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PHENOTYPIC METHODS FOR DETECTION OF METALLO-B-LACTAMASE (MBL) PRODUCTION IN MULTIDRUG RESISTANT GRAM NEGATIVE BACILLI – COMPARATIVE STUDY

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ABSTRACT

Introduction: One of the most common mechanism of resistance of bacterial pathogens to β-lactam antibiotics is production of β-lactamase, there are different types of Beta lactamases, which are expressed by drug resistant gram-negative bacteria. Carbapenemases (Metallo beta lactamases/MBL) are the β-lactamases with the widest spectrum of activity. Early detection of MBL-producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their interhospital and intrahospital dissemination. Several phenotypic methods are available for the detection of MBL producing bacteria. As there is no standardized method present study was done to screen MDR gram negative bacilli isolated from clinical samples for MBL-production by a low cost, convenient and sensitive procedure. **Methods:** All non-duplicate MDR gram negative isolates obtained from various clinical samples were screened for carbapenam resistance. All carbapenam resistant bacteria were screened for production of MBL by 3 phenotypic tests (Double disc synergy, combined disk test, Hodge test). The results were compared and analyzed on the basis of results obtained by E test. **Results:** During Study Period, 988 non duplicate gram negative bacilli were isolated, 70.64% (698) were multi drug resistant. Amongst Total number of MDR Isolates to carbapenam resistance was seen in 62(9.28%). These 62 isolates that were resistant to carbapenam were tested for MBL production. 54 (87%) of these 62 isolates showed MBL production by disc potentiation test whereas 41 isolates (66%) gave positive result by DDST. By Modified Hodge test, out of 62, 48 isolates (77.4%) were MBL positive. Compared to E- test, the Sensitivity Specificity and Accuracy for Disc potentiation test was 90%,100% and 90.32%, for Modified Hodge test was 80%,100% and 80.6% and for Double disc synergy test was 68.3%,100% and 69.3%. **Conclusion:** In our study, in comparison to MBL E test, disc potentiation test is more sensitive than double disc synergy test and Modified Hodge test for detection of MBL phenotypically.

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INTRODUCTION

A significant public health concern is emerging antimicrobial resistance. Very few effective antimicrobials against infections caused by these multi-drug resistant bacteria are available. Such bacteria are resistant to most of the available antimicrobial agents, they may be susceptible to agents which hepatotoxic/ nephrotoxic , leaving limited treatment options [1,2]**.** Most commonly encountered resistance mechanism in multi-drug resistant bacteria is production of β-lactamase. Different types of these enzymes are expressed by drug resistant gram negative bacteria. One of the most active β-lactamases enzymes, which hydrolyze most of beta lactum antibiotics and have wide spectrum of activity are carbapenemases [3].

The genes responsible for production carbapenemases (Metallo beta lactamases/MBL) are carried on large plasmids, these plasmids also carry resistance genes to most of other classes of antibiotics. Hence multi drug resistance is common in such organisms [2]. Production of carbapenemases (MBL- "Metallo Beta Lactamase Enzyme") previously was species-specific as they were chromosomally mediated carbapenemases which had limited potential for spread except in clonal manner. Recently, plasmid-mediated carbapenemases, such as KPCs have gained attention [2]. In absence of proper treatment, patients infected with such MBL producing organism have mortality rate of almost 50% [4]. Inter-hospital and intra-hospital dissemination, mortality and morbidity can be prevented by early detection of MBL-producing organisms [5]. Screening for MBL-producing isolates in Microbiology laboratories should be performed by a sensitive test which is low cost and convenient. Hodge test, disk test using EDTA – Imepenam and Imepenam alone & Epsilometer test (E test) are some of the methods by which MBL producing bacteria can be detected in laboratory.

Objectives to conduct present study were to find out prevalence of Multi drug resistant gram negative organism isolated from various samples from our hospital & their antibiogram and to evaluate four different phenotypic tests for detecting metallo beta-lactamase enzyme production in multidrug resistant GNR.

Methods

For the purpose of present study MDRO (Multi drug resistant gram negative organisms) were defined as "All gram negative organism showing resistant to at least one agent in three or more antimicrobial categories of antibiotics" [6].

Inclusion Criteria: All non repetitive gram negative organisms, complying with the definition of MDRO. The study was Hospital based cross sectional study.

