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Prevalence of Gram negative bacilli showing resistance to carbapenems

Medical Science

KEYWORDS : - Carbapenems, double disk diffusion test, Modified Hodge Test.

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ABSTRACT

Carbapenems are the β-lactam antibiotics used in the treatment of infections due to multidrug-resistant gram negative bacteria. The aim of study is to determine the prevalence of carbapenemase producing gram negative bacteria phenotypically among various clinical isolates received in the microbiology laboratory in a tertiary care hospital. Double disk diffusion test and Modified Hodge test (MHT) were employed for carbapenemase detection. Out of 849 isolates, 145 were resistant to meropenem and 92 were resistant to imipenem by disc diffusion method. 117 positive by MER-EDTA, 75 positive for IMP-EDTA and 79 positive for MHT. Use of both MHT and combined disk test with EDTA as a screening method can increase sensitivity of detection of carbapenemases and can aid timely intervention to initiate infection control practices and thereby improving the outcome of patient.

Introduction:

Infections caused by multidrug-resistant gram-negative pathogens impose a significant and increasing burden on both patients and healthcare providers. [1, 2] The term carbapenemase is used for any β-lactamase that hydrolyses carbapenems. Carbapenems are the β-lactam antibiotics used in the treatment of infections caused by β-lactam resistant Gram-negative bacteria due to the stability of carbapenems against majority of β-lactamases and their high rate of permeation through bacterial outer membranes. [3] Acquired carbapenem resistance due to the production of metallo-β-lactamases (MBLs) has been increasing. There are several mechanisms for carbapenem resistance such as the lack of drug penetration due to mutation in porins, loss of certain outer membrane proteins and efflux mechanisms.[4] The resistance mediated by MBL is plasmid mediated so it can spread faster. [5] Consequently, the rapid detection of carbapenemase production is necessary to initiate effective infection control measures to prevent their dissemination. Recently, CLSI has recommended Modified Hodge test for detection of carbapenemase activity in Enterobacteriaceae. Detection of genes coding for carbapenem resistance by PCR, usually give reliable and satisfactory results, but this method is of limited practical use for daily application in clinical laboratories because of the cost. Carbapenemase production is normally associated with generalized resistance to carbapenems, penicillins and cephalosporins, and these strains usually also harbour mechanisms of resistance to aminoglycosides and quinolones. Carbapenemase-producing pathogens have been associated with high rates of morbidity and mortality, particularly among critically ill patients with prolonged hospitalization. [6] The primary aim of the study is to determine the prevalence of carbapenemase producing gram negative bacteria using different phenotypic methods (double disk diffusion method and modified hodge test).

Materials and Methods

Bacterial Isolates

A total of 849 non-duplicate Gram negative bacterial isolates obtained from various clinical samples were processed at the Department of Microbiology by standard microbiological techniques for the isolation and the identification of the isolates.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing (AST) was done by the Kirby-Bauer disc diffusion method [7] according to the recommendations by Clinical and Laboratory Standards Institute(CLSI) [8]. The antimicrobial susceptibility test discs (Hi Media, Mumbai) used were namely, Amikacin 30 µg; Amoxy-clav 30 µg; Ampicillin 10 µg; Cefoperazone/Sulbactum 75/10 µg; Cefotaxime 30 µg; Ciprofloxacin 5 µg; Co-Trimoxazole 25 µg; Gentamicin 10 µg; Imipenem 10 µg; Meropenem 10 µg; Piperacillin /Tazobactum 100/10 µg; Aztreonam 30 µg; Cefepime 30 µg; Cefoxitin 30 µg; Ceftazidime 30 µg; Chloramphenicol 30 µg; Netilmicin 30 µg; Tetracycline 30 µg; Tigecycline 15 µg were used.

The antibiotic discs were placed on Muller-Hinton agar plates that were seeded with test organism, adjusted to a turbidity of 0.5 McFarland standard. The plates were incubated at 37°C for 18-24 hours after which zones of inhibition were measured and interpreted as per CLSI guidelines. The isolates resistant to imipenem and meropenem, with zone of inhibition ≤13 mm were used for phenotypic detection of carbapenemase production by double disk diffusion test using imipenem and meropenem with and without Ethylene Diamine Tetra Acetic acid(EDTA).

