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DETECTION OF CARBAPENEMASE PRODUCING GRAM NEGATIVE BACTERIA IN A TERTIARY CARE HOSPITAL, CHATTOGRAM

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ABSTRACT

Background: The global dissemination of carbapenemase in Gram negative bacteria is a severe public health concern worldwide because of their resistance to most antibiotics. Carbapenemase-producing Gram negative isolates are usually extensively drug resistant, and infections caused by these pathogens with significant morbidity and mortality present a serious clinical challenge. **Aim & Objectives:** This study was conducted to determine the prevalence of carbapenemase producers in urine and wound-swab along with antimicrobial resistance patterns of these organism. **Methodology:** Nonduplicate Gram negative bacteria were included in this study from inpatients of Chittagong Medical College Hospital. Antimicrobial susceptibility was done among Gram negative isolates. The modified carbapenem inactivation method was performed to see carbapenemase producers among meropenem resistant isolates. Finally carbapenemase genes were detected by multiplex PCR. **Result**: Among 300 samples 171 culture positive isolates showed 22.80% *Escherichia coli*, 26.90% *Klebsiella spp.*, 38.01% *Pseudomonas spp.*, 11.11% *Acinetobacter spp.* and 1.69% *Proteus spp.* Antimicrobial susceptibility tests revealed that the ciprofloxacin (78.36%), ampicillin (98.2%), ceftriaxone (71.34%), cefuroxime (77.77%), and ceftazidime (70.76%) were the most resistant, while amikacin (18.71%), pipercillin-tazobactam (25.73%), and meropenem (27.38%) had the lowest resistance. Among Gram negative isolates 21.63% were found to be carbapenemase producers by mCIM and 25% carbapenemase genes were detected by multiplex PCR. Multiplex PCR showed *bla*NDM gene in 86.04% isolates, *bla*-VIM in 2.32%, *bla*KPC+*bla*NDM+*bla*OXA-48 in 6.97%, *bla*KPC+*bla*OXA-48 in 2.32% and *bla*NDM+*bla*OXA-48 in 2.32% isolates. **Conclusion:** The study shows that rapid dissemination of *bla*NDM in Bangladesh demands the effective measures along with antibiotics policies in hospital which combact the spread of resistane strains. Moreover accurate detection of the genes related with carbapenemase production by molecular method like multiplex PCR overcome the limitation of phenotypic method.

KEYWORDS: Modified carbapenem inactivation method, New Delhi metallo-beta-lactamase, Oxacillinase, Verona Integron-encoded metallo-beta-lactamase, Multiplex Polymerase Chain reaction.

INTRODUCTION

Antimicrobial resistance (AMR) posses a complex threat to global health security and universal health coverage.[1] It is considered one of the most pressing issues in the global health care sector due to greater access to antibiotic drugs in developing countries. Gram negative bacteria have developed the broadest spectrum of resistance due to multiple structural adaptions and antibiotic degradation enzymes including Extended Spectrum Beta Lactamase, AmpC Cephalosporinase and Carbapenemase.[2] Among them, Enterobacteriaceae and non-fermentative Gram negative bacteria such as *Pseudomonas spp.* and *Acinetobacter spp.* are the most common pathogens of both community associated and health care associated infections.

Beta-lactamases with a variety of hydrolytic capabilities are known as carbapenemases. Penicillins, cephalosporins, monobactam, and carbapenems can all be hydrolyzed by them.[3] The Ambler classification system can be used to classify carbapenemases into class A, B, or D based on their molecular properties. The most common class A carbapenemmases are KPC enzymes, while notable transmissible class B carbapenemases include IMP, VIM and NDM enzymes. Common class D carbapenemases include OXA-23-like, OXA-24-like, OXA-48-like, OXA-58-like enzymes.[4] Three groups of carbapenemases – KPC, NDM, and OXA-48 are currently considered to be the three major beta-lactamases of epidemiological and clinical significance.[5]

