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Prevalence of Carbapenem Resistance and Comparison between Different Phenotypic Methods for Detection of Metallo-B-Lactamases in Gram Negative Non-Fermentative Bacteria in the Acute Wards of a Tertiary Care Centre

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A B S T R A C T

Non-fermentative Gram negative bacteria can cause life threatening infections in hospitalised patients. These organisms have high intrinsic antibiotic resistance. Carbapenems are potent agents against these. Production of carbapenemases especially MBLs can jeopardise its use. Study was done to detect the prevalence of carbapenem resistance in NFGNB and to compare the different phenotypic methods for detection of MBLs. Non-duplicate isolates were collected. Isolates were identified by using phenotypic methods and following standard laboratory protocols. Imipenem resistance was detected by using CLSI guidelines. A total of 296 isolates were collected. *Acinetobacter baumannii* was the predominant isolate (44.59%) followed by *Pseudomonas aeruginosa.*71.21% of *A.baumannii* and 52.06% of *P.aeruginosa* were imipenem resistant. 86.17% of *A.baumannii* and 82.53% of *P.aeruginosa* were modified Hodge test positive.67.02% of *A.baumannii* and 66.66% of *P.aeruginosa* were Double disk synergy test positive.76.59% of *A.baumannii* and 77.77% of *P.aeruginosa* were positive for combined disk test. 74.46% of *A.baumannii* and 76.19% of *P.aeruginosa* were positive for MBL by E-test. "Almost perfect agreement" was seen between CDT and E-test for *A.baumannii* whereas "Substantial agreement" was seen for *P.aeruginosa.* Hence CDT can be used as a cheaper method for phenotypic detection of MBLs.

Introduction

Non-fermentative Gram negative bacteria (NFGNB) like *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia* and *Burkholderia cepacia* can cause life threatening infections in hospitalised patients. They also have high intrinsic antibiotic resistance which places the patients at high risk (Hancock, 1998). Carbapenems are potent agents used for

treatment of serious infections caused by these bacteria. These are preferred because they have a broad spectrum of activity and are resistant to hydrolysis by most β-lactamases including extended spectrum beta lactamases (ESBLs). There is an increase in consumption in carbapenem antibiotics, especially in hospitals that have a high prevalence of ESBLs. Metallo-β-lactamases (MBLs) are an important group of carbapenemases and production of MBLs is increasingly being reported in NFGNBs, especially in *Pseudomonas aeruginosa.* MBLs are Ambler class B enzymes, can hydrolyse carbapenems and are inhibited by metal chelators. Its spectrum is broad but it can't hydrolyse Aztreonam (Franco *et al*., 2010). Genes of MBL like NDM, VIM, SIM etc. have the ability of horizontal transfer between pathogens in a hospital. PCR is accurate and reliable but its availability in resource limited setting is a constraint. Methods available for MBL detection take advantage of the enzyme's dependence upon Zn^{++}, by using chelating agents like EDTA and 2-mercaptopropionic acid to inhibit its activity (Behera *et al*., 2008). This study was carried out to detect the presence of MBLs in different imipenem resistant non-fermenters using various phenotypic methods and to compare these methods to ascertain which method can be used reliably in laboratories for fast identification of MBLs.

Materials and Methods

This study was carried out in the department of microbiology of a tertiary care level hospital in western Maharashtra for 1 year and 7 months. All consecutive, non-duplicate isolates of NFGNB were collected from various clinical samples like blood, sputum, tracheal aspirate, urine, pus etc. from hospitalised patients during the study period. All the isolates were identified taking in to account the colony morphology, pigment production and other phenotypic features. Further biochemical tests like oxidase test, fermentation of sugars, motility, nitrate reduction, decarboxylase tests and glucose oxidative fermentative test (OF test) etc. were done for speciation of the non-fermentative Gram negative bacilli according to standard protocol (Govan, 2011).

Antimicrobial Susceptibility

The antimicrobial susceptibility of all isolates was done by Kirby- Bauer disk diffusion method according to the CLSI guidelines 2012 (CLSI, 2012). Susceptibility of other antimicrobials was done under standard conditions. Carbapenem susceptibility was seen by using imipenem (10 μg) discs.