Phenotypic detection of Carbapenemases

Double disk diffusion test :

The imipenem and meropenem resistant strains were subjected to double disk diffusion test for detection of carbapenemases. [9, 10] An overnight broth culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on Muller Hinton agar plates. Two disks namely Imipenem (10 µg) and Imipenem-EDTA disks were placed at a distance of 30 mm centre to centre on the test organism inoculated plates. Imipenem-EDTA disks were prepared by applying 10 µl of a sterile 0.5 M EDTA (pH 8.0) solution on the Imipenem disk and allowed to dry. The plates were incubated at 37°C for 18-24 hrs. After incubation the zones of inhibition around the IMP and IMP-EDTA disks were measured, and increase in the zone size of ≥7 mm for test strains in the presence of EDTA were noted and interpreted as carbapenemase positive. The double disk diffusion test was also performed using Meropenem (10 µg) and Meropenem-EDTA disks (Figure 1).

Modified Hodge test (MHT):
The Imipenem and Meropenem resistant strains were tested for carbapenemases by Modified Hodge test. [11, 12] Briefly, a 0.5 McFarland turbidity standard of fresh overnight growth of the indicator organism *Escherichia coli* ATCC 25922 was inoculated on Mueller Hinton Agar (MHA) (Hi-Media, Mumbai). After drying, 10 µg Imipenem disk (Hi-media, Mumbai, India) was placed at the center of the plate. The test strains were heavily streaked from the edge of the disc to periphery. The plates were incubated overnight at 37°C. The presence of "clover leaf indentation" by the test strain was considered positive due to carbapenemase production. The procedure was repeated with 10 µg of Meropenem disk (Hi-media, Mumbai, India) with same test organism. In house known positive and negative controls were used.

Results:
Out of 3728 samples received, 1620 were urine, 339 pus, 480 sputum, 185 body fluid, 910 blood, 87 stool and 107 cerebrospinal fluid. A total of 849 Gram negative bacterial isolates were identified and included in this study. Of which *Escherichia coli* (41.22%) was the predominant followed by *Pseudomonas spp.* (15.78%), *Klebsiella spp.* (13.54%), *Enterobacter spp.* (12.24%), *Proteus spp.* (5.65 %), NFGNB (4.49%), *Moraxella spp.* (2.94%), *Citrobacter spp.* (1.88%), *Providencia spp.* (1.53%), *Morganella spp.* (0.58%). (Table: 1)

Antibiotic sensitivity pattern of Gram negative bacteria shown that 145(17.07%) isolates were resistant to Meropenem and 92(10.83%) isolates to Imipenem by disc diffusion method (Table 2).

117 (80.68%), 75 (81.52%) and 79 (68.27%) were positive for carbapenemases by MER-EDTA double disk diffusion test, IMP-EDTA double disk diffusion test and Modified Hodge test respectively (Table 3).

No significant difference was noted in the detection of Metallo beta-lactamase by DDST using either of Imipenem and Meropenem disks but as compared to Modified Hodge test(68%) the positivity was higher with DDST(80%).

Discussion:
Acquired carbapenem resistance is being increasingly reported among various Gram negative pathogens specially members of *Enterobacteriaceae*. Carbapenemases are classified as molecular class B (metallo beta-lactamases) or molecular classes A and D (serine carbapenemases). They are the last line of defense, used for treating multidrug resistant isolates associated with ESBL and AmpC production, so the emergence of carbapenemases is perceived as a real threat. [10] Carbapenemases are most commonly seen in non- lactose fermenting gram negative organisms namely *Pseudomonas spp.* and *Acinetobacter spp.* But from last few years the numbers of carbapenemases producing Enterobacteriaceae isolates are increasing. [11] In the present study majority of isolates showed resistance to other group of antibiotics including third generation cephalosporins, aminoglycosides and quinolones. Meropenem and Imipenem are the available carbapenems in India. There is an increasing resistance in Gram-negative bacteria towards Meropenem and Imipenem in India, but study in this area is limited. [12] Meropenem is well-tolerated and offers several potential advantages, including greater *in-vitro* activity against Gram-negative pathogens and the option of bolus administration. [13]Resistance to Meropenem was significantly higher to Imipenem which is similar to study by Gupta *et.al,* and others.[3, 14] Resistance to these drugs varies from 4-60%. [15-16] The resistance to carbapenems especially in *Pseudomonas spp*. results from reduced levels of drug accumulation or increased expression of pump efflux. [17]