Mobile genetic elements (MGE), also known as elements that encourage intracellular DNA mobility (for example, from the chromosome to a plasmid or between plasmids), as well as those that enable intercellular DNA mobility, play a significant role in the capture, accumulation, and dissemination of resistance genes. Resistance is mediated mainly by production of carbapenemase either serine based carbapenemase or metallo-beta-lactamases(MBLs). The carbapenemase encoding genes are often located on plasmid along with other resistance genes (i.e., hyperproduction of AmpC beta lactamase, certain ESBLs) resulting in multidrug drug resistance (MDR), extensive drug resistance (XDR) and pan-drug resistance (PDR).[6]

Clinical microbiology laboratories must identify isolates that produce carbapenemase in order to deliver targeted medication, practice antimicrobial stewardship, and update regional antibiotic guidelines for doctors. Furthermore screening of resistance mechanisms using of antimicrobial susceptibility test in addition to detect genes by phenotype and molecular analysis reveal that dissemination of carbapenemase including NDM, VIM, IMP, OXA-48 and KPC producers are rapid and wide - spread among healthcare facilities.

The classical phenotypic method cannot provide an efficient means of diagnosis of the carbapenemase producers. Additionally, multiplex PCR aids in the simultaneous detection of numerous genes, saves resources and labor, and assists in establishing the epidemiology of these genes and control of infection. Multiplex PCR also helps in simultaneous detection of various genes, reducing materials, manpower & helps in determining epidemiology related to these genes & infection control.[7] Hence this study was conducted to detect the presence of these enzymes obtained from wound-swab and urine from a tertiary care hospital and to compare the phenotypic method with genotypic method of resistant strains that guide clinicians in prescribing proper antibiotics, and in monitoring resistance trends.

MATERIALS AND METHODS

This was a cross-sectional type of study conducted in the Department of Microbiology of Chittagong Medical College in collaboration with different disciplines of Chittagong Medical College Hospital, (CMCH) Chattogram for a period of one year. A total of 300 samples (wound swab, urine) were collected after taking prior consent using predesigned data sheet and these samples were collected from admitted patients those had surgical or burn wound infections or suspected UTI irrespective of age, sex and antibiotic intake.

Bacterial strains

Samples were inoculated into sheep blood agar and Mac Conkey agar and incubated over night at 37°C. Phenotypic identification of the organisms were done by observing colony morphology, hemolytic criteria on blood agar media, staining characteristics, pigment production and relevant biochemical tests (oxidase, catalase, TSI, motility in soft agar and Simmon's citrate media) as per standard procedure.[8] A total of 171 non-duplicate Gram negative bacteria were isolated by conventional culture method.

Antimicrobial susceptibility testing

All bacterial isolates were tested for antimicrobial susceptibility testing by modified Kirby-Bauer disk-diffusion technique using Mueller-Hinton agar plates and the zone of inhibition were interpreted according to CLSI guidelines. Disks from each batch were first standardized by testing against reference strains of *Escherichia coli* ATCC-25922 and zones of inhibition were compared with standard value.[9]

Modified Carbapenem Inactivation Method for detection of Carbapenemase (CLSI, 2021)

In the mCIM, 1µL loopful of bacteria for Enterobacteriaceae or 10µL loopful of bacteria for *Pseudomonas spp*. or *Acinetobacter spp*.from an overnight blood agar plate were emulsified in 2mL trypticase soya broth (TSB). A 10µg meropenem disk was immersed in the suspension and incubated at 35°C±2°C in ambient air for 4 hours ±15 minutes. Just before or immediately following completion of TSB-meropenem disk suspension incubation, a 0.5 Mc-Farland suspension of *Escherichia coli* ATCC-25922 was prepared in nutrient broth or saline using the direct colony suspension method. A Mueller –Hinton agar (MHA) plate was inoculated with *Escherichia coli* ATCC-25922 using the routine disc diffusion procedure within 15 minutes. The plate was allowed to dry for 3-10 minutes before adding the meropenem disk. The meropenem disk was taken out of the TSB-meropenem disc suspension and put on an MHA plate that had already been contaminated with the indicator strain of *Escherichia coli* ATCC-25922. Then plate was incubated at 35°C±2°C in ambient air for 18-24 hours. Following incubation, the zone of inhibition was measured. No zone of inhibition or 6-15mm of zone diameter or presence of pinpoint colonies within 16-18mm zone considered as carbapenemase positive. On the other hand, zone dm of ≥19mm was carbapenemase negative or zone dm 16-18mm or presence of pinpoint colonies within ≥19mm zone was carbapenemase indeterminate.[9]

