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PHENOTYPIC AND GENOTYPIC DETECTION OF CARBAPENAMASE PRODUCTION AMONG ENTEROBACTERIACEAE IN A TERTIARY CARE HOSPITAL, PORT BLAIR, INDIA

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ABSTRACT **Background and Objective** Multidrug resistance is emerging worldwide at an alarming rate among a variety of bacterial species, causing both community-acquired and nosocomial infections, including Enterobacteriaceae. The clinical utility of carbapenems, the agents of last resort against multi-drug resistant Enterobacteriaceae, is under threat with the growing incidence of pan resistant isolates. This study aims to determine the prevalence of carbapenemase production among Enterobacteriaceae, as such data is not available from this area. **Materials and Methods** The study, prospective in design, was conducted during the period from April 2016 - August 2018. Any isolate reistant to IMP and/or MRP were subjected to phenotypic detection (Modified Hodge Test, MHT and Double Disk Synergy Test, DDST) and genotypic (blaIMP, blaKPC, blaVIM, blaOXA-48, blaOXA-23, blaOXA-51, blaOXA-58 and blaNDM) basis of carbapenamase production. **Result:** A total of 218 consecutive isolates of Enterobacteriaceae, which were resistant to IMP and/or MRP, were considered for the study. The most common isolates of Enterobacteriaceae were Klebsiella pneumoniae (n=122, 55.5%) followed by Escherichia coli (n=80, 36.4%). Modified Hodge Test (MHT) were positive for 12, 02 and Double Disc Synergy Test (DDST) were 11, 05 for Klebsiella and E.coli respectively. Multiplex PCR I and II, was performed for 36 isolates. The carbapenemase producing organisms were positive for 60% for single gene and 36% for multiple genes. **Conclusion:** Delay in detection of carbapenemase producing organisms result in longer hospitalization and increased health-care cost and this further affects the morbidity and mortality of patients in hospital set up.

KEYWORDS :

I. Introduction

Enterobactericeae are inhabitants of the intestinal flora and are among the most common human pathogens causing infections such as cystitis and pyelonephritis with fever, septiceamia, pneumonia, peritonitis, meningitis and device-associated infections. Enterobacteriaceae spread easily between humans by hand carriage as well as contaminated food and water and have a propensity to acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons. They are the most common source of both community and hospital-acquired infections, Escherichia coli being by far the most important pathogen.

Multidrug resistance is emerging worldwide at an alarming rate among a variety of bacterial species,causing both community-acquired and nosocomial infections. Carbapenems, the last line of therapy, are now frequently needed to treat nosocomial infections and increasing resistance to this class of β-lactams leaves the health care system with almost no effective drugs. These carbapenems are crucial for life-threatening hospital-acquired infections. A cross-sectional study from Nepal reported 95% multi-drug resistant enterobacteriaceae among isolates.(Yadav kp) These multi resistant bacteria acquire genes encoding multiple antibiotics resistant mechanisms, including β-lactamases, Extended spectrum β-lactamases (ESBL), AmpCs and carbapenemases. In β-lactamases, the primary cause of bacterial resistance by inactivating major class of antibiotics by cleaving the amide bond in the four atom of β-lactam ring structure in Penicillin, Cephalosporins, Monobactams and Carbapenems. Carbapenem hydrolysing β-lactamases belongs to molecular class-A, class-B and class-D, are the main source of antibiotic resistance in Enterobacteriaceae. These β-lactamases are encoded either by chromosomal genes (IMI and NMC of class A) or transferable gene located on plasmids and Transposons (KPC, IMP, VIM). Class A (KPC) capable of hydrolysing carbapenems, oxymino- cephalosporins and cephamycins, are inhibited by clavulanic acid or tazobactum. Class D (OXA-23, OXA-48) are able to hydrolyse carbapenems, but not inhibited by clavulanic acid or tazobactum. Both these class A and D are serine carbapenemases. Class B (IMP, VIM and NDM), are metallo-β-lactamases (MBL) use Zink cation for hydrolysis of β-lactam ring; are susceptible to ion chelators such as Ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid not by clavulanic acid, tazobactum or sulbactum. These metallo-enzymes confer resistance to all β-lactam antibiotics except monobactams. The KPC gene initially reported from *Klebsiella pneumoniae* isolates, have been found worldwide in multiple other gram negative species, such as E.coli, Citrobacter, Enterobacter, Salmonella and Pseudomonas. The New Delhi meallo-β-lactamase (NDM) has received the most attention recently as reported from different part of world. The emergence and spread of these carbapenemase producing Enterobacteriaceae have a significant impact on clinical outcome and public health concern. These are often resistant to all β-lactam drugs and frequently carry mechanisms conferring resistance to other anti microbials also. Infections with these resistance bacteria are associated with high mortality rate than those of carbapenem susceptible organisms. The clinical utility of carbapenems, the agents of last resort against multi-drug resistant Enterobacteriaceae, is under threat with the growing incidence of pan resistant isolates.[4]

