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Genotypic Detection of Carbapenemase Producing *Pseudomonas* species and *Acinetobacter* species by Multiplex PCR at a Tertiary Care Hospital in South-East Region of Bangladesh

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Abstract

Background: Increasing prevalence of carbapenem-resistant *Pseudomonas* species and *Acinetobacter* species in the nosocomial setting represents as emerging challenge to public health. The frequency of carbapenemase producing strains among clinical isolates has been steadily increasing over the past few years resulting in limitation of the therapeutic options. **Objective:** 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:** This was a cross sectional study which was done in the Department of Microbiology of Chittagong Medical College with 300 patients from February 2021 to June 2022. Nonduplicate *Pseudomonas* species and *Acinetobacter* species. were included in this study from inpatients of Chittagong Medical College Hospital. Antimicrobial susceptibility test was performed by Kirby Bauer disc diffusion method as per Clinical Laboratory Standard Institute-2021. The modified carbapenem inactivation method was done to see carbapenemase producers among meropenem resistant isolates. Finally carbapenemase genes were detected by multiplex PCR. **Results:** Out of 300 samples, 171 culture positive isolates showed 38.01% *Pseudomonas* species, 11.11% *Acinetobacter* species and along with 51.39% other species. Following CLSI guideline 2021, antimicrobial susceptibility tests revealed that highest resistance against ampicillin, cefuroxime, ciprofloxacin, aztreonam, ceftriaxone and ceftazidime were 98.80%, 77.38%, 73.80%, 70.23%, 69.04% and 67.85% respectively. The lowest resistance was observed against nitrofurantoin, amikacin, pipercillin-tazobactum, meropenem and ceropime 11.90%, 16.66%, 22.61%, 27.38% and 39.24% respectively. Also, 21.17% were found to be carbapenemase producers by mCIM and 24.70% carbapenemase genes were detected by multiplex PCR among meropenem resistance isolates. Multiplex PCR showed bla_{NDM} gene in 95.23% isolates, bla_{-VIM} in 4.77% among meropenem resistance isolates. **Conclusion:** In conclusion the frequency of carbapenemase producing *Pseudomonas* species and *Acinetobacter* species is increasing which causes a significant impact on patient′s management.

Keywords: Modified carbapenem inactivation method (mCIM); New Delhi metallo-beta-lactamase (NDM); Verona Integron-encoded metallo-beta-lactamase (VIM); Hospital acquired infection (HAI)

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Introduction

Carbapenem resistance *Pseudomonas* species and *Acinetobacter* species have been implicated in life threating nosocomial infections because they are frequently difficult to treat with available antibiotics associated with high death rates. These pathogens are more challenging for treatment of patients in ICU, burn centre and patients with cystic fibrosis. According to World Health Organization (WHO),

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Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) and Carbapenem-resistant *Acinetobacter baumannii* (CRAB) have been designated as a priority 1-critical pathogens for research and development of new antibiotics. They pose a significant therapeutic challenge due to lack of established treatment options because very few antimicrobial agents are active against them.

Carbapenem is a last resort drug for the management of multidrug resistant *Pseudomonas* species and *Acinetobacter* species infection. Carbapenem is a beta-lactam antibiotic which inhibits transpeptidases (penicillin-binding proteins) and prevents peptidoglycan synthesis, leading to lytic cell death[2]. Resistance is mediated mainly by production of carbapenemase either serine based carbapenemase or metallo-beta-lactamases (MBLs). Moreover, the combination of structural mutations with the production of other beta-lactamases, such as AmpC cephalosporinase and ESBL also causes resistance of carbapenem[3-4].

Carbapenem resistance in *Pseudomonas* species and *Acinetobacter* species have been demonstrated to be multifactorial, it may be due to carbapenemase synthesis, efflux pump or gene overexpression[5]. The carbapenemase encoding genes are often located on plasmids along with other resistance genes resulting in multidrug resistance (MDR), extensive drug resistance (XDR) and pan-drug resistance (PDR)[3].

Carbapenemases can be divided based on their molecular characteristics into class A, B or D using the Ambler classification system. The most common class A carbapenemase is KPC enzyme, 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[6]. Three groups of carbapenemases – KPC, NDM, and OXA-48 are currently considered to be the three major beta-lactamases of epidemiological and clinical significance[7].

