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Molecular detection of carbapenemase producing genes in gram negative bacilli isolated from clinical specimens at a tertiary care center in rural population in Northern India.

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Abstract

The global spread of carbapenemase-producing Gram negative bacilli is a critical medical and public health issue. These bacteria are often resistant to all beta-lactam agents and are frequently co-resistant to multiple classes of other antimicrobial agents, leaving very few treatment options. This study was undertaken to optimize phenotypic methods as screening and confirmatory test to detect carbapenemase production and their genetic support system. A total of 800 Gram negative bacilli were screened from various clinical specimens for meropenem and imipenem resistance by Kirby-Bauer disk diffusion method. Resistant isolates were further checked for carbapenemase production including metallo-β-lactamases production by Modified Hodge test and imipenem-EDTA combined disk test. Carbapenemase production was further confirmed by PCR. Out of 800 Gram negative isolates 110 (13.75%) were found resistant to carbapenem by screening test which included 84 (76.36%) isolates from Enterobacteriaceae, 13 (1.6%) Pseudomonas aeruginosa and 13 (1.6%) isolates of Acinetobacter spp. 90 isolates were found positive for carbapenemase production by phenotypic methods, were further subjected to PCR. NDM (3.63%) was the most common gene isolated in this study. Another two genes detected were IMP and VIM, 2.72% and 1.81% respectively. This study shows a clearer spectrum of the current CRGNB (Carbapenem Resistant GNB) scenario in the hospital setup. Multi drug resistance is emerging as serious threat and becoming a major health concern in the era of infectious diseases.

Keywords: Carbapenemase, Modified Hodge Test, metallo-β-lactamases, beta lactam antibiotics

Introduction

The global spread of carbapenemase-producing Gram negative bacilli is an emerging medical and public health issue. These bacteria are often found resistant to all beta-lactam agents and are frequently co-resistant to other antimicrobial agents, leaving very few treatment options in hand [1]. There are several mechanisms responsible for carbapenem resistance such as efflux mechanisms, loss of certain outer membrane proteins and lack of drug penetration due to mutation in porin proteins. Among the β-lactamases, the carbapenemases especially transferrable metallo-β-lactamases (MBLs) are the most feared because they have the ability to hydrolyze virtually all drugs in that class, including the carbapenems [2]. Infection caused by CRGNB are associated with high morbidity and mortality, and also have the potential for wide spread transmission of carbapenem resistance via mobile genetic elements [3].

The carbapenemases producing Gram negative bacteria are able to transmit easily among humans. These bacteria

exchange their genetic material to each other through horizontal gene transfer by the means of plasmids and transposons [4]. Resistance to carbapenems is now frequently increasing in many hospitals acquired infections [5].

The present study was done for the detection of Carbapenem resistant gram-negative bacilli and prevention of their nosocomial outbreak as patients colonized with these bacteria could be an important source of transmission in a healthcare setting. These tests may help early detection of Carbapenemase producing Gram negative bacilli and thus wastage of antibiotics could be prevented. This research is novel in our area in India and results were informative about the epidemiological issues.

Material and methods

This is a cross-sectional study carried out in the Microbiology Department of Uttar Pradesh University of Medical Sciences, Saifai during Jan. 2015 to June 2016. A total of 800 non duplicative gram negative isolates from all clinical samples of urine, pus, sputum, blood, and other body fluids included in this study. All clinical samples were received from OPD and patients admitted in different clinical departments.

All gram-negative isolates were identified as lactose fermenting or non-lactose fermenting on the basis of colony characteristics on MacConkey Agar (HiMedia), Gram staining and a panel of biochemical reactions. The antimicrobial susceptibility of these isolates was performed by the Kirby Bauer's disc diffusion method on Mueller-Hinton Agar (MHA), as per standard protocol. Isolates found with reduced susceptibility to meropenem and imipenem (zones of inhibition ≤19 mm as per CLSI 2015 guidelines) by disc diffusion method, were further screened for the production of carbapenemase. Phenotypic screening of carbapenemase production of meropenem or imipenem resistant isolates was done by Modified Hodge Test (MHT) and Combined disk test.

Modified Hodge test

In the Modified Hodge test Mueller Hinton Agar (HiMedia) surface was lawn cultured with the overnight growth of Escherichia coli ATCC 25922. 10µg meropenem disk was placed at the center of the plate and then test organism and QC strains were inoculated in a straight line out from edge of the disc. A clover-leaf like indentation of the zone of inhibition of the test strain considered as a positive screening test for carbapenemase production [6]. (figure 1)

Combined disk test

In combined disk test two 10 µg imipenem disks (HiMedia) placed on the plate, and 10 µl of EDTA solution added to one of them to obtain the desired concentration (750 µg). Increase in inhibition zone size of the Imipenem with EDTA disk was ≥7 mm more than the Imipenem disk alone, which was considered as MBL positive [7]. (figure 2)

Gene identification

Isolates found carbapenemase producer on phenotypic screening test were subjected to PCR assay for detection of various genes encoding for carbapenemase production. Total DNA targeting both genomic and plasmid DNA was extracted by using QIAamp DNA Mini Kit (QIAGEN, Germany) as per manufacturer instructions.