After sanction from the Institutional Ethical committee (Ref No: IEC/NKPSIMS/1/2017), study was conducted in microbiology lab of our tertiary care hospital from Sept 2017 –Sept 2019, Isolation and identification of all non duplicate gram negative organisms obtained from clinical samples from admitted patients were done by "standard bacteriological methods "and sensitivity testing was done by disc diffusion methods according to "CLSI guidelines". Imepenam resistant isolates were screened for MBL by following tests for detection of MBL. Statistical analysis was done by using SPSS. V. 24

Metallo Beta Lactamase detection:

(I) Disc Potentiation test/Combined Disc test (Imipenem (IMP)-EDTA combined disc test)[7]

Lawn culture of test organism was done onto Mueller Hinton agar plate. Two 10 µg Imipenem discs were placed on inoculated plates and 5 µl of 0.5 M EDTA solution was added to one Imipenem disc. After incubation at 35°C for 16 to 18 hrs, the zone of inhibition around Imipenem discs was recorded. An increase in zone size of at least 7 mm around the Imipenem EDTA disc was recorded as a positive result.

(II) Double Disc synergy test[8]

Lawn culture of test organism was done onto Mueller Hinton agar plate. After 15 min, a 10-µg imipenem disc and a blank filter paper disc was placed 10 mm apart from edge to edge, and 10 µL of 0.5 M EDTA solution was then applied to the blank disc, which resulted in approximately 1.5 mg/disc. After overnight incubation, the presence of an enlarged zone of inhibition around the blank disc was interpreted as EDTA-synergy test positive.

III) Modified Hodge Test for Carbapenemase Detection [9]

A lawn culture of 1:10 dilution of *E. coli* ATCC 25922 to a Mueller Hinton agar plate was made and allowed to dry for 3–5 minutes. A 10 µg Imepenam susceptibility disk was placed in the center of the test area. In a straight line, four strains of test organisms from the edge of the disk to the edge of the plate were streaked. After 16–24 hours of incubation, the plate was examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of

inhibition of the carbapenem susceptibility disk. Test organisms showing indentation were considered positive.

IV) E-Test[10]

In the presence of EDTA if MIC reduction of Imepenam was by three or more 2-fold dilutions test was interpreted as positive for metallobeta lactamase enzyme production. Amongst phenotypic tests, E- test was considered to be gold standard. Sensitivity and specificity of other phenotypic tests were derived by taking E test to be the gold standard.

RESULTS

988 isolates of gram negative organisms from clinical samples of admitted patients were included for the study. Antibiotic sensitivity by disc diffusion test revealed that 70.64% (698) were multi drug resistant. The samples were obtained from critical care areas of hospital. (ICU, SICU, Burn ward, PICU/NICU and Surgical wards). Identification of isolates was done; maximum isolates were Klebsiella Species, 260 (39%), followed by *E. coli* 194 (29%). Pseudomonas, Acinetobacter and other Non fermenters contributed (25.7%). Forty (6%) isolates were other enterobacterales (Citrobacter, Enterobacter, Serratia) Table 1

Table 1: MDR Isolates

ISOLATES	No of Strains (698)
Klebsiella	272 (39%)
E. coli	202 (29%)
Pseudomonas	112 (16%)
Acinetobacter	43 (6%)
Non-fermenters	26 (3.7%)
Others	43 (6%)

Antibiotic sensitivity pattern of all multi drug resistant isolates by the disc diffusion (CLSI) method showed 80% isolates were resistant to Beta lactum antibiotics, 74% to BL- BLI combinations, 80% isolates were resistant to Fluroquinolones, 66% to aminoglycosides, 71% to Pipracillin-tazobactum and 9.2% were resistant to carbapenams. The antibiogram of Carbapenam resistant isolates is shown in table 2. Pseudomonas (29.62%) and Acinetobacter (24.39%) were the most common isolates showing resistance to carbapenams as mentioned in table 3

All 62 carbapenam resistant isolates were tested for production of metallo beta lactamase enzyme. 87% of these isolates showed production of MBL enzyme by disc potentiation test, 66% isolates showed production MBL enzyme by DDST and combined disc test. Further, out of 62, 48 isolates (77.4%) showed production MBL enzyme production by Modified Hodge test. All Fifteen Klebsiella pneumonia showed production MBL enzyme by Modified Hodge test. Again, By E-test (Ezy MIC Strip -Hi-Media) 96.7% isolates showed production MBL enzyme. Compared to E- test, the Sensitivity Specificity and Accuracy for Disc potentiation test was 90%,100% and 90.32%, for Modified Hodge test was 80%,100% and 80.6% and for Double disc synergy test was 68.3%,100% and 69.3%. The results are summarized in table 4.

DISCUSSION

In infections caused by multi drug resistant organisms, carbapenams are used as the last resort for treatment. There are reports to show that the resistance to this life saving antimicrobial is increasing since last fifteen years. Chromosomal genes were known only in P. *aeruginosa* and Acinetobacter spp, and spread was mostly clonal, but now members of Enterobacteriaceae have acquired resistance to carbapenams which is plasmid mediated and is disseminated in inter and intraspecies [11]. Infection with Metallo-beta-lactamase (MBLs) producing organisms leads to higher rates of Morbidity, Mortality, apart from higher health care costs [12].