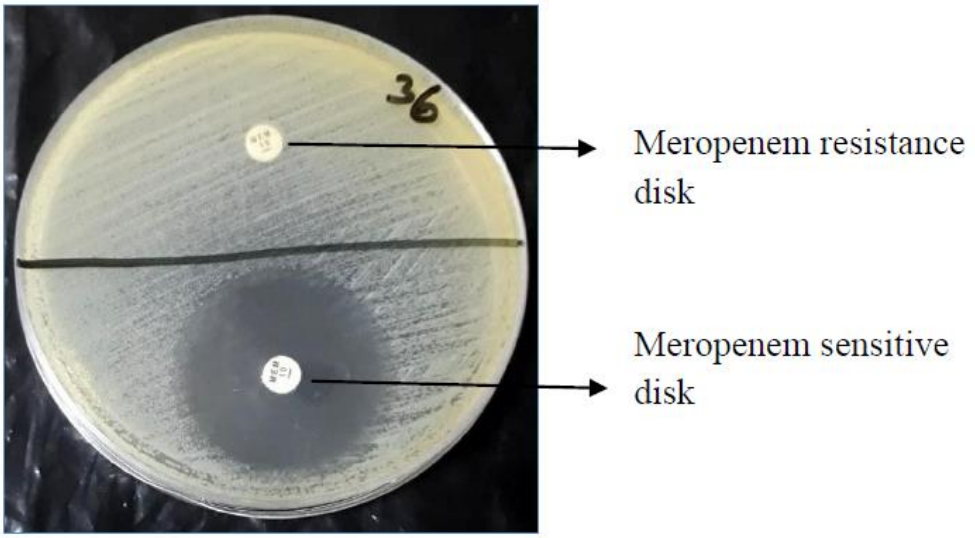

Fig 1: Modified Carbapenem Inactivation method shows Carbapenemase positive isolates.

Molecular characterization of carbapenem resistance genes

Polymerase chain reaction (PCR)-based detection of Ambler class B MBLs (*bla*IMP, *bla*VIM and *bla*NDM), Ambler class D (*bla*OXA-48), and class A, (*bla*KPC) was carried out on meropenem resistant isolates. Coexistence of carbapenemase encoding genes**,** namely, NDM, OXA-48 and KPC were also evaluated by PCR. The boiling procedure was used to extract the genomic DNA. To produce PCR products, the following primer pairs that had previously been utilised were used.

Primer	Sequence	Gene	Product Size (bp)
IMP-F	GGAATAGAGTGGTCGCTTAAYTCTC	*bla* IMP	232
IMP-R	GGTTTAAYAAAACAACCACC		
VIM-F	GATGGTGTTTGGTCGCATA	*bla*VIM	390
VIM-R	CGAATGCGCAGCACCAG		
OXA-F	GCGTGGTTAAGGATGAACAC	*bla*OXA	438
OXA-R	CATCAAGTTCAACCCAACCG		
NDM-F	GGTTTGGCGATCTGGTTTTC	*bla*NDM	621
NDM-R	CGGAATGGCTCATCACGATC		
KPC-F	CGTCTAGTTCTCTGCTGTCTTG	*bla*KPC	798
KPC-R	CTTGTCATCCTTGTTAGGCG		

The following cycling parameters were used: Initial Denaturation**:** at 94° C for 10 minutes followed by 36 cycles of amplication consisting of denaturation: at 94°C for 30 s, primer annealing**:** at 52°C for 40 s, extension**:** at 72°C for 50s and final extension. at 72°C for 5 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 120 volts for 20 minutes, stained with 1% ethidium bromide andvisualized under UV light.[10]

Statistical analysis

Data were analyzed by using IBM-SPSS statistics v.20.0 for windows. Comparison were performed using chi-square test by chi-square calculator. Tables and figures were used to illustrate the results.