Modified Hodge Test (MHT)

All the isolates that were imipenem resistant were subjected to Modified Hodge test (MHT) as described by Lee *et al.,* (2001). In a plate of Mueller Hinton agar an inoculum of 0.5 McFarland of *E.coli* ATCC 25922 was applied. After waiting for 15 mins, imipenem disc (10μg) was placed onto the inoculated MHA plate in the centre and gently pressed down so as to ensure complete contact with the agar plate. 3-4 colonies of the test isolate were taken with a sterile loop and was streaked on the plate from centre to the periphery of the plate. Positive and negative controls were streaked on the same plate at 90° angulations from centre to periphery for quality control. Carbapenemase producing isolates were detected, when the test isolate produced the enzyme and allowed growth of the carbapenem susceptible strain (*E.coli* ATCC 25922) towards the imipenem disc. The result was a characteristic cloverleaf- like

indentation in case of carbapenemase production. The plates were examined after 16-24 hours of inoculation for a clover-leaf type indentation at the intersection of the test organism and the *E.coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disc. If a clover-leaf indentation of the *E.coli* ATCC 25922 growing along the test organism growth streak within the disk diffusion zone was produced, then this isolate was considered as carbapenemase producing (Fig 1). If no growth of the *E.coli* ATCC 25922 along the test organism growth streak within the disk diffusion zone was observed, then this isolate was considered as non-carbapenemase producing isolate.

Phenotypic Methods for Detection of Metallo-β-lactamases (MBL)

Imipenem EDTA Double Disc Synergy Test (DDST)

Double disc synergy test was done as described by Lee *et al.,* (2003). Three to five well isolated colonies of same morphology were selected. A 0.5 McFarland solution was made and with the help of a swab the inoculum was applied to a MHA plate with the help of a rotor. A 10 μg imipenem disc was applied to the plate. Another sterile filter paper disc was then applied 10 mm away to the imipenem disc (edge to edge). 10 μl of 0.5M EDTA solution was then added to the filter paper disc (750μg). The plates were then incubated and the results were interpreted after 16-18 hours. The tests were interpreted as positive if a large synergistic zone was formed in between the discs (Fig 2).

Imipenem-EDTA Disc Method (Combined Disc Method)

Combined disc test was done as described

by Yong *et al.,* (2002). This test was done to confirm the production of MBLs phenotypically. Three to five well isolated colonies of same morphology were selected. A 0.5 McFarland solution was made and with the help of a swab the inoculum was applied to a MHA plate with the help of a rotor. Two imipenem discs (10 μg) were applied apart to the plate. To one of the discs 10 μl of 0.5M EDTA solution (750 μg) was then added. The plates were then incubated and the results were interpreted after 16-18 hours. The test was interpreted as positive for MBL if the difference in zones of inhibition of ≥7mm is produced (Fig 3).

Epsilometer Test (E-test)

E-test was done as described by Lee *et al.,* (2005). This test was done to confirm the production of MBLs phenotypically and to determine the MIC. Three to five well isolated colonies of same morphology were selected. A 0.5 McFarland inoculum was made and applied to a MHA plate. After about fifteen minutes the E-strip (imipenem and imipenem+ EDTA) was applied to the plate at the centre. The results were interpreted after 16-18 hours. A tear drop zone of inhibition was taken as the result. The zone edge intersecting the graded strip was taken as the minimum inhibitory concentration of that isolate. IP and IPI readings were taken respectively for each isolate. MIC ratio of IP/IPI of ≥ 8 (3 log dilutions) was interpreted as MBL producing. Phantom zone or deformation of the ellipses was also interpreted as positive for MBL regardless of IP/IPI ratio (Fig 4).

