

**EVALUATION OF VARIOUS PHENOTYPIC METHODS FOR DETECTION OF
CARBAPENEMASE PRODUCTION IN CARBAPENEM RESISTANT GRAM NEGATIVE
BACTERIA.**

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ABSTRACT

Introduction : A carbapenemase is a β -lactamase that hydrolyses carbapenems. This study was undertaken to compare the various phenotypic methods to detect carbapenemase production in carbapenem resistant Gram negative bacteria obtained from clinical samples in a tertiary care hospital, Kolkata. **Aim** - Evaluation of various phenotypic methods for detection of carbapenemase production. **Materials and methods** – Those Gram negative bacilli that were found to be carbapenem resistant or intermediate sensitive were tested by automated system. All the Gram negative bacilli with reduced susceptibility to carbapenems were further subjected to phenotypic tests for detection of carbapenemase. The results of the phenotypic tests were compared with that of an automated system. **Results:** 1404 Gram negative bacilli were isolated, out of which 148(10.54%) were found to have reduced susceptibility. *E.coli* was isolated in 37.16% cases followed by *Klebsiella spp* 26.35%, *Acinetobacter spp.* 22.3%, *Pseudomonas spp.* 7.43%, *Citrobacter spp.* 3.38% and *Enterobacter spp.* 3.38%. 35(23.6%) were found to produce carbapenemase on testing with an automated system. Out of the total 35 carbapenemase producing GNB, 32 were correctly detected by double disc synergy test, 29 were correctly detected by modified Hodge test, 25 were correctly detected by E – test and 19 were correctly detected by combined disc test. The sensitivity was found to be 91.42% for double disc synergy test (DDST), 82.86% for modified Hodge test (MHT), 71.43% for E – test and 54.29% for combined disc test (CDT). **Conclusion** - Our study showed that DDST was the most effective test to detect carbapenemase, particularly MBL production, followed by MHT.

KEYWORDS: Metallo – β - lactamase, clinical isolates, β -lactams.

INTRODUCTION

A carbapenemase is a β -lactamase that hydrolyses carbapenems ie any one or all of imipenem, ertapenem, meropenem and doripenem. Carbapenemases are the most versatile family of β -lactamases which lead to resistance to several carbapenems and to other β -lactams as well. Failure to detect and treat severe infections caused by carbapenemase producers may lead to increased mortality.^[1] The carbapenemases which are plasmid borne and transferrable (e.g. the metallo- β -lactamases) are particularly feared because of their ability to hydrolyze many β -lactams, including the carbapenems. The metallo- β -lactamase (MBL) producing strains are not only resistant to all β -lactams but also to the aminoglycosides and fluoroquinolones.^[2] Other mechanisms responsible for carbapenem resistance

include failure of the drugs to penetrate due to mutation in porins, loss of certain outer membrane proteins and efflux pumps.^[3] The CLSI has recommended the modified Hodge test (MHT) for detection of carbapenemases in Enterobacteriaceae. However, this test has not been recommended in non-fermenters.^[4] Other phenotypic methods for detection of carbapenemases include the double disc synergy test (DDST)^[5], MBL E-test^[3] and the combined disc test. The presence of carbapenemase encoding genes can be confirmed by molecular techniques like PCR which is costly and hence not feasible in resource limited countries whereas detection of carriers is possible by using simple screening tests like the various phenotypic methods which have already been mentioned. This study was therefore undertaken to compare the various

phenotypic methods to detect carbapenemase production in carbapenem resistant Gram negative bacteria obtained from clinical samples in a tertiary care hospital, Kolkata.

MATERIALS AND METHODS

This study was conducted between Jan 2014 to Dec 2014 in the Department of Microbiology, Medical College & Hospital, Kolkata after getting the approval of Institutional Ethical Committee. All the samples of critically ill patients collected in the Department were processed. The samples were pus, sputum, urine, blood and endotracheal tube aspirate (collected according to the site of infection or clinical features).

The samples were inoculated on blood agar (containing 5% defibrinated blood) and Mac Conkey agar which were then incubated at 37°C for 24 hrs. The Isolation and identification of the microorganisms was done by standard microbiological procedures (colony morphology, Gram staining, motility and biochemical tests).