RESULTS

A total of 300 samples 57% Gram negative bacteria were isolated and 21.63% carbapenemase producers were detected by mCIM and carbapenemase genes in 25.14% isolates.

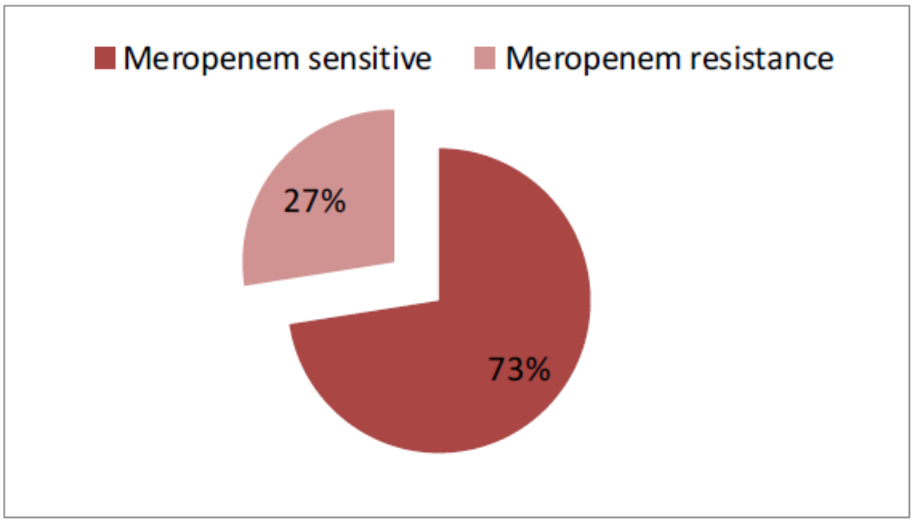

Figure I: Meropenem resistant isolates among the Gram negative bacteria.

Figure I: shows among 171 Gram negative bacteria, meropenem resistant isolates were found 47(27%). Table 1 - shows out of 47(27%) meropenem resistant isolates, 37 isolates were found to be carbapenemase producers by mCIM. Higher rate of carbapenemase was observed in *Pseudomonas spp.* 14(37.83%) followed by *Escherichia coli* 10(27.02%), *Klebsiella spp.* 9(24.32%) and *Acinetobacter spp*. 4(10.81%). Table 2 - shows among 47 meropenem resistant isolates, 19 uropathogens harbored 18(94.73%) *bla*NDM and 1(5.26%) *bla*NDM+blaOXA-48 and 24 isolated bacteria in wound-swab carried 19(79.16%) *bla*NDM, 1(4.16%) *bla*VIM and co-existence of 3(12.50%) *bla*KPC+*bla*NDM+*bla* OXA-48, 1(4.16%) *bla*KPC+ *bla*OXA-48. No *bla*IMP gene detected in any isolates. Table 3 -shows out of 43 carbapenemase genes producing isolates highest rate of carbapenemase gene was observed in *Pseudomonas spp.* 15(34.88%) followed by *Escherichia coli* 11(25.58%), *Klebsiella spp.* 11(25.58%) and *Acinetobacter spp*. 6(13.95%). Considering PCR as the gold standard, the results of the mCIM test were compared with PCR by Chi-square tests (Table 4). The difference between the mCIM and PCR to detect carbapenemase-producers was statistically highly significant ($p < 0.001$).

Table 1: Distribution of Carbapenemase Producing bacteria (n=37).

Name of Organism	Carbapenemase detected by mCIM		Total(n=37)
	Urine(n=17)	Wound swab(n=20)	
Escherichia coli	6 (35.29%)	4 (20.00%)	10 (27.02%)
Klebsiella spp.	4 (23.52%)	5 (25.00%)	9 (24.32%)
Pseudomonas spp.	7 (41.17%)	7 (35.00%)	14 (37.83%)
Acinetobacter spp.	0 (0.00%)	4 (20.00%)	4 (10.81%)
Total	17(100%)	20 (100%)	37 (100%)

Table 2: Detection of Carbapenemase genes among the isolates in urine and wound-swab(n=43).