Results and Discussion

A total of 296 samples were collected during the study period. One hundred thirty two isolates were *Acinetobacter baumannii*, 121 were *Pseudomonas aeruginosa* and 43 other

NFGNB like *Stenotrophomonas maltophilia*, *Alkaligenes faecalis*, *Burkholderia cepacia*, *Sphingomonas paucimobilis*, *Achromobacter denitrificans*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Burkholderia pseudomallei* were isolated. Out of these 296 isolates, 187 (63.1%) were imipenem resistant. Ninety four out of 132 (71.21%) of *A. baumannii,* 63 out of 121 (52.06%) of *P.aeruginosa* and 30 out of 43 isolates of other non-fermenters were found to be imipenem resistant. MHT was positive in 81 (86.17%) isolates of imipenem resistant *A.baumannii* (IRAB)*,* 52 (82.53%) isolates of imipenem resistant *P.aeruginosa* (IRPA) and 7 (23.33%) of other imipenem resistant non-fermenters isolates. Overall 140 (74.86%) isolates were positive for MHT.

In DDST, overall 109 (58.28%) imipenem resistant isolates were found to be DDST positive. Sixty three imipenem resistant *A.baumannii* isolates (67.02%), 42 *P.aeruginosa* (66.66%) and 4 (13.33%) isolates of other NFGNB were DDST positive.

By Combined disk test, overall 126 (67.37%) isolates were positive by CDT for MBL. Among individual isolates 72 of imipenem resistant *A.baumannii* (76.59%)*,* 49 (77.77%) of *P.aeruginosa* and 5 (16.66%) of other non-fermenters were CDT positive.

By E-test, overall 123 (65.77%) imipenem resistant isolates were positive for MBL by E-test. 70 (74.46%) isolates of imipenem resistant *A.baumannii,* 48 (76.19%) of *P.aeruginosa* and 5 (16.66%) isolates of other non-fermenters were MBL positive by E-test.

With the global spread and increase in the occurrence of different types of carbapenememase producing strains, their early detection is crucial. This will help significantly in timely implementation of strict infection control practices and treatment with alternative antibiotics. Different molecular techniques are available for detection of genes responsible for carbapenem resistance but they are not available in smaller centres. In these centres phenotypic characterisation can be done with limited resources and they are very sensitive in detection of MBLs etc. (Deshmukh *et al*., 2011).

A total of 296 samples were collected and analysed during the period from Dec 2012 to Jul 2014. The most common isolate in the present study was *Acinetobacter baumannii* (44.59%), followed by *Pseudomonas aeruginosa* (40.87%). Other non-fermenters like *Stenotrophomonas maltophilia* (6.7%), *Alcaligenes faecalis* (2.7%), *Burklolderia cepacia* (1.35%), *Sphingomonas paucimobilis* (1.35%), *Achromobacter denitrificans* (0.67%)*, Pseudomonas fluorescens* (0.67%), *Pseudomonas putida* (0.67%) and *Burkholderia pseudomallei* (0.33%) were present in decreasing order (Fig 5). Joseph *et al.,* and Irfan *et al.,* also had *A.baumannii*as their predominant isolate among non-fermenters (Joseph *et al*., 2011; Irfan *et al*., 2008). However Kalidas*et al.,* showed higher prevalence of *P.aeruginosa* (50.24%) among the non-fermenters from a study in Kolkata (Rit *et al*., 2013).

Imipenem resistance was seen in 187 isolates (63.1%) by Kirby-Bauer disk diffusion method. There are very few literatures from India and they mention prevalence of carbapenem resistance in non-fermenters varying from 36 to 90% (Goel *et al*., 2011). Out of these 187 imipenem resistant isolates, 50.26% isolates were of *A.baumannii,* 33.68% of *P.aeruginosa* and 16.04% of isolates were of other NFGNB.

Amudhan*et al.,* found that 64.08% and 34.07% of his imipenem resistant NFGNB isolates to be due to *A.baumannii* and *P.aeruginosa* respectively (Amudhan *et al.*, 2012). Our study also has *A.baumannii* as predominant organism having imipenem resistance among all non-fermenters.