Thereafter, antimicrobial susceptibility test was performed by Kirby-Bauer disc diffusion method and interpreted according to CLSI guidelines.^[4] Those Gram negative bacilli that were found to be carbapenem resistant (CRGNB) or intermediate sensitive (CIGNB)^[4] (zone size – 16 – 21mm for meropenem and 19 – 21mm for ertapenem) were further tested by automated system (VITEK 2 – compact) for antibiotic sensitivity testing and carbapenemase production.

All the CRGNB or CIGNB were further subjected to phenotypic tests for detection of carbapenemase. The results of the phenotypic tests were compared with that of the VITEK 2 – compact.

Modified Hodge Test (MHT)

The detection of carbapenemases was done by the modified Hodge Test. An overnight culture suspension of *E. coli* ATCC 25922 adjusted to 0.5 McFarland standard was inoculated evenly on the surface of a Muller-Hinton agar (MHA) plate using a sterile cotton swab.

After a brief drying at room temperature, an imipenem disc (10µg) was placed in the centre of the plate and the four strains (two test, one positive control and one negative control) were streaked from the edge of the disc to the periphery of the plate in four different directions. After incubating the plate overnight at 37° C, the presence of a cloverleaf shaped zone of inhibition due to carbapenemase production by the test strain was considered as positive^{[5][6][7]}(Fig 1).



Fig 1 : Modified hodge test showing cloverleaf shaped zone of inhibition

The EDTA disc synergy test/ Double disc synergy test (DDST)

- A 0.5 M EDTA solution was made by dissolving 186.1 g of disodium EDTA. 2H₂O (REACHEM, Chennai, India) in 1,000 ml of distilled water. The pH was adjusted to 8.0 with the help of Sodium hydroxide (HI-MEDIA, Mumbai, India). Thereafter, the EDTA solution was sterilized by autoclaving^[8]. The test was done by simultaneous testing of two different β – lactams (meropenem and ceftazidime). An overnight culture suspension of the test isolate was adjusted to a turbidity of 0.5 Mac Farland standard and was inoculated on the surface of a Mueller Hinton agar plate. A 10 µg meropenem disc and 30 µg ceftazidime disc (HIMEDIA, Mumbai, India) were placed on the agar. A blank disc (6mm in diameter) was kept on the inner surface of the lid of the MHA plate and 10 µl of 0.5M EDTA was added to that disc. The EDTA disc was then transferred to the surface of agar and was kept 10 mm edge – to – edge apart from the meropenem and ceftazidime discs. After incubating overnight at 37°C, the presence of an expanded growth inhibition zone between the two discs was interpreted as positive for carbapenemase (MBL) production.^[8] (Fig 2)



Fig 2 : Double disc synergy test showing expanded growth inhibition zone between the two discs

The combined disc test (CDT) - An overnight culture suspension of the test isolate adjusted to a turbidity of 0.5 Mac Farland standard, was inoculated on the surface of a Mueller Hinton agar. A 10 µg imipenem disc and

IMP – EDTA (10/750 μ g) discs were placed on the agar. After a 24 hr incubation of the plate, the result was read. A ≥ 7 mm increase in the inhibition zone around the IMP – EDTA disc as compared to the IMP disc alone was interpreted as positive.^[7] (Fig 3)

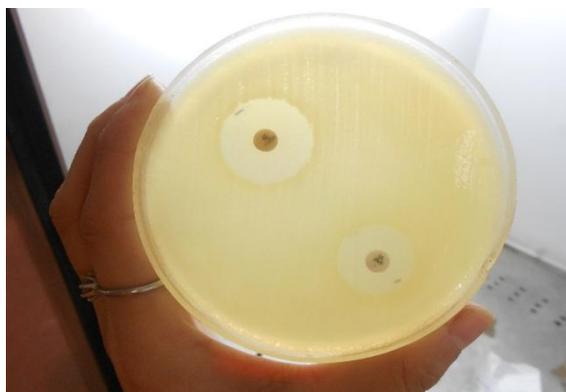


Fig 3: Combined disc test showing a ≥ 7 mm increase in the inhibition zone around the Imipenem– EDTA disc as compared to the Imipenem disc alone