Carbapenemase genes	Clinical samples		Total
	Urine	Wound swab	
*bla*NDM	18 (94.73%)	19 (79.16%)	37 (86.04%)
*bla*VIM	0	1 (4.16%)	1 (2.32%)
*bla*IMP	0	0	0
*bla*KPC	0	0	0
*bla*OXA 48	0	0	0
*bla*KPC + *bla*NDM + *bla*OXA 48	0	3 (12.50%)	3 (6.97%)
*bla*KPC + *bla*OXA 48	0	1 (4.16%)	1 (2.32%)
*bla*NDM + *bla*OXA 48	1 (5.26%)	0	1 (2.32%)
Total	19 (100%)	24 (100%)	43 (100%)

Table 3: Distribution of Carbapenemase genes among the isolated bacteria (n=43).

Name of organism	*bla*NDM	*bla*VIM	*bla*KPC + *bla*NDM + *bla*OXA 48	*bla*KPC + *bla*OXA 48	*bla*NDM + *bla*OXA 48	Total
Escherichia Coli	11 (29.72%)	0	0	0	0	11 (25.58%)
Klebsiella spp.	6 (16.21%)	0	3 (100.00%)	1 (100.00%)	1 (100.00%)	11 (25.58%)
Pseudomonas spp.	14 (37.83%)	1 (100.00%)	0	0	0	15 (34.88%)
Acinetobacter spp.	6 (16.21%)	0	0	0	0	6 (13.95%)
Total	37(100%)	1(100%)	3 (100%)	1 (100%)	1 (100%)	43(100%)

Table–4: Association between mCIM andMultiplex PCRtest results among the Meropenem resistant isolates (n=47).

mCIM	Multiplex PCR Test			χ^2 *test Significance*
	Positive	Negative	Total	
Positive	37	00	37	$\chi^2 = 11.447$ P = 0.001 **HS**
Negative	06	04	10	
Total	43	04	47	
●Figures within parentheses indicate percentage ● HS = Highly *Significant (P < 0.01)*				

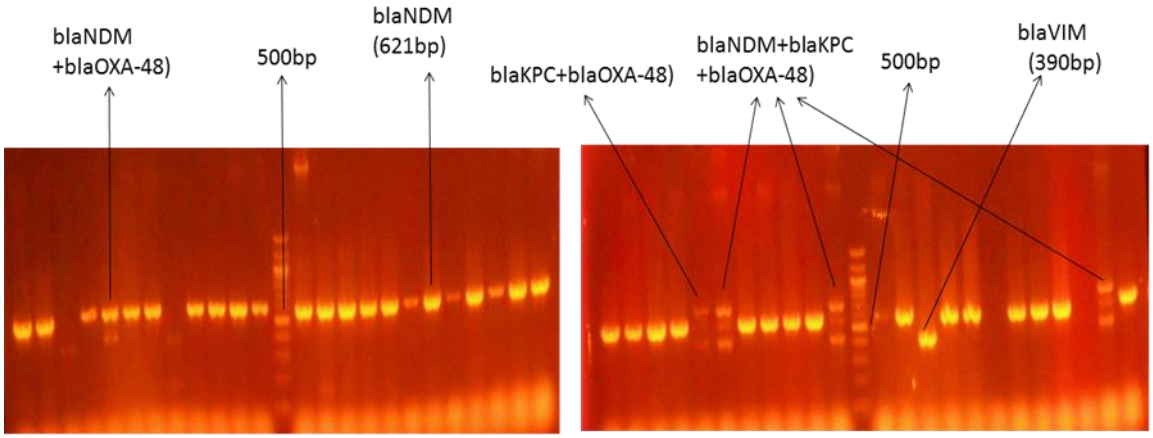

Figure II- Snapshot is showing multiplex PCR gel electrophoresis of amplified DNA. 500bp- DNA ladder, 621bp shows definite band of *bla*NDM gene, 621bp+438bp indicating co-existence of *bla*NDM and *bla*OXA-48, 798bp+438bp indicating co-existence of *bla*KPC and *bla*OXA-48, and 621bp+798bp+438bp indicating co-existence of *bla*NDM,*bla*KPC and *bla*OXA-48, 390bp shows a positive band of the *bla*VIM gene.