The PCR conditions and the primers (Imperial Life Sciences Pvt Ltd, Gurgaon Haryana India) for the genotypic characterization of carbapenem resistant strains, were used as previously described by Poirel L., Timothy R., et al. Details of the primers used are given in Table 1. Cycling conditions was 10 min at 94°C and 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52°C, and 50 s at 72°C, with 5 min at 72°C for the final extension [8].

Results

By disk diffusion method, 110 (13.75%) out of total 800 isolates were found resistant to either imipenem or meropenem during the study period. In this study Klebsiella puemoniae was the most common bacteria isolated among 110 carbapenem resistant strains which were 30 (27.27%). Majority of the CRGNB cases were from indoor of different clinical departments and ICU of the hospital (89.09%), followed by OPDs (10.9%). Maximum number of resistant isolates were from urine sample 29 (26.36%), followed by pus 27 (24.54%), blood 22 (20%) and 32 (29.08%) from other body fluids.

In the present study, out of 110 carbapenem resistant isolates, 90 (81.81%) were found positive for carbapenemase production by modified Hodge Test (MHT). Carbapenemase production by MHT was highest with Acinetobacter spp. with 12/13 (92.3%), followed by Pseudomonas spp. 11/13 (84.61%). 52 isolates exhibited a ≥ 7 mm zone size enhancement in the combined disc test considered metallo-β-lactamases (MBL) producers. NDM (3.63%) was the most common gene isolated in this study. Other two genes detected were IMP and VIM 2.72% and 1.81% respectively (table 2).

Discussion

Carbapenems are most potent β-lactam antibiotics and presently considered as the agents of treatment of multidrug resistant gram negative bacterial infections due to the stability of these agents against the majority of β-lactamases.

In the present study, the overall resistance to carbapenems was 13.75% which is coherent to the study by Kaur M and Gupte S et al. (2015) who reported 17% resistance to carbapenems in Gram negative bacilli. Also, Manoharan et al.,(2011) and Gupta E et al.(2006) showed 17% and 17-22% resistance to carbapenems respectively [9-11]. From India various studies have found different rates of carbapenem resistance. A study conducted in Meerut showed 5-6% carbapenem resistant in Enterobacteriaceae [12].

Maximum number of samples were urine 29 (26.36%) followed by pus 27 (24.54%), blood 22 (20%) and 32 (29.08%) from other infections. Another study from north India has the comparable results as this, where most of the carbapenem resistant organism were isolated from urine 47.1% (n=20) followed by pus 27.1% (n=13) [13]. The MHT method is easy to perform, but diverse specificity values have been reported by different authors, so there is always a chance of false-positive results, which we needs to be aware [6]. In this study 90/110 (81.81%) CRGNB isolates were positive by Modified Hodge test. Carbapenemase production by MHT was highest with Acinetobacter spp. with 12/13 (92.3%). Similar study done by Sahin et al. found concordant results with 85% sensitivity of MHT [14]. Other studies of Delphine G et al. and Sanjeev K et al. found positive results 68.57% and 34% respectively by MHT [15-16]. In this study, we used a PCR-based assay as an alternative to verify carbapenem resistance among CRE (Carbapenem resistant Enterobacteriaceae) isolates.

In our study, we screened only carbapenem resistant isolates with combined disk test in which 52 (47.27%) isolates found to be MBL positive. In India, published reports indicate the prevalence of MBLs (metallo-beta-lactamases) range from 7-65%. A study done in India found the prevalence of MBL between 31% and 55% among multi drug resistance bacteria [17-18].

PCR methods were employed to find the resistant genes, we detected 9.09% prevalence of CRDG (carbapenem resistance determining gene) among multi drug resistance gram negative bacteria. This is a rather worrisome finding in the poor population of India. Their prevalence is also corresponding to another data reported from India which

shows the prevalence of CRDG among gram negative bacterial isolates to be 43% [19-20]. This can be due to difference in efficacy of antibiotic used in our settings. The results obtained by PCR methods in our study indicated that 9 (8.18%) CRGNB isolates harbored class 2 metallo-beta-lactamase gene. The New Delhi metallo-β-lactamase (blaNDM-1) gene was found in 4 (36.36%) CRGNB isolates in our study, 2 were from K. peumoniae, 1 from E. coli and 1 from A. baumannii. Until recently, the most common MBLs found worldwide in Enterobacteriaceae were VIMs (Verona integron-encoded MBLs) and IMPs (active on imipenem) [21].VIM was found in 2 (1.81%) isolates in our study. This gene was detected in K. pneumoniae and P. aeruginosa. This data corresponds to the findings of a study done in South India where VIM was reported in 1 isolate out of 24 Gram negative bacilli [22]. We found 3 (2.72%) isolates which carry blaIMP-1 gene, 2 isolated from Pseudomonas aeruginosa and 1 from Acinetobacter baumannii.