Isolate wise distribution showed that a total of 132 isolates collected during the study period were of *A.baumannii*. 71.21% of *A.baumannii* isolates were imipenem resistant. Study by Khajuria *et al.,* reported a prevalence of imipenem resistance in *A.baumannii* from a centre in western Maharashtra to be at 42.11 % and Shivaprasad *et al.,* have reported prevalence of carbapenem resistance in *A.baumannii* at 50.59% (2014). Baran *et al.,* found the prevalence of IRAB to be at 53.7% from a hospital in Turkey (2008). Kalidas *et al.,* however found IRAB to be at a lower level at 10%. A total of 121 *P.aeruginosa* were collected during the study period. Out of these 52.06% were imipenem resistant. Bhalerao *et al.,* have reported imipenem resistance in *P.aeruginosa* at 67.5%, Behera *et al.,* showed a prevalence of 69%, Onguru *et al.,* had a prevalence of 44.1%, Singh *et al.,* had a prevalence of 21% and Carmeli*et al.,* had a prevalence of 13% and Lin *et al.,* had a prevalenceof 10.2% (Onguru *et al.*, 2008; Bhalerao *et al.*, 2010; Carmeli *et al.*, 1999; Singh *et al.*, 2009; Lin *et al.*, 2014). Kalidas*et al.,* in his study from Eastern India reported lower resistance to imipenem (9%) in *P.aeruginosa.*

All imipenem resistant isolates were screened for production of carbapenemases by MHT. 74.86% of all imipenem resistant isolates were MHT positive. Amudhan *et al.,* found 94.4% of imipenem resistant isolates to be MHT positive in NFGNB from a study in south India. In present study 86.17% of imipenem resistant *A.baumannii*

isolates were MHT positive. Khajuria *et al.,* reported that 60% of imipenem resistant *A.baumannii* was MHT positive and Amudhan *et al.,* reported 97.41% positivity in MHT. 82.53% of imipenem resistant isolates of *P.aeruginosa* were MHT positive. Singh *et al.,* had 71% of their IRPA as MHT positive. In isolates which were negative for MHT other mechanisms of carbapenem resistance like efflux pump activation or closure of ion channels may responsible. Only 23.33% of other non-fermenters were MHT positive. This highlights that in other non-fermenters, carbapenemase production may not be a predominant mechanism of imipenem resistance.

All imipenem resistant isolates were screened for production of metallo-β-lactamases (MBL) by doing double disk synergy test (DDST), combined disk test (CDT) and E-test (MBL and MIC determination) (Fig 6, Table 2). 58.28% of all isolates were MBL positive by DDST whereas 67.37% were MBL positive by CDT and 65.77% were found to be MBL positive by E-test method. Amudhan *et al.,* found that 80.4% of his imipenem resistant isolates were positive for MBL screening which is higher than the present study (15). Farzana R had 68.5% positivity for DDST and 85.71% for CDT in imipenem resistant isolates overall (Farzana *et al.*, 2013). The finding is matching ours in the sense that CDT is more sensitive than DDST. 67.02% of *A.baumannii* was positive by DDST, 76.59% by CDT, and 74.46% by E-test. Khajuria *et al.,* reported DDST, CDT, E-test positivity in 57.41%, 47.09% and 67.74% of imipenem resistant isolates of *A.baumannii* respectively. Shivaprasad *et al.,* reported 67.05%, 81.8% and 100% sensitivity in detecting MBLs by DDST, CDT and E-test respectively. Overall 82.97% isolates of imipenem resistant *A.baumannii* were

positive for MBL by all phenotypic methods. DDST was positive for 5 isolates which were negative for both CDT and E-test and E-test was positive for an isolate which was negative by CDT and DDST. None of the MHT negative isolates were found to be MBL screen positive.