A study conducted on *Acinetobacter baumanii* for the prevalence of metallo beta lactamase using double disk synergy test and PCR among the isolates resistant to one or both of Meropenem and Imipenem 9.3% were positive by imipenem-EDTA Microbiological assay and 6.97% by extended Imipenem-EDTA disk synergy test. [18]

A study from north India reported 7.87% carbapenem resistance which is lower as compared to our study as the carbapenem resistant strains at our centre were 17.07% (145/849). Metallo beta lactamase resistance in that study was 5.75%. [19] This could be due to our institute being a tertiary care centre, there is increased use of Carbapenems in patients with life threatening diseases.

A study on nonfermenters for the production of carbapenemases from Pondicherry reported that 50% of *Pseudomonas aeruginosa* that were resistant to Meropenem were MBL producers by disk synergy test but only 28.1% were positive by Modified Hodge test. One isolate positive by Modified Hodge test was found negative for MBL and AmpC β-lactamase by other methods. Of the Meropenem resistant *A. baumannii*, 6.5% were found to be MBL producers, of which one (14.3%) was positive for carbapenemases by Modified Hodge test. [20]

Although different phenotypic methods have been described for a long time there are no standard guidelines for screening of carbapenemases. [18] We have used EDTA as chelating agent as it was reported to be more sensitive than Sodium Mercaptoacetic Acid(SMA) disk for *Pseudomonas spp*. Proposed by Galani et al., and also reported that using ceftazidime was better for detecting MBLs compared to Imipenem disk by Mendiratta *et al* and Hemalatha. [21-23] But Balan *et al* reported that both imipenem and ceftazidime are equally efficient in detecting MBLs.[24] We have detected carbapenemases phenotypically using IMP-EDTA double disk diffusion test, MER-EDTA double disk diffusion test and Modified Hodge test using Imipenem. Our study shows that IMP-EDTA double disk test and MER-EDTA double disk test are equally effective for detection of carbapenemases.

CLSI has recommended Modified Hodge test (MHT), a generic phenotypic test that can be useful to demonstrate the production of carbapenemase enzymes in Enterobacteriaceae, especially for Klebsiella pneumoniae Carbapenemase(KPC) in *Klebsiella spp*., but not in non-fermenters. A study carried out in Greece in 2007 showed that 98 % cases were positive and 0.03 % were false positive considering PCR as gold standard.

A study was done comparing DDST with Imipenem-EDTA and Meropenem-EDTA which has reported higher specificity, sensitivity and positive predictive value using imipenem with EDTA as compared to Meropenem with EDTA. In our study no difference was noted among the DDST using the two carbapenems. But detection of metallo-beta lactamases was higher with DDST using either of the two carbapenemase with EDTA as compared to Modified Hodge test. [25]

Conclusion:
The Gram negative bacteria cause clinically important infections which may lead to severe morbidity and mortality. The presence of carbapenemase producing gram negative bacteria have rapidly emerged causing significant therapeutic problems. Clinical microbiology laboratories must take

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an aggressive approach to detect carbapenemases in order to provide clinicians with clinically relevant susceptibility results. The double disk diffusion test with either of Meropenem or Imipenem has clear advantage for detection of metallo beta lactamase detection as compared to Modified Hodge test.

Table 1: Number of Gram negative bacteria from various clinical samples.

	Number of Organisms in each sample								(%)
Organisms	Urine	Pus	Sputum	Body fluid	Blood	Stool	CSF	Total	
Escherichia coli	211	54	23	24	25	13	00	350	41.2%
Enterobacter spp.	61	10	12	05	13	00	03	104	12.2%
Klebsiella spp.	43	19	43	02	05	01	02	115	13.5%
Citrobacter sp.	08	02	01	02	03	00	00	16	1.8%
Providencia sp.	08	05	00	00	00	00	00	13	1.5%
Proteus sp.	32	13	00	00	03	00	00	48	5.6%
NFGNB	08	09	03	12	05	00	02	39	4.6%
Pseudomonas sp.	39	33	25	09	23	00	05	134	15.7%
Moraxella	01	00	23	01	00	00	00	25	2.9%
Morganalla	03	00	02	00	00	00	00	05	0.58%
Total	414	145	132	55	77	14	12	849	

Antibiotic sensitivity pattern of Gram negative bacteria shown that 145 isolates were resistant to Meropenem and 92 isolates to Imipenem by disc diffusion method. These were selected for testing carbapenemase production (Table 2).