E – Test - An overnight culture suspension of the test isolate was adjusted to a of 0.5 Mac Farland standard and was inoculated on the surface of a Mueller Hinton agar. An E – test strip with Meropenem on one side and MRP – EDTA on the other side were placed on the agar. After a 24 hr incubation of the plate, the result was read as positive if MIC for MRP /MRP EDTA > 8 , or reduction of meropenem MIC by ≈ 3 log 2 dilutions in the presence of EDTA or appearance of a phantom zone was interpreted as positive for carbapenemase production^[9] (Fig 4)

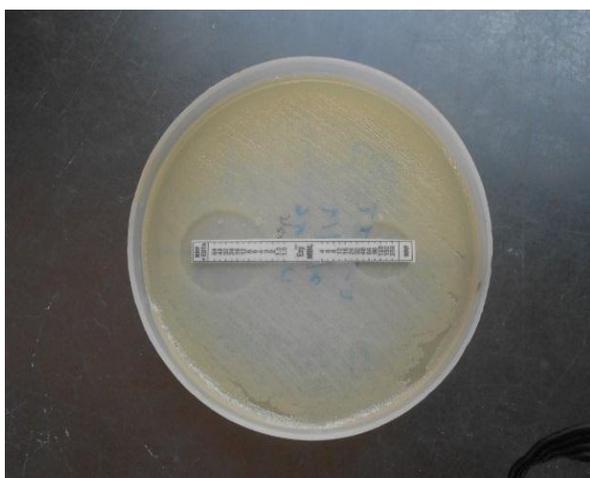


Fig 4: E – test showing MIC for MRP /MRP EDTA > 8 .

RESULTS

1404 Gram negative bacilli were isolated from different clinical samples received in the Department of Microbiology, out of which 148(10.54%) were found to have reduced susceptibility (zone size 16 – 21mm for meropenem and 19 – 21mm for Ertapenem) by Kirby - Bauer disc diffusion method. Out of these 148 CRGNB

(carbapenem resistant Gram negative bacilli) or CIGNB (carbapenem intermediate sensitive Gram negative bacilli) isolated, *E.coli* was isolated in 55 (37.16%) cases followed by *Klebsiella spp* 39(26.35%), *Acinetobacter spp.33* (22.3%), *Pseudomonas spp.11* (7.43%), *Citrobacter spp.5* (3.38%) and *Enterobacter spp.5*(3.38%). (fig 5)

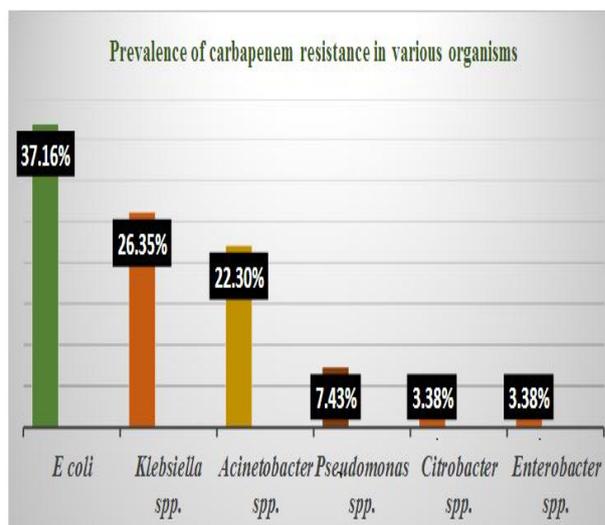


Fig 5: Prevalence of carbapenem resistance in various organisms

Among those Gram negative bacilli that were found to be resistant or intermediate sensitive to Carbapenem, 35 (23.6%) were found to produce carbapenemase on testing with an automated system. (VITEK 2 compact). Out of the total 35 carbapenemase producing GNB, 32 were correctly detected by double disc synergy test(DDST), 29 were correctly detected by modified Hodge test (MHT), 25 were correctly detected by E – test and 19 were correctly detected by combined disc test(CDT). (Fig 6)