Table 2: Antibiotic sensitivity pattern of Carbapenam resistant MDRO

Antimicrobial agents	Sensitive (%)
Cefuroxime	0%
Ceftazidime	0%
Cefepime	0%
Pipracillin Tazobactum	2%
Amikacin	8%
Ciproflox	0%
Levoflox	1%
Ceftriaxone-Sulbactum	0%
Ceftriaxone-Sulbactum	0%
Imepenam	0%

Detection of resistance mechanism for carbapenam is important for selecting the antimicrobial for proper treatment and it also helps in applying infection control measures. Hence Screening for MBL-producing isolates in microbiology laboratories in hospitals with a high prevalence of MBL-producing isolates should be performed by a sensitive test which is low cost and convenient a phenotypic test which is accurate and easy-to-perform [13,14]. Currently there are no standardized recommendations for MBL screening [15].

In countries where carbapenam resistance amongst clinical isolates is high simple sensitive screening test in laboratory to detect MBL production in Gram-negative bacilli is useful. Antimicrobial resistance (AMR) is global challenge, due to increasing antimicrobial resistance treatment of common pathogens has become challenging. Rising AMR has resulted in morbidity, mortality, increased duration of hospital stays thus increasing the costs and number of complications. It has been projected that worldwide 10 million deaths will occur due to AMR by 2050 [16].

Table 3: Presence of carbapenam resistance in gram negative isolates

	ISOLATES	MBL	%
Klebsiella	272	15	5.76
E. coli	202	0	0
Pseudomonas	112	32	29.62
Acinetobacter	43	10	24.39
Non-fermenters	26	5	20
Others	43	0	0
Total	668	62	9.28

Table 4: Analysis of Phenotypic tests

Method	Total strains tested	Positive	Sensitivity (%)	Specificity (%)	Accuracy (%)
Disc Potentiation	62	54 (87%)	90	100	90.3
Double Disc Synergy Testing	62	41(66%)	68.3	100	69.3
Modified Hodge Test	62	48 (77.4%)	80	100	80.6

MDRO have been defined as "All gram negative organism showing resistant to at least one agent in three or more antimicrobial categories of antibiotics" [7]. In the present study amongst 988 gram negative bacilli studied 70% isolates were MDRO as per the definition. Most of these isolates were obtained from patients admitted in areas where maximum antibiotics are used. Increasing rates of multi-drug resistance in gram-negative bacteria has been reported by various authors. In a study done Siwakoti et al. [17], 47% admitted patients were infected by multi-drug resistant gram-negative bacteria. In a systematic scoping review by Saharman et al. most of the isolates obtained from ICU from Low and Middle income Countries were resistant to multiple classes of antibiotics.

In list of priority pathogens (PPL) prepare by WHO, drug resistant bacteria are classified as "critical, high, and medium priority organism" [18]. According to this list 'Critical priority' pathogen includes Enterobacteriaciae (Carbapenam/Third generation Cephalosporin Resistant), Acinetobacter and Pseudomonas. In present study most of the isolates showing multi drug resistance belonged to critical priority organisms, *Enterobacteriacie* (74%), followed by *Pseudomonas* (16%), Acinitobacter and non fermenters. Resistance to carbapenam was seen in 62(9.28%) isolates. Most common isolates producing carbapenamases were *Pseudomonas* (29.62%) and *Acinetobacter* (24.39%) The 2017 guidance document, WHO reported that in the Indian subcontinent carbapenem resistance, is seen in *Acinetobacter baumannii* (more than 50% isolates) and Pseudomonas aeruginosa accounts (31% to 50% isolates). ICMR-AMRSN surveillance data for antimicrobial resistance from 22 sites in India showed approximately 80% isolates of *Acinetobacter baumannii* and around 30% isolates of *Pseudomonas aeruginosa* are resistant to carbapenem. In a contrasting study in children by Thacker et al only 15% of Pseudomonas aeruginosa were resistant to Carbapenem [22]. In study by Mogasale et al, 12% and 5 % *Acinetobacter baumannii* and *Pseudomonas aeruginosa* respectively showed carbapenem-resistance [20].

Different studies have shown varying number of isolates which are resistant to carbapenam, most of the studies have focused on *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Non fermenters, very few studies have included Enterobacteriaceae isolates. Phenotypic test for MBL enzyme production, which indicates production of carbapenamases, is based on the principle of metal ions chelation. Chelation of metal ions is by the presence of EDTA / thiol based compounds which are metal chelators. MBL enzyme activity is inhibited in absence of metal ion based on this principal, MBL enzyme production can be detected by different phenotypic tests [23]. *Metallobeta lactamase* enzyme production was tested by various phenotypic tests in all 62 isolates showing resistance to carbapenams. MBL enzyme production was shown by 54 (87%) carbapenam resistant isolates by disc potentiation test **(**Imipenem-EDTA

combined disc test), whereas 41 isolates (66%) showed positive result by DDST and disc potentiation test. By Modified Hodge test, out of 62, 48 isolates (77.4%) were positive for *Metallobeta lactamase* enzyme production. All the Enterobacteriaceae isolates (100%) showed production of metallobeta lactamase enzyme by Modified Hodge test.