This study aims to determine the prevalence of carbapenemase production among Enterobacteriaceae by both Phenotypic and Genotypic methods, as such data is not available from this area.

II. Materials and Methods

A prospective study was performed in the Department of Microbiology. Enterobacteriaceae isolated from various clinical samples (pus, blood, body fluid and urine) received at the clinical Microbiology laboratory between April 2016 - October 2018 was considered for the study. Identification of Enterobacteriaceae isolates to the species level was based on Automated Identification System BD Phoenix. Antimicrobial susceptibility of the isolates were screened by Kirby-Bauer disc diffusion method using Amikacin (AK 30µg), Gentamicin(GEN 10 µg), Ceftriaxone (CTR, 30µg), Cefipime (CPM, 30µg), Ciprofloxacin(CIP,5µg), Cotrimoxazole (COT, 1.25/23.75µg), Imipenem(IMP, 10 µg), Meropenem (MRP,10µg), Piperacillin-Tazobactum (PIT, 100/10µg), and interpreted as per CLSI guidelines. Susceptibility to Cefoxitin (30 µg) disc was used for presumptively identification of Amp C beta lactamase production.

Screening for carbapenems resistance was done using Imipenem (IMP) or Meropenem (MRP) disc (10 mcg, Hi-Media Lab) as per CLSI guideline 2016. Minimum Inhibitory Concentration (MIC) of carpapenems against these isolates was determined by Automated Identification System BD Phoenix. Phenotypic detection of carbapenemse production was done by Modified Hodge Test (MHT)[10] and Metallo beta lactamase was done by Double Disk Synergy Test

(DDST). All strains resistant to either IMP or MRP was further subjected to genotypic analysis for carbapenamase producing genes by using two set of multiplex PCR. The first set, Multiplex I consists of blaKPC, blaVIM, blaOXA-48 and another set, Multiple II with blaIMP, blaNDM, blaOXA-23 as shown in table 1.

DNA extraction of isolates were performed by using HiPurA Bacterial Genomic DNA Purification Kit from Himedia as per manufacturer instructions. The PCR reaction mixtures for Multiple I and II, each contained 12.5 microl master mix (Taq, dNTP, $MgCl_2$ and buffer), 6 µl primers (3 set of forward and backward primers), 5 µl of template DNA and distilled water. The PCR conditions were as follows: initial denaturation at 95°C for five minutes, 35 cycles at 95°C for one minute, at different annealing temperatures for one minute and 72°C different annealing temperatures for one minute and 72°C for one minute, followed by a single, final elongation step at 72°C for five minutes. The annealing temperature was optimal at 56°C for amplification of the blaKPC, blaOXA and blaVIM genes and 52°C for amplification of the blaNDM, blaIMP and blaOXA genes (Table 1). Amplicons were visualized after running at 100 V for 90 minutes on a 1.5% agarose gel containing ethidium bromide. A 100 bp DNA ladder (Himedia) was used as a size marker.

Statistical analysis of data were performed by using SPSS 17 (SPS Inc., Chicago, IL, USA)

III. Result

A total of 2489 consecutive isolates of Enterobactericeae were obtained during the study period, with *Escherichia coli* (1466, 58.89%) and *Klebsiella* spp (887, 35.63 %) being the commonest comprising 95% of the total. The other isolates found were *Proteus* spp (72, 2.89%) and *Enterobacter* spp (32, 1.28%) and others (1.31%) as rare isolates as *Citrobacter* (08), *Morganella* spp(07), *Providencia* spp(05), *Salmonella*e (04), *Serratia* spp(03), *Pantoea* spp (03) and *Edwardsiella* spp (02).