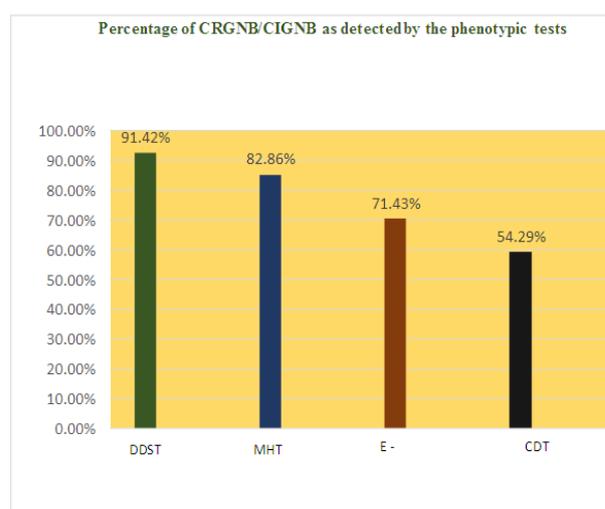


Fig 6: Percentage of CRGNB/ CIGNB as detected by the detected by the phenotypic tests

Statistical analysis –

The sensitivity was found to be 91.42% for double disc synergy test (DDST), 82.86% for modified Hodge test (MHT), 71.43% for E – test and 54.29% for combined disc test (CDT). Specificity of all the tests was calculated to be 100%. Thus, the double disc synergy test (DDST) was found to be the most sensitive test to detect carbapenemase production.

DISCUSSION

Carbapenems are often used as a drug of last resort for treatment of infections due to multi drug resistant Gram-negative bacilli. However, Carbapenem resistance is an emerging problem in infections caused by Gram-negative bacilli and is a matter of concern as they are found to be resistant to most other antibiotics. The first metallo- β -lactamase-producing *Pseudomonas aeruginosa* strain was isolated in Japan in 1988.^[10] Now, the metallo- β -lactamase producing isolates have disseminated worldwide.^{[11][12][13]} In India, MBL producing *Pseudomonas aeruginosa* was first reported in 2002.^[14]

In our centre, we found the prevalence of carbapenem resistance to be 10.54%. A similar study conducted by Datta *et al.* reported a CRE (carbapenem resistant enterobacteriaceae) prevalence rate of 7.87% in a tertiary care hospital^[15] whereas Gupta *et al.* reported carbapenem resistance varying from 17 to 22% among the various *Enterobacteriaceae* strains at their centre.^[16]

In contrast to our study, meropenem resistance was reported to be 30% in a study conducted by Mulla S *et al.*^[17] and 31.81% by Mahajan G *et al.* by Kirby bauer disc diffusion method.^[18]

We also found that the most common Gram-negative bacilli responsible for carbapenem resistance was *Escherichia coli* in our Hospital. This finding differs from the study conducted by Porwal *et al.* where they found *Klebsiella pneumoniae* to be the most common (44%) carbapenem resistant Gram-negative bacilli in their ICU setting.^[19]

Among those Gram-negative bacilli that were found to have reduced susceptibility to Carbapenem, 35 (23.6%) were shown to produce carbapenemase on testing with an automated system. (VITEK 2 compact). However, in a study conducted by Amjad A *et al.*^[20], 69% of the isolates showed the presence of carbapenemase by MHT. In a study by Mahajan G *et al.*^[19], 47.6% of the isolates were found to produce carbapenemase enzyme by MHT.

In our study, among these carbapenemase producing GNB, 32 (91.42%) were correctly detected by double disc synergy test (DDST), 29 (82.86%) were correctly detected by modified Hodge test (MHT), 25 (71.43%) could be detected by E – test and 19 (54.29%) were correctly detected by combined disc test (CDT). This finding is similar to the other studies where DDST was found to be more reliable and the rates of positivity for MBL ranged from 14.8% - 72%.^[21 - 23] while studies conducted by Behera *et al.*^[24] and Qu *et al.*^[25] suggested that CDT

was a better method for detecting MBL production than DDST. However, in this study, Carbapenemase production could not be confirmed by any molecular method like PCR which was a limitation of our study.

CONCLUSION

Our study showed that DDST was the most effective test to detect carbapenemase, particularly MBL production, followed by MHT. Presence of different carbapenemases is a matter of concern and should be routinely detected for development of newer therapeutic strategies and strict infection control practices. However, considering the cost constraints in resource limited countries, DDST (which is easy to perform and economical) can be included into routine testing protocols in laboratories to monitor the emergence and spread of MBLs and carbapenemases.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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