Table.1 Comparison of DDST, CDT, E-test

Test	*A.baumannii*	*P.aeruginosa*	Others
DDST	63 (67.02%)	42 (66.66%)	4 (13.33%)
CDT	72 (76.59%)	49 (77.77%)	5 (16.66%)
E-test	70 (74.46%)	48 (76.19%)	5 (16.66%)
TOTAL	78 (82.97%)	51 (80.95%)	6 (20.00%)

Table.2 Comparison of Different Phenotypic Tests for MBL with E-test

Comparison of Tests	k value (95% C.I)	Interpretation
DDSTand E-test (*A.baumannii*)	0.464 (0.271-0.657)	Moderate agreement
CDT and E-test (*A.baumannii*)	0.885 (0.775-0.995)	Almost perfect agreement
DDST and E-test (*P.aeruginosa*)	0.538 (0.314-0.763)	Moderate agreement
CDT and E-test (*P.aeruginosa*)	0.776 (0.589-0.963)	Substantial agreement

Fig.1 MHT Test for Detection of Carbapenemases (PC-Positive Control, NC- Negative Control, PT- Positive Test, NT- Negative Test)
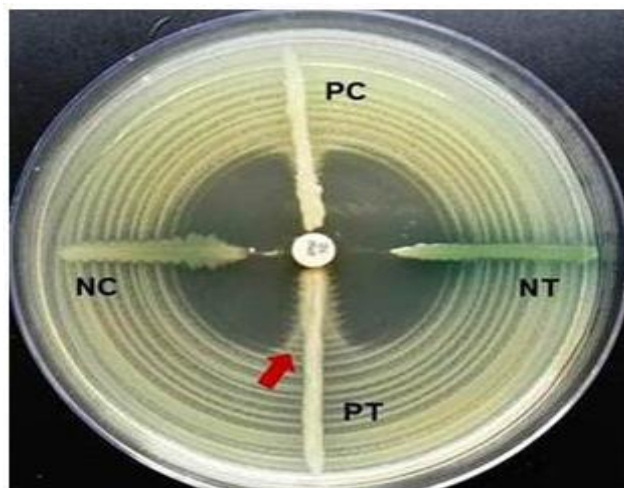
Fig.2 Imipenem-EDTA DDST Test for MBL Screening; Zone of Accentuation between Imipenem and EDTA

Fig.3 Imipenem EDTA Combined Disc Test (CDT) for MBL Screening; Imipenem and EDTA Zone Size > 7mm than IPM Zone Size

Fig.4 MBL Test for *Pseudomonas aeruginosa* by E-test; MIC> 256 and MBL Positive
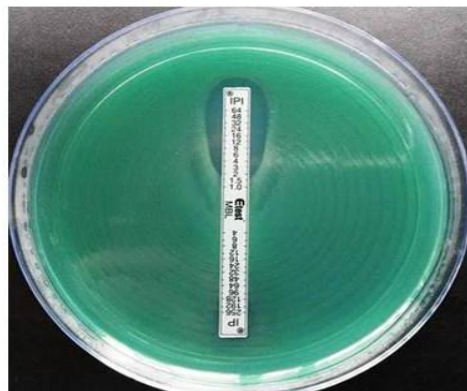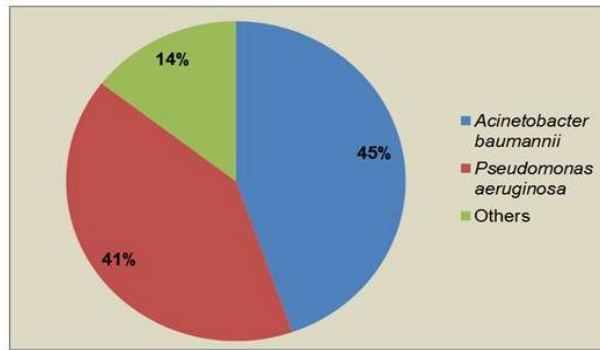
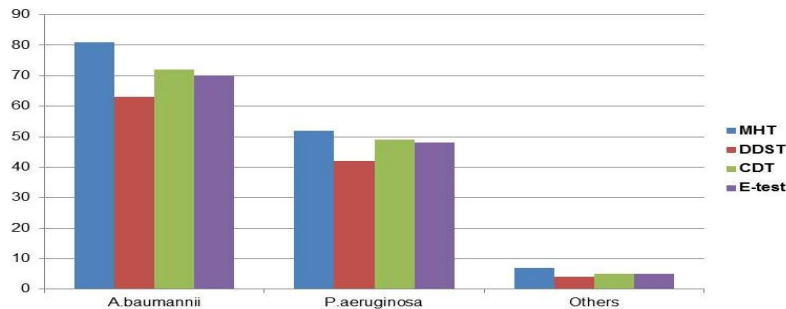**Fig.5** Species Wise Distribution in all Samples