Table 2- Gram negative bacteria showing resistance to carbapenemases

Organisms		Meropenem (M) resistance	% M resistance for organism	Imipenem (I) resistance	% I resistance for organism
Escherichia coli	n= 350	38	10.8%	22	6.28%
Enterobacter sp	n= 104	16	15.38%	10	9.61%
Klebsiella sp.	n= 115	26	22.6%	15	13.04%
Citrobacter sp.	n= 16	03	18.75%	00	0%
Providencia sp.	n= 13	04	30.76%	01	7.69%
Proteus sp.	n=48	05	10.4%	02	4.16%
NFGNB	n= 39	11	28.2%	09	23.07%
Pseudomonas sp.	n= 134	42	31.34%	33	24.62%
		145		92	

117 were positive for carbapenemases by MER-EDTA double disk diffusion test, 75 were positive for IMP-EDTA double disk diffusion test and 79 were positive for Modified Hodge test respectively. Percentages were given in relation to individual organism. (Table 3)

Table 3: Results of Double disk diffusion test and Modified Hodge test for detection metallo beta lactamase (MBL).

Bacteria		Number of positives (%)		
		Double Disk Diffusion Test		Modified Hodge test using imipenem
		MER-EDTA	IMP-EDTA	
Escherichia coli	n=350	34 (9.71%)	18 (5.14%)	18 (5.14%)
Enterobacter sp	n=104	09 (8.65%)	07 (6.73%)	08 (7.69%)
Klebsiella sp.	n=115	23 (20.0%)	11 (9.56%)	12 (10.43%)
Citrobacter sp.	n=16	01 (6.25%)	00	00
Providencia sp.	n=13	02 (15.38%)	01(7.69%)	00
Proteus sp.	n=48	02 (4.16%)	00	01 (2.08%)
NFGNB	n=39	10 (25.64%)	08 (20.51%)	07 (17.94%)
Pseudomonas sp.	n=134	36 (26.86%)	30 (22.38%)	33 (24.62%)
Total		117	75	79

Figure legends:

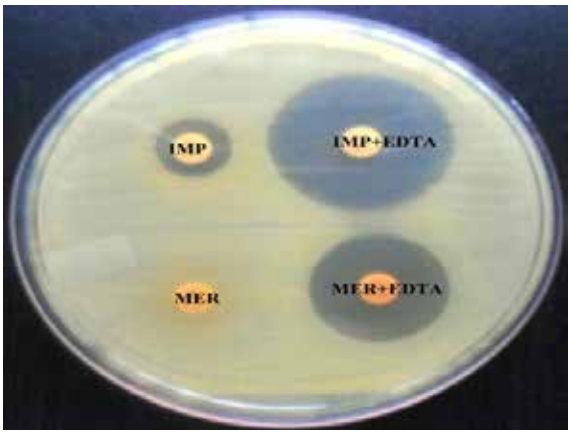

Figure 1: Showing carbapenemases production ≥7mm of IMP (Imipenem) - EDTA than IMP and MER (Meropenem) - EDTA than MER.

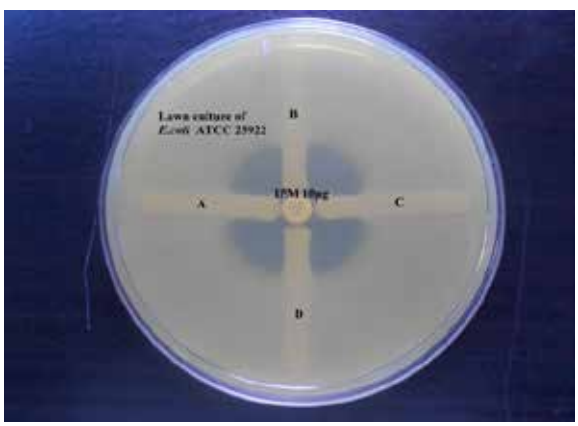

Figure 2: Modified Hodge test: Clover leaf shaped inhibition zone indicating carbapenemases production.

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