Out of these 252 (10.12%), which were resistant to IMP and/or MRP, were considered for the study. Resistant to Imipenem (n=224) or Meropenem (n=169) and both (142) were found during study period. The most common isolates were Klebsiella (n=148) followed by Escherichia coli (n=86). These resistant isolates were shown in figure 1. Male was slightly greater than female, 55% male vs 45% female. Adult child ratio was 13:1 with minimum age 7 days to maximum age 92 years. Resistance pattern of two common isolates, Klebsiella and E.coli, were shown in figure 2.

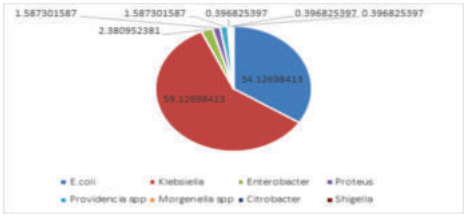

Figure1. Distribution Of Carbapenems Resistant Isolates.

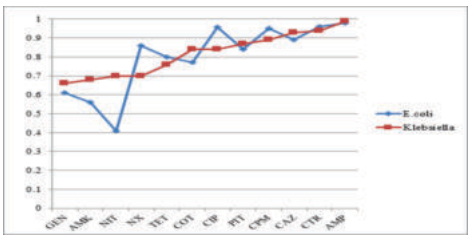

Figure2. Antimicrobial Resistance Pattern Of Two Common Isolates Escherichia Coli And Klebsiella Pneumoniae

A total of 99 MHT and DDST were performed, in which 39 isolates were *E. coli* and 55 isolates, Klebsiella. MHT found positive in 25 isolates for klebsiella and 08 for *E. coli*, DDST in 32 isolates for Klebsiella, 17 for *E.coli* and one isolates of Providencia. Both MHT and DDST were positive, 11 of Klebsiella and 4 of *E.coli*. The sensitivity of DDST with respect to detect metallo-β-lactamases genes was 77.78% (CI 52.36% to 93.59%) and specificity was 20.00% (CI 4.33% to 48.09%). The sensitivity of MHT with respect to detection of carbapenemase genes were 100.00% (CI 75.29% to 100.00%) and specificity was 25% (CI 8.66% to 49.10%) and DDST was 53.85%(45.03% to 62.43%). The positive predictive value of MHT was 46.43%(CI 40.22% to 52.75%). The accuracy of the performed MHT test was 54.55% (CI 36.35% to 71.89%) and DDST was 51.52% (CI 33.54% to 69.20%).

Multiplex PCR I and II, was performed for 36 isolates and their primers used are shown in Table 1. Result of these Carbapenemase producing genes were shown in Table 2 (a) and (b). The carbapenemase producing organisms were positive for 60% for single gene and 36% for multiple genes.

Table1. Details of Primers used for Carbapenemase producing genes in Multiplex I & II

Targeted Gene	Primer 15 pmol/µl	Sequence (5' to 3' Direction)	Length (Bases)	Amplicon Size bp	References
Multiplex I (KPC, OXA-48 and VIM)					
blaKPC	Forward	TGTTGCTGAAG GAGTTGGGC	20	340	13
	Reverse	ACGACGGCATA GTCATTTGC	20		
blaOXA-48	Forward	AACGGGCGAAC CAAGCATTTT	21	585 or 597	13
	Reverse	TGAGCACTTCT TTTGTGATGGCT	23		
blaVIM	Forward	CGCGGAGATTG ARAAGCAAA	20	247	13
	Reverse	CGCAGCACCRG GATAGAARA	20		
Multiplex II (NDM, IMP and OXA)					
blaIMP	Forward	TAAAATACCTTG AGCGGGC	19	439	13
	Reverse	AAATGGAAACT GGCGACC	18		
blaNDM	Forward	GAGTGGCTTAA TTCTCRATC	20	183	13
	Reverse	CCAAACYACTA SGTTATCT	19		
blaOXA-23	Forward	GTGGTTGCTTCT CTTTTTCT	20	736	13
	Reverse	ATTTCTGACCGC ATTTCCAT	20		

Table2(a). Organism wise Carbapenemase producing single gene in Multiplex PCR I & II

Organism	Multiplex PCR I			Multiplex PCR II		
	blaKPC	blaVIM	blaOXA-48	blaNDM	blaIMP	blaOXA-23
Klebsiella	02	10	05	03	04	03
E.coli	04	01	04	02	06	05
Proteus		01				
Providentia				01		

Table2(b). Distribution of Carbapenemase producing multiple genes in Multiplex PCR I & II