Fig.6 Comparison between Different Phenotypic Tests

In *P.aeruginosa* 66.66% of isolates were positive for MBL by DDST, 77.77% by CDT and 76.19% by E-test. Behera *et al.,* reported that 85.71% and 64.28% of imipenem resistant isolates were positive for screening by CDT and DDST respectively which matches our study in the sense that CDT proved to be more sensitive for screening for MBL than DDST. They also reported 100% sensitivity by E-test for MBL which is in contrast to the present study in which we are reporting 76.19% IRPA by E-test. This discrepancy may be because of low number of isolates tested by them. However in our study E-test was able to pick up some MBLs which were negative by CDT and DDST, which matches with that of Behera *et al.,* Bhalerao *et al.,* reported DDST to screen 50% of MBLs and CDT picked up 66.7% of MBLs which is lower than what we found in present study. The possible explanation for this may be the highly subjective interpretation of results in DDST or there may be low incidence of MBLs in their setting. In all 80.95% of isolates showed MBL production by all three screening methods. Varying prevalence of MBL is seen worldwide, 39% in Italy and 44% in Brazil. All isolates for MBL screen were positive by CDT except two, which were positive by E-test only. In rest of imipenem resistant isolates other mechanisms may be responsible for imipenem resistance. No MHT negative isolate was positive for any MBL screening test positive.

In other non-fermenters 13.33% were MBL

producing by DDST, 16.66% by CDT and 16.66% by E-test. In all 20% of isolates were positive for MBL by all screening tests. This suggests that mechanisms other than MBL may be responsible for carbapenem resistance in them.

DDST and CDT were compared with E-test for MBL detection using kappa co-efficient (k) (25). According to Khosravi *et al.,* E-test is 100 % sensitive and specific in detection of MBLs in IRPA (Khosravi *et al*., 2012). So in the present study we did comparison to see the agreement between CDT and E-test and DDST and E-test. We found moderate agreement between DDST and E-test in both *A.baumannii*(k=0.464) *andP.aeruginosa* (k=0.538) whereas absolutely perfect agreement was seen between CDT and E-test in *A.baumannii*(k=0.885) and substantial agreement (k=0.776) was seen between CDT and E-test in *P.aeruginosa* (Table 2). Based on this finding we can safely say that CDT can be used as a suitable alternative for E-test as it's cost effective and can be relied upon for detection of MBLs in routine clinical laboratories. Behera *et al.,* also recommended the use of CDT for the phenotypic detection of MBL in laboratories routinely.

In conclusion, Carbapenem resistance is emerging as a major problem in treatment of patients in many clinical settings around the globe. The problem is getting compounded due to the rapid spread of strains harbouring these resistant genes. Carbapenem is being used as a broad spectrum antibiotic especially in critically ill patients. Only few reserve drugs are present for carbapenem resistant isolates but it is associated with many side effects. Multiple mechanisms of carbapenem resistance are present in NFGNB. The carbapenem resistance mechanism in NFGNB in our hospital

setting is mostly because of metallo-β-lactamases and other carbapenemases enzyme. Just by doing a few simple phenotypic tests like MHT, CDT the presence of carbapenemases and MBL can be detected. Since there are limited treatment options against these, continuous vigilance, early identification and treatment is very important to prevent further spread. Constant and regular monitoring of the incidence of such organisms in various critical areas of the hospital like ICU and acute medical units, prompt recognition of potential areas of colonisation and getting rid of them is the only important preventive strategy. To keep this problem in check, simple infection control measures like proper hand washing, adherence to infection control guidelines and antibiotic stewardship must be followed and time to time revision must be done.

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