DISCUSSION

The emergence and spread of beta lactamases producing Gram negative bacteria is a world-wide public health threat.[11] This could be a serious issue, especially in hospitals, as carbapenems are increasingly required to treat infections brought on by Gram-negative bacteria that develop extended spectrum beta lactamases.[12,13] But the wide spread use of carbapenem in clinical practice has led to the development of resistance among Gram negative bacteria.

In our study, Gram negative bacteria, displayed resistance against meropenem 27%. Similar study was found by Kumari et al. (2022) in India, where meropenem resistance was 22%.[14] Factors that play a critical role in the emergence of meropenem resistance are improper antibiotic prescription, uncontrolled public access to antimicrobials, poor sales regulation, lack of infection control measures within healthcare centers.[15]

In the present study, mCIM detected 17 carbapenemase producing uropathogens where the most prevalent was *Pseudomonas spp*. 7(41.17%) followed by *Escherichia coli* 6(35.29%), and *Klebsiella spp.* 4(23.52%). Again, mCIM also detected 20 isolated bacteria in wound-swab where the most predominant was *Pseudomonas spp*. 7(35.00%) followed by *Klebsiella spp*. 5(25.00%), *Escherichia coli* 4(20.00%),and *Acinetobacter spp*. 4(20.00%). Similar studies were found by Awoke, T.et al. (2019) who reported that carbapenemase producing *Klebsiella spp.* was 21.20% and Pragasam et al. (2018) found 40.00% *Pseudomonas spp*. as carbapenemase producers.[16,17] Our study was in accordance with Juan Li et al. (2019) in China and Tsai et al. (2020) in Taiwan

who noted that carbapenemase producing *Escherichia coli* were 27.84% and 30.47% respectively.[18,19] Another study by Elbadawi et al (2021) found that carbapenemase producing *Acinetobacter spp.* was 50% that was not correlated with our study.[1] In *Pseudomonas spp.* carbapenem resistance is typically multifactorial and produced by a variety of mechanisms. Generally, *Pseudomonas aeruginosa* exhibit resistance to carbapenems by acquisition of transferable genes encoding carbapenemases or inactivation of the carbapenem porin OprD or the overexpression of efflux pump system such as MexAB-OprM.[20] In recent years, the rate of carbapenem resistance in *Pseudomonas aeruginosa* have increased worldwide and have become of great concern since they significantly restricts the therapeutic options for patients.[21] However the discripency of carbapenemase producing *Acinetobacter spp.* expressed that prevalence of carbapenemase producers vary country to country and different samples, but its increasing is very alarming for us.

Khan et al. (2017) reported that in the Asian continent, especially China and India were a reservoir of NDM in which about 58.20% abundance of the *bla*NDM-1 variants.[22] In Ethiopia, the prevalence of carbapenemase producing isolates was 21.20% by mCIM and the most dominant gene was *bla*NDM 92.90%[16] whereas our study noted that the result of mCIM was 21.63% from total gram negative bacteria with predominant gene *bla*NDM was 86.04%. Similar finding also found by Khatun et al. (2015) where *bla*NDM was 73.70%.[23] The result of the current study reflects the emergence of carbapenemase gene mainly *bla*NDM is alarming for therapeutic options and its rising in Bangladesh like India, Pakistan and Nepal indicating inappropriate and nonprescription antibiotic use as a probable cause of development of resistance in this subcontinent.[4] Furthermore, rapid dissemination of *bla*NDM producing organisms might be facilitated by conditions such as overcrowding, over the counter availability of antibiotics, low level of hygiene and weak hospital antibiotic policies.[24]Our findings differ with several other studies where Begum et al. (2016) in Bangladesh and Anjana et al. (2020) in Nepal revealed that predominant *bla*NDM were 55% and 45.94% respectively[25,26] The discripency of these result revealed that there were differences in samples number and various samples and area variation.