Antibiotic was considered as a magic bullet when first introduced. But unfortunately, the genes expressing resistance to antimicrobials have emerged in many bacterial strain have seriously interfered with therapy, allowing infections to progress despite of antibiotic administration.

Conclusions

This study was done for Carbapenem resistant gram negative bacilli and prevention of their nosocomial outbreak. Carbapenase-producing Gram negative bacilli are an emergency public health issue. These bacteria are becoming co-resistant to several other antimicrobial agents, restricting the antimicrobial options for treatment.

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Legends Tables and Figures

Table 1: Primer sets

Primer	Sequence (5'⇒3')	Gene	Product size (bp)
IMP-F	GGAATAGAGTGGCTTAAYTCT	blaIMP	232bp
IMP-R	CGGTTTAAYAAAACAACCACC		
VIM-F	GATGGTGTTTGGTCGCATA	blaVIM	390bp
VIM-R	CGAATGCGCAGCACCAG		
KPC-Fm	CGTCTAGTTCTGCTGTCTTG	blaKPC	798bp
KPC-Rm	CTTGTCATCCTTGTTAGGCG		
OXA-F	GCGTGGTTAAGGATGAACAC	blaOXA-48	438bp
OXA-R	CATCAAGTTCAACCCAACCG		
NDM-F	GGTTTGGCGATCTGGTTTTC	blaNDM	621bp
NDM-R	CGGAATGGCTCATCACGATC		

Table 2: Distribution of different carbapenem resistance determining genes among the multidrug resistance gram negative bacteria

Bacteria	Carbapenem resistance determines gene			Total
	NDM	IMP	VIM	
K. pneumoniae	2 (50%)	0 (0%)	1 (50%)	3 (33.34%)
E. coli	1 (25%)	0 (0%)	0 (0%)	1 (11.12%)
P. aeruginosa	0 (0%)	2 (67%)	1 (50%)	3 (33.34%)
A. baumani	1 (25%)	1 (33.34%)	0 (0%)	2 (22.23%)
Citrobacter spp.	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Enterobacter spp.	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	4 (44.45%)	3 (33.34%)	2 (22.24%)	9 (100%)

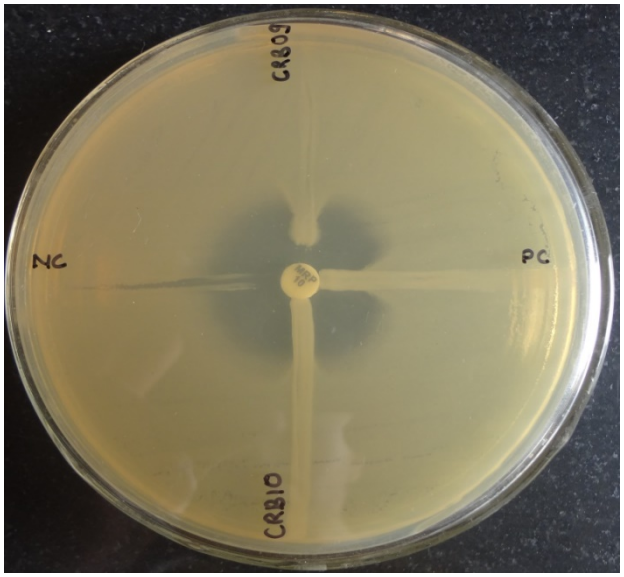

Figure 1 showing clover leaf formation in Modified Hodge test

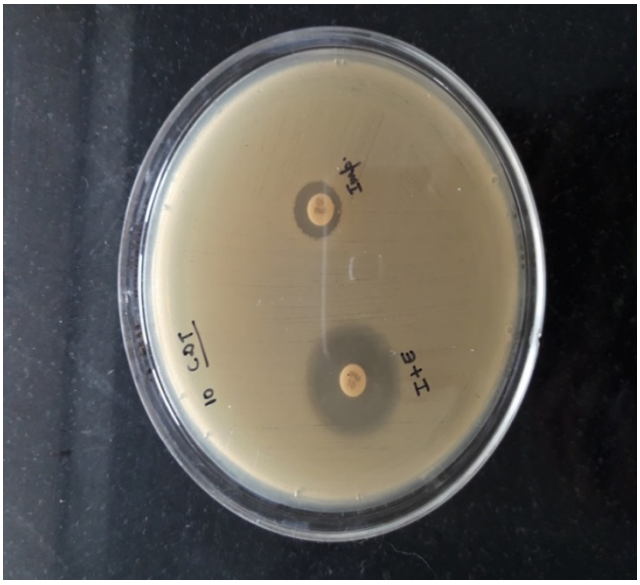

Figure 2 showing increased zone size with imipenem-EDTA disk in Combined Disk Test

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