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PHENOTYPIC DETECTION OF METALLO-BETA-LACTAMASES AND THEIR SUSCEPTIBILITY PATTERN IN ENTEROBACTERIACEAE FROM CLINICAL SPECIMEN IN A TERTIARY CARE HOSPITAL, NORTH INDIA.

Microbiology

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ABSTRACT

BACKGROUND: The recent global emergence and dissemination of Carbapenemases-producing GNB (Enterobacteriaceae) that are resistant to carbapenems is a significant concern with respect to patient care. It is important to find carbapenem resistance among routine clinical isolates of family Enterobacteriaceae so as to guide the clinicians in the selection of appropriate antimicrobial treatment and infection control measures.
METHODS: The present study was conducted in the Department of Microbiology in the National Institute of Medical Science and Research (NIMSR), Jaipur, Rajasthan over a period of 5 months study from October 2019 to February 2020. All the clinical isolates of Enterobacteriaceae were screened for Imipenem and Meropenem resistance as per CLSI guidelines 2019 and phenotypic confirmation was done by EDTA disk diffusion test and Modified Hodge Test.
RESULTS: A total of 335 isolates of Enterobacteriaceae were included in this study. Out of 335 isolates, 144 (42.9 %) were male patients and 191 (57.1%) were female patients. The most common organisms isolated were E.coli, Klebsiella spp., Citrobacter spp. and Proteus spp. 59.70%, 27.16%, 6.5% and 4.26% respectively. Out of total isolates, clinical specimen distribution was as following Urine 158 (47.16%) followed by Pus 50 (14.92%) and sputum 44(13.13%). Imipenem and meropenem resistance was seen in 78(23.28%). The two methods used for confirmation of MBL production in our study were EDTA Disk Synergy Method and MHT (Modified Hodge Test) and they showed MBL production in 68 (87.18%) & 61 (78.21%) respectively. A more specific method was found to be the EDTA disk synergy method. Antibiotic sensitivity pattern of non-MBL producers & MBL producers by EDTA Disk Synergy Method was observed and compared. Non-MBL producers showed 100% sensitivity towards Polymyxin B and Colistin followed by Meropenem, Imipenem, and Tetracycline 78.65%, 74.15%, and 71.16% respectively. In MBL producers only Polymyxin B and Colistin showed 100% sensitivity while none other class showed any significant sensitivity.
CONCLUSIONS: Detection of MBLs production is a critical issue because it is often associated with expensive antibiotic treatment, treatment failures, and infection-associated mortality.

KEYWORDS

Enterobacteriaceae, MBL, Modified Hodge test, EDTA, CRE.

INTRODUCTION:

The rapid and irrepressible increase in antimicrobial resistance of Enterobacteriaceae is widely accepted as a major problem that has been observed over the last decade [1]. Enterobacteriaceae members have become one of the most important causes of nosocomial and community-acquired infections [2].

Enterobacteriaceae are normally found as intestinal normal flora and its members include E.coli, Klebsiella spp. Citrobacter spp. Salmonella spp. Proteus spp. etc [3].

These bacteria are capable enough to become resistant to antibiotics by a number of mechanisms both intrinsic and acquired and most common of which include enzymatic degradation of antibiotics [4]

Enterobacteriaceae can be seen with extended-spectrum beta-Lactamases (ESBL) and AmpC-type beta Lactamases with cephalosporins Carbapenem-resistant Enterobacteriaceae is defined as Enterobacteriaceae that test resistant to at least one of the Carbapenem antibiotics (Ertapenem, Meropenem, Doripenem, and Imipenem) or produce a Carbapenemase (an enzyme that can make them resistant to carbapenem antibiotics). [5, 6].

The carbapenemases producing GNB (Enterobacteriaceae) are easily transmitted among humans. The MBL producing Enterobacteriaceae possesses genetic material transferred from each other through horizontal gene transfer by the means of plasmids and by transposons [7]. Increasing resistance to carbapenems is now frequently being observed in many hospital-acquired and several community-acquired infections [8].

Ambler and others have classified carbapenemases broadly into two types based on the reactive site of the enzymes. One is Serine carbapenemases and the other is Metallo-β-Lactamases [9]. MBLs producers spread easily via plasmid transfer and cause hospital-acquired infections. Such infections like pneumonia, blood infection, urinary tract infection, etc. mainly concern patients admitted in ICU with several co-morbidities and a history of long time administration of antibiotics [10]. Early detection of MBL-producing organisms is strategic to establish appropriate antimicrobial therapy [11]. So the present study was undertaken to detect screening for MBL-producing isolates by a low cost, convenient and sensitive antibiotics for Enterobacteriaceae.