	Multiplex I All Negative	Multiplex II All Negative	blaKPC & blaVIM	blaOXA-48 & blaKPC	blaOXA-48 & blaVIM	blaNDM & blaIMP	blaIMP & blaOXA-23	blaOXA-23 & blaNDM
Klebsiella	06	13	0	0	02	0	02	01
E.coli	06	04	0	01	00	0	03	0
Proteus	0	0	0	0	0	0	0	0
Providentia	0	0	0	0	0	0	0	0

DISCUSSION

A total of 252 (10.12%) were suspected of carbapenemase production because of non susceptibility to IMP and/or MRP(intermediate or resistant zone diameter according to CLSI clinical break point (CBP)) and not suspected of carbapenemase production (Susceptible to IMP and /or MRP) served as a negative control group. In this study Klebsiella spp (n=148) and E.coli (n=86) which were resistant to carbapenem by disk diffusion method had been evaluated.These were further confirmed by obtaining MIC of Imipenem and meropenem by automated system, BD Phoenix(BD, USA). The MIC was >16 mcg/ml

for both Imipenem and Meropenem, thus there was concordance between disk diffusion and MIC. These isolatesresistance to most of antimicrobials were tested and resistant rate varies from 60 % to 100 % for both Klebsiella and E.coli. Most resistant antibiotic was Ampicillin/Ampicillin sulbactum, third generation Cephalosporins mainly Ceftriaxone and Cefotxime and least resistant was aminoglycosides Amikacin and Gentamycin. Similar type of resistant pattern was found in previous study of this institute for Klebsiella and Acinetobacter. Resistant to both imipenem and meropenem is a strong indicator of carbapenemase production rather than resistance to either one of the carbapenems, as this may imply a different resistance mechanism. The majority of carbapenem resistant isolates were from pus, followed by urine, respiratory and blood. This contrast the findings by Nagaraj et al, study from south India.

Phenotypic Analysis

Modified Hodge Test is the phenotypic method of detection of Class-A (KPC) and class-D carbapenemase (OXA-48 , OXA-23), which are predominant cause of resistance in Enterobacteriaceae and DDST using Imipenem and Imipenem EDTA disk, is used for detection of MBL. The MHT is simple and inexpensive to perform and is well established in many clinical microbiology laboratories in the United States based on its ability to detect KPC producers. The MHT also demonstrates good sensitivity for many other carbapenemases, including VIM, IMP, and OXA-48-like enzymes. For U.S. collections of Enterobacteriaceae, sensitivity has been documented between 93 and 98%. Similar result was obtained in our case also as of high sensitivity. Unfortunately, the MHT performs poorly in the detection of MBL producing isolates.[1] In addition, the MHT also suffers from poor specificity because bacteria producing AmpC enzymes combined with porin mutations can give a false-positive result. MHT and DDST were performed on 99 resistant isolates. MHT were found positive in 45 % of Klebsiella and 23% in E.coli. These findings were similar as Datta S et al study from north India. DDST were found positive in 58% of klebsiella and 44% of E.coli. However some of isolates both MHT and DDST were positive suggesting co-existence of both mechanism of resistance. Both negative isolates were suggestive of resistance mechanisms other than carbapenem hydrolysing enzymes such as efflux pump and porin channel loss.

Genotypic Analysis

The combination of primers set presented in this study had covered most of carbapenemase genes found in Enterobacteriaceae. These set of primer taken for previous study had covered all known carbapenemase genes that have been identified in clinical isolates of Enterobacteriaceae. Primers were well tested in vitro used in multiplex PCR for screening Carbapenemases.

In this study small subset of reactions were used for detecting carbapenemases gene in Enterobactericeae but the result were conclusive. KPC enzymes are currently the most clinically signifi- cant enzymes among class A β-lactamases. The first KPC- producing strain (KPC-2 in K. pneumoniae) was identified in 1996 in the Eastern part of the USA. Since then, many KPC variants have been identified (KPC-2 to KPC-13) (www.lahey.org). KPC gene exhibit activity against a wide spectrum of β-lactams, including penicillins, older and newer cephalosporins, aztreonam, and carbapenems.

CONCLUSION:

The emergence of Carbapenem drug resistance in Enterobacteriaceac has become a substancial clinical problem since detection of Carbapenamase production not evident from the routine antimicrobial resistance profile by disk diffusion method. Delay in detection of these organisms result in longer hospitalization and increased health-care cost and this further affects the morbidity and mortality of patients in hospital set up.

Conflict of interest:

The authors declare no conflict of interest

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