.	MBL E-Test	85	100	00	-

[Table/Fig-5]: Comparative evaluation of MBL production by various Phenotypic tests (85 Isolates)

and have been reported from several countries and also from various hospital settings in India [10-14]. Colistin resistant isolates are rare but occurrence is reported [15]. By acquiring various kinds

of resistance mechanisms, *A.baumannii* has evolved as one of the most difficult nosocomial pathogens to control and treat.

Simple and accurate tests are needed to detect MBL producers. There are no standard guidelines available for detection of MBL. Different studies have reported the use of various methods. Most of the studies have used Imipenem-EDTA double disc synergy test, Imipenem-EDTA combined disc test, Modified Hodge test and MBL E-test. According to those studies, MBL production ranged from 7% to 65%. [Table/Fig-6] shows the comparative study.

Several studies have reported the use of DDST as one of the convenient methods for the detection of Ambler class B MBL production and the positivity has varied from 14.8% - 72%. [16-18]. According to their findings, DDST was found to be more reliable and reproducible with high rate of positivity. However, in our study DDST showed the lowest positivity and our result was similar to the study conducted by Behra et al., The disadvantage of DDST was subjective interpretation of result in some instances. In our study, CDT was found to be superior to DDST and was in accordance with other published studies [9].

Modified Hodge test is a simple method for screening MBL producing isolates. It was originally described by the Centre for Disease Control for Carbapenemase detection in *Enterobacteriaceae*. It has displayed high efficiency for detection of Carbapenemase. However, occasional isolates show false positive results which is may be due to minor carbapenem hydrolysis by CTX-M or AmpC enzymes.[1] Performance of MHT can be more reliable when Imipenem discs with 50mM ZnSO4 and 0.5M EDTA is used [7].

Previously published data has shown low positivity of MHT when compared to other tests, which vary from 14.8% - 56.16%. [7,17,18] However our study has shown high positivity which is in accordance with the results of Amudhan SM et al., Though CLSI does not advocate the use of MHT for detection of Carbapenemase production in non-fermenting gram negative bacilli, several authors have found MHT with Imipenem, EDTA and ZnSO4 as a useful screening test for Carbapenemase production [3].

E-test is a quantitative technique for determining the MIC of antimicrobial agents against microorganisms and for detection of resistance mechanisms. E-test MBL strip based on a combination of a β-lactam substrate and a β-lactam or MBL inhibitor was specifically designed to detect clinically relevant MBLs. The E-test MBL strips (Imipenem-Imipenem+EDTA [Ip-Ipl]) has the ability to detect MBLs (both chromosomal and plasmid mediated) in aerobic and anaerobic bacteria. We, in accordance with other published studies,[9,19] found that MBL E-test to be very sensitive for detection of MBLs. Since we screened only Carbapenem resistant isolates with MBL E-test, it might have accounted for increased rate of positivity. Another possibility of higher positivity in our study might be the disseminated multi-drug resistance in *Acinetobacter* and its counteracting mechanisms occurring in the recent scenario, compared to the earlier times.

Sl.No	Authors and year of publication	Number of Strains tested	DDST	CDT	MHT	E-Test
1.	Lee K, et al., 2003 [7]	73	45 (61.64%)	-	41 (56.16%)	-
2.	Walsh TR, et al., 2002 [19]	130	-	-	-	83 (63.85%)
3.	Jesudasan M, et al., 2005 [17]	50	36 (72%)	-	28 (56%)	-
4.	Behera B, et al., 2008 [9]	63	36 (57.14%)	48 (76.19%)	-	30 (47.62%)
5	Irfan S et al., 2011 [2]	100	-	83 (96.6%)	-	-
6	Amudhan SM et al., 2011 [3]	116	-	92 (79.3%)	113 (97.4%)	-
7	John S et al., 2011 [18]	242	36 (14.8%)	-	36 (14.8%)	-
8	Present study	168	57 (67.05%)	69 (81.18%)	85(100%)	85 (100%)

[Table/Fig-6]: Comparative Studies by other authors

CONCLUSION

Our study showed that MHT and E-test were equally efficient to detect MBL production, followed by Combined disc test. Simultaneous existence of different carbapenemases is a problem to reckon with and should be seriously considered for alternative and newer therapeutic strategies, strict infection control measures and continuous surveillance. Easy detection methods are required for each of these in routine clinical labs. However, considering the cost constraints of E-test, simple CDT and MHT can be used which are easy, economical and can be incorporated into routine testing in labs to monitor the emergence of MBLs and carbapenemases in MDR *AB*. Initial screening of the putative carbapenemase producers will help to organise intervention and early directed therapy.

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