MATERIAL AND METHOD:

A total of 335 clinical isolates of Enterobacteriaceae, which were isolated from various samples (blood, urine, sputum, pus, E.T., and swab) were identified by standard procedures.

Samples were inoculated on Blood agar, MacConkey agar, and Nutrient agar Identification of isolates was done by using standard biochemical tests. The susceptibility testing of isolates to antibiotics was determined by the Kirby-Bauer disc diffusion method using the Clinical and Laboratory Standard Institute (CLSI) guidelines (2020) [9]. Antibiotics included were Ampicillin (10μg), Ceftazidime (30μg), Gentamycin (10μg), Imipenem (10μg), Meropenem (10μg), Ciprofloxacin (5μg), Tetracycline (30μg), Polymyxin-B (300 units) and Colistin (10μg).

Phenotypic detection of MBLs: To screen MBL producers in Enterobacteriaceae, Imipenem and Meropenem resis¬tant isolates were considered for phenotypic detection by two methods.

Modified Hodge Test (MHT):

In the Modified Hodge Test, the growth was suspended in Normal saline and matched to McFarland standard (0.5). It was diluted 1:10 by adding 0.5 ml of the McFarland to 4.5 ml of saline. The standard strain of E. coli (ATCC 25922) was first inoculated on the Mueller Hinton Agar (MHA) plate as lawn culture. A 10μg Imipenem disk was placed at the center of the plate and each clinical isolate was streaked from the disk to the edge of the plate and the later was incubated at 37˚C for 12 hours [12].

After an incubation period, the plates were examined for a clover-leaf type of pattern of indentation at the intersection of growth of the test organism and the standard strain *E. coli* ATCC 25922, within the zone of inhibition of the Imipenem disc [9].

EDTA Disk synergy test: EDTA Disk synergy test was used for the detection of Metallo-β - Lactamases in the Imipenem and Meropenem resistant clinical isolates. First of all, an EDTA solution with 0.5 M strength was prepared by dissolving 186.1 g of Disodium EDTA in one litter (1000 ml) of distilled water. The adjusted pH to 8.0 and the solution was sterilized by autoclaving. An overnight culture broth of the test isolate was adjusted to a turbidity of 0.5 McFarland standards and was spread on the surface of a Mueller Hinton Agar plate. A 10μg Imipenem disk (HI - MEDIA) was placed on the agar surface. A blank disk zone size (6 mm diameter) was then kept on the inner surface of the lid of the Muller Hinton Agar(MHA) plate and 10μl of 0.5 M EDTA was poured onto it with the help of an auto pipette. This EDTA disk was then placed on the surface of the agar and was kept about 10 mm edge-to-edge apart from the Imipenem disk. After overnight incubation at 37°C, the presence of an expanded growth inhibition zone between the two disks was interpreted as positive for MBL production [13].

RESULTS:

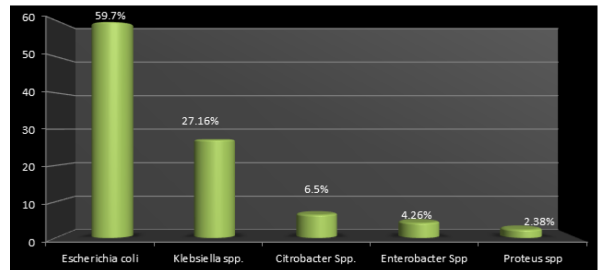
A total no. of 335 isolates of Enterobacteriaceae was included in this study. Out of 335 isolates which are 144 (42.9 %) were Male patients and 191 (57.1%) Female patients.

Table 1: Distribution of male and female patients.

S. no.	Gender	Number of isolates	Percentage
1	Female	191	57.1
2	Male	144	42.9
3	Total	335	100

Total 335 isolates included 200 (59.70%) *E.coli* followed by 91 (27.16%) *Klebsiella* species, 22 (6.5%) *Citrobacter* species, 14 (4.26%) *Enterobacter* species and 8 (2.38%) Proteus species (Figure No.1).

Figure 1: Distribution of Total Enterobacteriaceae with organism wise.



Out of the 335 bacterial isolates, most of the specimen were Urine 158 (47.16%) followed by Pus 50 (14.92%), Sputum 44 (13.13%) and Swab 30 (8.93%) respectively.