E- test was done by Imipenem with and without EDTA Ezy MIC Strip^{R} (Hi-Media) Sixty isolates (96.77%) were positive for metallobeta lactamase enzyme production. In a similar study 250 imipenem resistant Pseudomonas isolates for metallobeta lactamase enzyme production by three phenotypic methods were studied by Sachdeva et al [21]. Results of their study showed that out of 147 isolates which showed production of metallobeta lactamase enzyme production by E-test 92(62.5%) showed metallobeta lactamase enzyme production by Modified Hodge test, 122 (82.3%) by DDST and 144(97.9%) by Combined disc method. Similar sensitivity for detection of metallobeta lactamase enzyme by Imipenem-EDTA combined disc test and imipenem-EDTA MBL E test was reported in the study by Behra and collegues [22]. In a similar study out of 84 genotyping confirmed MBL-producing isolates, Combined-disk test was able to detect all 84 (100%) isolates (100% Sensitivity) 66 (79%) were detected by Double-disk synergy test [24,25].

Both chromosomally and plasmid mediated metallo β lactamases, can be detected by the E test MBL strip (IP-IPE) in aerobic and anaerobic bacteria [26].Hence E.-Test was used as gold standard to determine Sensitivity, Specificity and accuracy**.** Compared to E- test, the Sensitivity Specificity and Accuracy for Disc potentiation test was 90%, 100% and 90.32%, for Modified Hodge test was 80%, 100% and 80.6% and for Double disc synergy test was 68.3%, 100% and 69.3%. (Table 5), depicts comparison of findings of sensitivity between disc potentiation and double disc synergy testing in studies conducted on phenotypic tests for MBL by various authors.

Present study showed that disc potentiation is more sensitive than double disc synergy testing in detection of MBL producers. Similar findings were seen in studies by Clare franklin et al, Nirav et al, B. Behra et al [22,23]. Few studies have shown better sensitivity with DDST, the reason for the difference may be because the interpretation of result in DDST is subjective. In disc potentiation results are seen more clearly than double disc synergy testing [25,26]. Also, in most of the studies cited above, Enterobacteriaceae group was not included and the tests were performed on Pseudomonas and Acinetobacter/Nonfermenter species only.

Table 5: Comparison of disc potentiation and double disc synergy testing in various studies

Study	Disc Potentiation	Double Disc Synergy Testing
Galani et al.[24]	94.7%	100%
Picao et al.[25]	80	82.6
Franklin et al.[23]	100	79
Nirav et al.[26]	96.3	81.4
B Behera et al.[22]	85.7	64.2
Present study	90	68.3

All the Enterobacteriaceae isolates (100%) showed MBL production with Modified hodge test, It confirms the CDC guidelines which recommends MHT for detection of MBL in Enterobacteriaceae.

CONCLUSION

Around 70% isolates in the present study are Multi drug resistant gram negative organisms, and they is increasing rapidly. It is imperative to detect and control the spread of these MDR pathogens. Treatment of infections caused by MDR ("Multi drug resistant") pathogens is difficult to treat causing high mortality and morbidity.

Carbapenams are the mainstay of treatment for these MDR pathogens, around 10% of the isolates in present study were resistant to carbapenams and this is resistance is being transmitted from *Psedomonas* and Acinetobacter to Enterobacteriaceae isolates. Phenotypic tests are the cost-effective solution in resource limited settings, apart from E test which is gold standard, disc potentiation test for *Psedomonas* and *Acinetobacter* and Modified Hodge test for *Enterobacteriaceae* isolates can be used to detect *metallobetalactamases* in clinical isolates. In isolates producing *metallobetalactamases* instead of carbapenams other alternative antibiotics can be used. The drawbacks in present study are absence of molecular detection of various genes responsible for MBL enzyme production

FINANCIAL ASSISTANCE

The study was internally funded by the college and no external grant or financial assistance was received.

CONFLICT OF INTEREST

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

Umesh Santlal Hassani contributed in conceptualizating of the study. He also contributed in comparing antibiograms for Multi drug resistant organisms. Performing different Phenotypic tests. Rashmi Mahalle prepared the first draft of manuscript and checked it. She did the necessary corrections in the manuscript and she also contributed in doing statistical analysis.

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