The prevalence of carbapenemases producers still remain obscure because of the lack of proper detection method in many countries, especially those with limited resources and poor laboratory settings[8]. In this situation, there is a growing demand for the rapid and accurate detection of carbapenemase producing bacteria to determine the precise antibiotic therapy and prevent the hospital-based outbreaks[9]. Several approaches have been developed to identify the presence of carbapenemases in Gram negative bacteria. These include phenotypic and genotypic (nuclear amplification-based) tests. Timely and accurate detection of carbapenemase producing Gram negative bacteria is essential for both clinicians and infection control practitioners to prevent the progression of hospital-based outbreaks. In 2017, based on the Carbapenem Inactivation method (CIM), Clinical laboratory and standards institute (CLSI) recommended the modified Carbapenem Inactivation Method (mCIM) for detection of carbapenemase.

On the contrary the multiplex PCR was found to be more sensitive than existing phenotypic methods. Multiplex PCR also helps in detection of various genes, reducing materials, manpower and helps in determining epidemiology related to these genes and infection control[10]. This study was conducted to determine the prevalence of carbapenemase producers in urine and wound-swab along with antimicrobial resistance patterns of these organisms.

Methodology

Study Settings and Population: This was a cross-sectional type of study conducted in the Department of Microbiology of Chittagong Medical College, Chattogram, Bangladesh 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 having surgical or burn wound infections or suspected UTI irrespective of age, sex and antibiotic intake.

Laboratory Procedure: Samples were inoculated into sheep blood agar and Mac Conkey agar and incubated over night at 37ºC. 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, MIU and Simmon's citrate media) as per standard procedure[11]. A total of 84 non-duplicate non fermentative 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 2021. 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[12].

Modified Carbapenem Inactivation Method for Detection of Carbapenemase: In the mCIM, 10µL loopful of bacteria for *Pseudomonas* species or *Acinetobacter* species 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 35ºC±2ºC 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 disk 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 disk suspension and put on an MHA plate that had already been contaminated with the indicator strain o*f Escherich*ia *coli* ATCC-25922. Then the plate was incubated at 35ºC±2ºC in ambient air for 18 to 24 hours. Following incubation, the zone of inhibition was measured. No zone of inhibition or 6 to 15mm of zone diameter or presence of pinpoint colonies within 16-18mm zone were considered as carbapenemase positive. On the other hand, zone dm of ≥19mm was considered carbapenemase negative or zone dm, 16 to 18mm or presence of pinpoint colonies within ≥19mm zone were considered carbapenemase indeterminate[12].

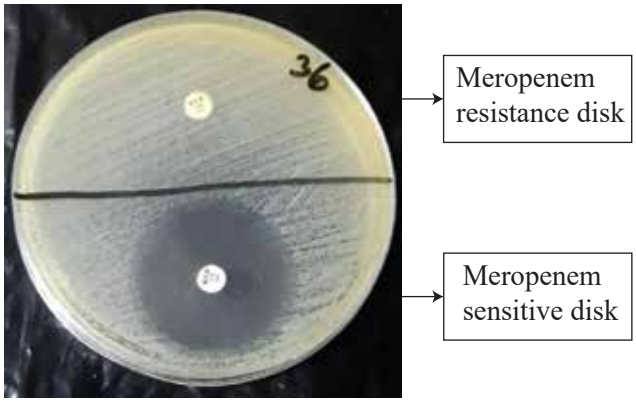

Figure I: Modified Carbapenem Inactivation Method

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 were used (Table-1).

The following cycling parameters were used. Initial denaturation was done at 94°C for 10 minutes followed by 36 cycles of amplication consisting of denaturation at 94°C for 30s, primer annealing at 52°C for 40s, 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 1.0% ethidium bromide and visualized under UV light[13].

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Categorical data were summarized in terms of frequency counts and

Table 1: List of Primers of Genes

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		

percentages. Continuous data were expressed as mean, standard deviation, minimum and maximum.