Table No. 2:- Distribution of all samples with organism wise.

S.N.	Samples	*E.coli*	*Klebsiella spp.*	*Citrobacter spp.*	*Enterobacter spp*	*Proteus spp*	Total
1	Urine	119 (35.53%)	27 (8.06%)	6 (1.79%)	4 (1.19%)	2 (0.59%)	158 (47.16%)
2	Pus	30 (8.9%)	14 (4.18%)	3(0.89%)	2 (0.59%)	1 (0.29%)	50 (14.92%)
3	Sputum	15 (4.48%)	20 (5.97%)	6 (1.79%)	2 (0.59%)	1 (0.29%)	44 (13.13%)
4	Swab	15 (4.48%)	10 (2.98%)	3(0.89%)	1 (0.29%)	1 (0.29%)	30 (8.93%)
5	E.T.	10 (2.98%)	8 (2.34%)	2(0.59%)	2 (0.59%)	2 (0.59%)	24 (7.16%)
6	Blood	5 (1.51%)	9 (2.69%)	1(0.29%)	1 (0.29%)	0 (0%)	16 (4.78%)
7	Others	6 (1.79%)	3 (0.94%)	1(0.29%)	2 (0.59%)	1 (0.29%)	13 (3.92%)
8	Total	200(59.70%)	91 (27.16%)	22 (6.53%)	14 (4.17%)	8(2.38%)	335 (100%)

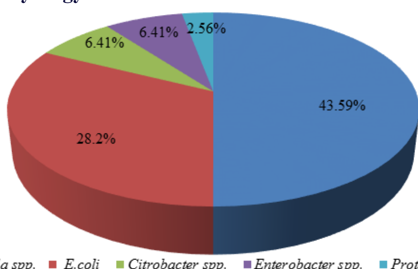
Out of total, Imipenem and Meropenem resistance, used as screening method, was seen in 78 isolates. Out of these, distribution of organisms was as following, Klebsiella spp. 38 (42.30%), followed by *Escherichia coli* 26 (33.33%), Citrobacter spp. and Enterobacter spp. 6 (7.7%) each and Proteus spp. 2(2.5%). (Table no. 3).

Among the two methods used for confirmation of MBL production in our study, were EDTA Disk Synergy Method and MHT (Modified Hodge Test) and they showed MBL production in 68 (87.18%) & 61 (78.21%) isolates respectively. More specific method was found to be EDTA disk synergy method (Table no. 3).

Table No. 3: Comparison of MBL detection through confirmatory methods (EDTA Disk Synergy Method & MHT).

S.	Total MBL producer with screening method (Imipenem & Meropenem resistance)	EDTA Disk Synergy Method	MHT (Modified Hodge Test)
1	Klebsiella spp. n= 38 (48.72%)	34 (43.59%)	30 (38.46%)
2	E.coli n= 26 (33.33%)	22 (28.20%)	20 (25.64%)
3	Citrobacter spp. n= 6 (7.7%)	5 (6.41%)	5 (6.41%)
4	Enterobacter spp. n= 6 (7.7%)	5 (6.41%)	4 (5.13%)
5	Proteus spp. n= 2 (2.5%)	2 (2.56%)	2 (2.56%)
6	Total 78	68 (87.18%)	61 (78.21%)

Figure No. 2: Distribution of MBL detection through detection by EDTA Disk Synergy Method.



Antimicrobial susceptibility pattern of non-MBL producers & MBL producers by EDTA Disk Synergy Method was found out. Non-MBL producers were more sensitive showing 100 % sensitivity against Polymyxin B and Colistin followed by Meropenem, Imipenem, and Tetracycline 78.65%, 74.15%, and 71.16% respectively. MBL producers by EDTA Disk Synergy Method showed 100 % sensitivity against Polymyxin B and Colistin. Sensitivity against other classes of drugs was low. (Table No.4).

Table No. 4: Comparison of Antibiotic Sensitivity in Non-MBL producers and MBL producers by MHT method.