There were several studies , where Jaggi et al. (2019) in India, reported 29.54% *Escherichia coli*, Khater et al. (2022) in Saudi Arabia, found 37.50% *Pseudomonas spp*. and Elbadawi et al. (2021) in Sudan noted 15.80% *Acinetobacter spp.* harbored *bla*NDM and all these studies were correlated with our study.[27,28,1] Whereas dissimilar study was noted by Sattar et al. (2020) in Bangladesh found 28.57% *Klebsiella pneumoniae* carried *bla*NDM.[24] In our hospital setting, increased uses of carbapenem derivatives reflects *bla*NDM positive isolates and they exhibited multi and extensive drug resistance.[29]

Copresence of multiple resistance genes is a big concern in the spread of antibiotic resistance It has been observed that majority of Gram negative bacteria contained more than one carbapenemase gene, where co-existence genes were mostly found in *Escherichia coli* and *Klebsiella spp.*[14] In the present study, 1(100%) *Pseudomonas spp.* carried *bla*-VIM gene, coexistence of *bla*KPC+*bla*NDM+*bla*OXA genes, *bla*KPC+*bla*-OXA48 genes and *bla*-NDM+*bla*-OXA-48 genes were present in 5 different isolates of *Klebsiella spp*. In Bangladesh, Begum et al. (2016) reported that more than one carbapenemase genes in nine of the isolates, where coharbouring of *bla*NDM and *bla*OXA-48 in 4 isolates.[25] In China, Han et al. (2020) found that the coharbouring of bla-NDM+*bla*-OXA-48 was present in 1 *Klebsiella spp.* which also correlated to our study.[30] In Sudan, Elbadawi et al.(2021) revealed out of 45 *Pseudomonas spp.* isolates, 14 *Pseudomonas spp.* contained *bla*NDM and 1 harbored *bla*VIM which were similar to our study.[1], Furthermore, in the current study showed no *bla*IMP that was supported by Solanki et al.(2014) in India where no *bla*IMP was found in any of the isolates.[7] Treatment of infections brought on by carbapenem-resistant strains faces a new difficulty due to the co-existence of carbapenem-resistant genes with beta-lactamases and other resistance genes on plasmids.[31] In addition to this, co-existence to carbapenem retains genes make resistance to other antimicrobials, which threatens global antibiotic therapy, patient recovery and the economy.[32]

In the present study the comparison between mCIM and Multiplex PCR revealed,out of 47 Meropenem resistant isolates, 37 carbapenemase producers were detected by mCIM and 43 carbapenemase genes were found by Multiplex PCR which was statistically highly significant. It also showed that PCR detected additional six isolates which were negative by mCIM. Though phenotypic tests are specific, they do not differentiate between chromosomal and plasmid encoded genes.[26] So, the short comings of phenotypic approaches are resolved by precise detection of the genes associated with carbapenemase synthesis by molecular methods such Multiplex PCR.[7]

The current emergence of carbapenemase-producing Gram negative bacteria is of concern because very few antibiotic options remain available and its containment is more challenging in developing countries due to poor antimicrobial resistance surveillance and irrational use of antibiotics.[33] By the year 2050, drug-resistant infections may cause about 10 million deaths annually, with approximately 90% of the predicted deaths to happen in Asia and Africa.[34,35]

So effective measures such as the establishment of active surveillance and infection control programmes, emphasizing hand hygiene together with coherent

antibiotic policies in hospitals and clinics should be implemented to stop and manage the spread of carbapenemase in hospitals and communities.

CONCLUSIONS

Phenotypic method has a disadvantage of inability to discriminate the type of carbapenemase and indeterminant result of mCIM requiring more incubation time where further test needed to establish presence or absence of carbapenemase production. So accurate detection of the genes related with carbapenemase production by Multiplex PCR is a reliable assay for rapid screening and identification of resistance traits that helps in determining epidemiology related to genes and infection control.

RECOMMENDATIONS

- As Carbapenem is a WATCH group of drug, it should not be used indiscriminately without antibiotic sensitivity test.
- PCR method can be used as superior diagnostic tool where infrastructure and facilities are available.

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