Ethical Clearance: Ethical Clearance committee of Chittagong Medical College has reviewed and approved the research proposal entitled "Genotypic Detection of Carbapenemase Producing *Pseudomonas species* and *Acinetobacter species* by Multiplex PCR at a Tertiary Care Hospital in South-East Region of Bangladesh" under the memo no CMC/PG/202/700 on14.02.2021. All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information maintained. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

A total of 84 non-fermentative Gram-negative bacteria, 65(77.4%) *Pseudomonas* species and 19(22.6%) *Acinetobacter* species were isolated and 21.4% carbapenemase producers were detected by mCIM and 25.0% carbapenemase genes were found in meropenem resistance isolates. The bacterial isolates were tested for antimicrobial susceptibility following CLSI guideline 2021 where 65 *Pseudomonas species* displayed highest resistance towards ampicillin, cefuroxime, ciprofloxacin, aztreonan, ceftriaxone and ceftazidime (around 99.0% to 68.0%) and lowest resistance against nitrofurantoin, amikacin, pipercillin-tazobactum, meropenem and cefepime (around 9.0% to 43.0%). On the other hand, 19 *isolated Acinetobacter species* exhibited highest resistance against ampicillin, ciprofloxacin, cefuroxime, ceftriaxone and ceftazidime (around 100.0% to 74.0%) and lowest resistance against meropenem. amikacin, pipercillin-tazobactum and cefepime (around 31.0% to 42.0%) (Figure II).

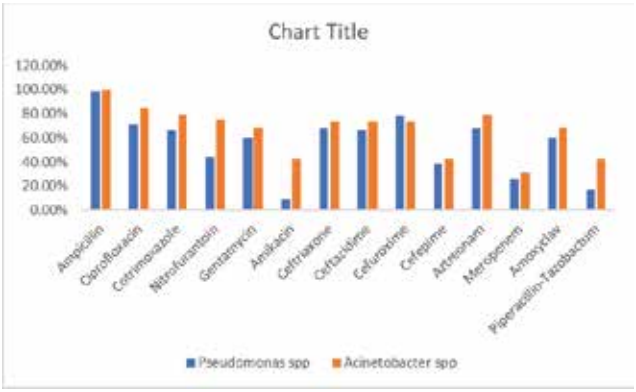

Figure II: Antibiotic Resistance Pattern between *Pseudomonas* speciesand *Acinetobacter* species

Out of 23 meropenem resistant isolates, 18 isolates were found to be carbapenemase producers by mCIM and higher rate of carbapenemase was observed in *Pseudomonas* species, 14(78.0%) followed by *Acinetobacter* species, 4(22.0%) (Table 1).

Table 2: Distribution of Carbapenemase producing bacteria (n=18)

Name of Organism	Carbapenemase detected by mCIM		Total
	Urine	Wound swab	
Pseudomonas species	7(100.0%)	7(64.0%)	14(78.0%)
Acinetobacter species	0(0.0%)	4(36.0%)	4(22.0%)
Total	**7(100.0%)**	**11 (100.0%)**	**18(100.0%)**

Among 23 meropenem resistant isolates, 20(95.2%) isolates harbored bla_{NDM} and 1(4.8%) bla_{VIM} (Table 2).

Table 3: Detection of Carbapenemase genes among the isolates in urine and wound-swab (n=21)

Carbapenemase Genes	Clinical samples		Total
	Urine	Wound swab	
bla_{NDM}	**8(100.0%)**	12(92.3%)	20(95.2%)
bla_{VIM}	**0(0.0%)**	1(7.7%)	1(4.8%)
Total	**8(100.0%)**	**13(100.0%)**	**21(100.0%)**

Out of 21 carbapenemase genes producing isolates highest rate of carbapenemase gene was observed in *Pseudomonas* species 15(71.4%) followed by *Acinetobacter species* 6(28.6%) (Figure III).

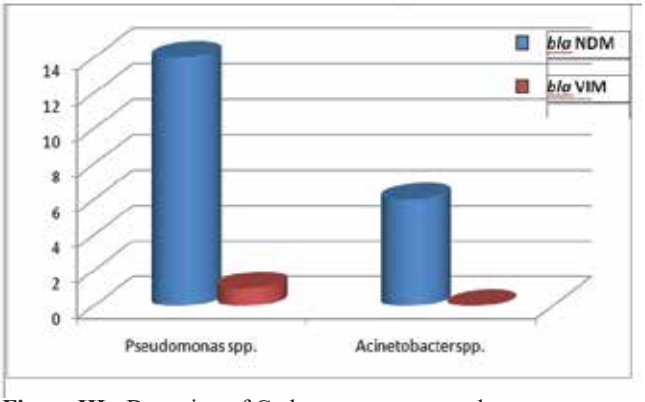

Figure III: Detection of Carbapenemase genes between *Pseudomonas species* and *Acinetobacter species*

Considering PCR as the gold standard, the results of

the mCIM test were compared with PCR by Chi-square tests. The difference between the mCIM and PCR to detect carbapenemase-producers was statistically significant ($p<0.05$) (Table 3).