S.N.	Antibiotics	Non-MBL producers	MBL producers by EDTA Disk Synergy Method
		Sensitivity n=267	Sensitivity n=68
1	Ampicillin (10μg)	73 (27.34%)	0 (0%)
2	Ceftazidime (30μg)	159 (59.55%)	0 (0%)
3	Meropenem (10μg)	210 (78.65%)	0 (0%)
4	Imipenem (10μg)	198 (74.15%)	0 (0%)
5	Gentamycin (10μg)	140 (52.43%)	19 (27.94%)
6	Ciprofloxacin (5μg)	153 (57.30%)	23 (33.82%)
7	Tetracycline (30μg)	190 (71.16%)	27 (39.71%)
8	Colistin (10μg)	267 (100%)	68 (100%)
9	Polymyxin-B (300 units)	267 (100%)	68 (100%)

DISCUSSION: -

The worldwide spread of acquired MBL in clinically important pathogens such as Enterobacteriaceae family and other non-fermenting gram-negative bacilli are a great concern [14]. The MBL producer isolates of the Enterobacteriaceae family are found in all geographical regions. MBL producer in the Enterobacteriaceae family is increasingly encountered in hospital infections in India. Bacteraemia due to Enterobacteriaceae carries a higher rate of mortality shown by previous studies from other countries [15].

In October 2019 to February 2020 this study was conducted in a tertiary care center at Jaipur Rajasthan. In our study total isolates from

the family, Enterobacteriaceae were 335 and their MBL producer was 68 (20.3%). A similar result was found in another study by Mita D.Wadekar et.al. 2013 [15] who found it 18.0%.

In our study of 335 bacterial isolates, most of the specimens were 158 (47.16%) urine followed by Pus 50 (14.92%), Sputum 44 (13.13%), and Swab 30 (8.93%). Our findings are similar to those of Nachimuthu Ramesh et. al. 2008 [16].

The most common organisms found in this study were Escherichia coli 200 (59.70%) followed by Klebsiella species 91 (27.16%), Citrobacter species 22 (6.5%), Enterobacter species 14 (4.26%) and Proteus species 8 (2.38%). This result is comparable to a study conducted by Mandeep Kaur et.al. 2015 [17]. Their findings were Escherichia coli 180 (60%), Klebsiella species 50(16.16%) respectively.

Out of the total, Imipenem and Meropenem resistance, used as a screening method, was seen in 78 (23.28%) isolates. The result in our study is comparable to another study by Manoharan et al. 2011 [18]. Who reported 17% resistance to Imipenem in Enterobacteriaceae.

After screening of MBL production, confirmation was done by using two methods, Disk Synergy Method and MHT (Modified Hodge Test). Out of total screen positive 78 isolates, these tests showed MBL production in 68 (87.18%) & 61 (78.21%) isolates respectively. A more specific method was found to be the EDTA disk synergy method. Nearby finding was reported by Nidhi Agarwal et al. 2019 [19] where they observed 71.93% and 68.42% detection of MBLs by EDTA Disk Synergy Method and MHT (Modified Hodge Test). EDTA disc synergy test is found to be more reliable with a high rate of positivity when compared to MHT in our study which is also in concordance with the study by Aparna et al. 2014 [20].

Out of these 68 confirmed isolates, distribution of organisms was as following, Klebsiella spp. 34 (43.59%), Escherichia coli 22(28.20%), Citrobacter spp. and Enterobacter spp. 5 (6.41%) each and Proteus spp. 2(2.56%). The distribution of organisms has been a bit different in other studies. A study conducted by Mita D. Wadekar et.al. 2013 [15] found distribution as Klebsiella spp. 33.3%, Enterobacter spp. 16.6%, E.coli 13.4%.

Antimicrobial sensitivity pattern in non-MBL producers showed 100% sensitivity against Polymyxin B and Colistin followed by Meropenem, Imipenem, and Tetracycline 78.65%, 74.15%, and 71.16% respectively. And more sensitive antibiotics in confirmatory MBL producers were Polymyxin B and Colistin at 100%. Our results are comparable to other studies by Pramila Pathak et al 2017 [21] and Behera B et.al, 2008 [22]. It is the sensitive, reliable, cheap and easy to perform. Therefore, it can be recommendation for routinely used in clinical laboratories for effective screening of carbapenemase producing bacterial isolates, which can help in accurate and timely detection of MBL producing Enterobacteriaceae, so that correct intervention and early directed emperical treatment can be instituted.

CONCLUSION:

The presence of MBL has a grave influence on treating regimens. The reckless use of broad-spectrum antimicrobials increases this problem. Simple phenotypic screening tests are proved to be rapid and convenient for their detection in-hospital care units. To overcome the problem of emergence and the spread of multi-drug resistance Enterobacteriaceae, a combined antibiotic interaction and cooperation between the microbiologists, clinicians, and the infection control team is needed.

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