Table 4: Association between mCIM and Multiplex PCR Test results among meropenem resistant isolates (n=47)

mCIM	Multiplex PCR Test			P value
	Positive	Negative	Total	
Positive	18	00	18	
Negative	03	02	05	0.05
Total	**21**	**02**	**23**	

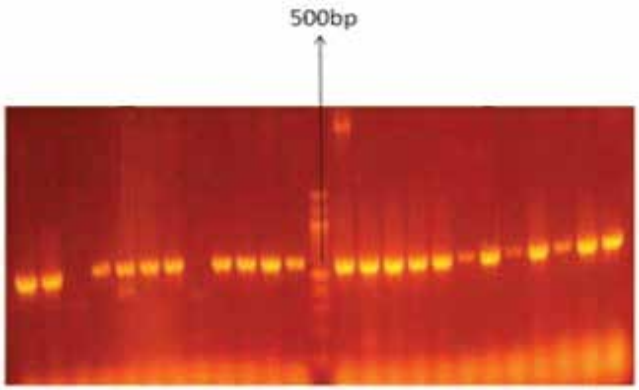

Figure IV: Snapshot is showing multiplex PCR gel electrophoresis of amplified DNA. 500bp- DNA ladder, 621bp shows definite band of ***bla$_{NDM}$*** gene and 390bp shows a positive band of the *bla$_{VIM}$* gene.

Discussion

Carbapenemase producing *Pseudomonas species* and *Acinetobacter species* are emerging worldwide because of their capacity to become increasingly resistant to all available antibiotics. So, this review focused on detection of carbapenemase genes as well as antibiotic resistance pattern among the isolates which is key to the initiation of early effective therapy.

Regarding our resistance profiles, *Pseudomonas species* were resistance (68.0%) against third generation cephalosporins followed by 17.0% pipercillin- tazobactum and 26.0% meropenem. Similar study were found by Nithyalaksmi et al[14] and Nasir et al[15] in India, where *Pseudomonas species* displayed resistance 75.9% against third generation cephalosporins, 13.8% against piperacillin–tazobactam and 20.4% against meropenem. The current study showed that among the isolated *Acinetobacter species*. major resistance was observed to third generation of cephalosporin (73.7%) while the least resistance rate was observed in meropenem (31.57%) and amikacin, pipercillin-tazobactum and cefepime (42.1%). Tilahun et al[16] in North East Ethiopia noted that *Acinetobacter* species showed resistance to amikacin (37.9%) and meropenem (34.5%) that was supported by our study. However a study by Fatema et al[17] Dhaka, Bangladesh revealed the highest rate of resistance to ceftriaxone (99.6%).

Pseudomonas species and *Acinetobacter species* are both intrinsically resistant to many antibiotics due to complementary mechanisms, the main ones being the low permeability of their outer membrane, the production of the AmpC beta-lactamase or carbapenemases and the production of several efflux systems belonging to the resistance-nodulation-cell division family. In addition, *Pseudomonas species* may also acquire resistance to antibiotics due to permeability barrier of the cell surface in the form of biofilm production[18]. Moreover higher antibiotic resistance in this study might be due to the fact that common antibiotics are sold over the counter of Bangladesh and anybody can buy them without doctor′s advice[19].

In the current study, 41.17% carbapenemase producing *Pseudomonas species* was found in uropathogen and 35.00% carbapenemase producing *Pseudomonas species* and 20.00% *Acinetobacter species* were detected in the isolated bacteria in wound-swab. by mCIM. Similar study was found by Pragasam et al[5] in India where 40.00% *Pseudomonas species* were found as carbapenemase producers. Another study by Elbadawi et al[9] in Sudan found that carbapenemase producing *Acinetobacter species* was 50.0% that was not correlated with our study. Generally, *Pseudomonas aeruginosa* can acquire 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 restrict the therapeutic options for patients[21]. On the contrary, the mechanisms of carbapenem resistance *Acinetobacter species* consist of natural membrane impermeability, basal efflux activity, and the presence of two chromosomally encoded beta-lactamases, an ADC cephalosporinase and an OXA-51 oxacillinase[22]. However, the discrepancy of carbapenemase producing *Acinetobacter species* expressed that prevalence of carbapenemase producers vary from country to country and different samples, but its increasing rate is very alarming for us.

In the present study, 27.4% meropenem resistant isolates were subjected to multiplex PCR and 25.0% carbapenemase genes were found among the isolates from which 38.09% were uropathogen and 61.9% were found in wound-swab. Carbapenemase producing genes were detected as 95.2% bla_{NDM} and (4.8%) $bla\text{-}_{VIM}$. Among 21 carbapenemase producing isolates, the most prevalent was *Pseudomonas species* 15 71.4% followed by *Acinetobacter species* 6(28.6%). In the current study, 20 bla_{NDM} were harbored by different isolates such as 14(70.0%) *Pseudomonas species* and 6(30.0%) *Acinetobacter species*.

Khan et al[23] in India, reported that in the Asian continent, especially China and India are reservoir of NDM in which about 58.2% presence of the $bla_{NDM\text{-}1}$ variants were found. In Ethiopia[24], the prevalence is carbapenemase gene of bla_{NDM} was 92.9% that was similar to our study where the predominant gene bla_{NDM} was 95.2%. The other study by Khatun et al[25] Dhaka, Bangladesh noted that bla_{NDM} was 73.7% that is similar to our study in *Pseudomonas species* whereas dissimilar study was noted by Elbadawi et al[9] in Sudan which found that 15.8% *Acinetobacter species* harbored bla_{NDM} .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[6]. 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[26]. In this hospital setting, the carbapenem-resistant (CR) phenotype is very common for MDR and particularly for XDR isolates because carbapenemase-encoding genes are carried on mobile genetic elements that usually carry genetic determinants for resistance to other antibiotics. So treating patients with HAI caused by these pathogens is usually a challenge due to intrinsic and acquired resistance to the commonly used and affordable antibiotics, which has been heightened by the emergence of bla_{NDM27}.

In the present study, we observed that two meropenem resistante isolates showed negative amplification by PCR. The plethora of meropenem resistance among non-carbapenemase producers might be modification of target site, change in membrane permeability or upregulation of efflux pump. Furthermore, selective pressure and/or simultaneous presence of other drug resistance genes such as gene cassettes may be reason for non-carbapenemase producers.

In the present study, the comparison between mCIM and multiplex PCR revealed, out of 23 meropenem resistant isolates, 18 carbapenemase producers were detected by mCIM and 21 carbapenemase genes were found by multiplex PCR which was statistically significant. It also showed that PCR detected additional three isolates which were negative by mCIM. Though phenotypic tests are specific, they do not differentiate between chromosomal and plasmid encoded genes and hence genotypic characterization should be considered[28]. So accurate detection of the genes related with carbapenemase production by molecular methods like multiplex PCR overcome the limitations of the phenotypic methods[29].

Conclusion

The current emergence of carbapenemase-producing *Pseudomonas species* and *Acinetobacter species* are becoming a major public health crisis worldwide 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. So, accurate detection of carbapenemase-producing glucose-nonfermenting Gram-negative bacilli (CPNFs), including *Pseudomonas species* and *Acinetobacter species*, is necessary to prevent their dissemination within health care settings. So the need of the hour includes strong antimicrobial stewardship program followed by the establishment of active surveillance and infection control programmes, emphasizing hand hygiene together with coherent antibiotic policies in hospital and clinics to stop and manage the spread of carbapenemase in hospital and communities. As Carbapenem is a WATCH group of drugs, 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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Conflict of Interest

The authors declare no conflict of interest.

Financial Disclosure

This research received no external funding

Authors' contributions
Md. Ehsanul Hoque conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Abul Kalam and Abu Hena Md. Saiful Karim contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Jahanara Rosy involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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