Original Article

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Evaluation of Phylogenetic Diversity and Molecular Profiling of blaNDM-1 Gene Harbored in Clinical Carbapenem Resistant *Pseudomonas spp.* in Chattogram

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ABSTRACT

Background: *The emergence of the blaNDM-1 gene, a potent metallo- β-lactamase conferring resistance to carbapenems, has become a major global public health concern, particularly among multidrug-resistant Pseudomonas aeruginosa strains, which complicates treatment options and exacerbates the burden of antimicrobial resistance. This research is performed to determine the prevalence of the blaNDM-1 gene in Pseudomonas spp.and the phylogenetic diversity pattern of the blaNDM-1 gene.*

Materials and methods: *This cross-sectional study was conducted in the Department of Microbiology, Chittagong Medical College where Pseudomonas spp. was identified conventionally and the antimicrobial susceptibility pattern was performed by modified Kirby Bauer disc diffusion method. Carbapenemase-producing Pseudomonas spp. was determined by phenotypically (mCIM) and molecular tests (PCR). Sequencing of the blaNDM-1 gene was done by automated Sanger sequencing.*

Results: *From urine, wound swab, endotracheal aspirate, sputum and blood Pseudomonas spp. (21.86%) was identified phenotypically. Antimicrobial susceptibility tests revealed resistance to Imipenem (24.53%) and Meropenem (30.19%) with a total of carbapenem resistance (33.33%). Carbapenemase producers were determined by mCIM (24.07%) and the detected blaNDM-1 gene (37.04%). Nucleotide sequencing of randomly selected blaNDM-1 gene was done and a phylogenetic tree was constructed.*

Conclusion: *This study shows the existence of silent AMR genes with an association between phenotypic and molecular methods. To combat the limitation of phenotypic tests, molecular methods may be a good alternative to detect resistance cases to overcome treatment failure where facilities are available.*

Key words: blaNDM-1 gene; Metallo- β-lactamase; Phylogenetic diversity; Silent AMR gene.

Introduction

Globally, Pseudomonas aeruginosa is now a major contributor to Gram-negative infections, particularly in patients with compromised immunity.[1,2] It results in diseases of the urinary tract, respiratory tract, bloodstream, surgical sites, eyes, wounds (Particularly burn patients) and the external ear. Hospital-Acquired Infections (HAI) are often caused by P. aeruginosa, in patients hospitalized for over a week.[3] Nosocomial infections brought on by this bacterium have gained attention recently as a serious issue because of the organism's inherent resistance to numerous antibiotic classes and its ability to develop practical resistance to all clinically available, effective medicines.[4] Because of this, P. aeruginosa has been included to the first list of ESKAPE pathogens (Which also includes Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, as a top priority (Critical) organism for study, discovery and new drug development.[5] The genome of P. aeruginosa is incredibly complex with a vast array of virulence genes and sophisticated regulation and signaling systems, contributing to the pathophysiology of both acute and chronic infections.[6] Notably, P. aeruginosa has produced Extensively Srug-Resistant (XDR) clinical isolates due to its capacity to build resistance through a variety of processes, such as

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the selection of chromosomal mutations and the accumulation of transferable resistance genes.[7] Specifically, a combination of the following mechanisms frequently leads to carbapenem resistance in P. aeruginosa: (a) Overexpression of efflux pump systems (b) overexpression (Through derepression) of chromosomal cephalosporinase and (c) Loss or reduced expression of the outer membrane protein OprD.[8-10]

Furthermore, P. aeruginosa may develop carbapenem resistance as a result of acquiring carbapenemases, primarily Ambler class B Metallo- β-lactamases (MBLs).[11] Notably, the most recent multi-national ERACE-PA Surveillance study demonstrated that carbapenemase producers are becoming more common among isolates of P. aeruginosa.[12] Although the exact origins of MBLs are unknown, environmental bacteria such as Enterobacteriaceae are most probably the source.[13] Mobile genetic elements such as integrons that reside on plasmids or bacterial genomic DNA are the primary means by which the genes encoding MBLs can spread.[13,14] Most MBLs are classified as subclass B1[15,16]. The three most prevalent MBLs, IMP (Imipenemase) VIM (Verona integron-encoded metallo- β-Lactamase), and NDM (New Delhi metallo-β-Lactamase), are well known for their epidemiological and clinical significance.[13,17,18] Of the different kinds of MBLs in Asia, the most prevalent carbapenem resistance mechanism in Pseudomonas aeruginosa and Enterobacterales is New Delhi metallo- β–Lactamases (NDMs).[19] The prevalence of these MBLs has significantly increased globally, especially in Southeast Asian nations like Bangladesh, India, and Pakistan as well as the United Kingdom.[14,20,21]

Nucleotide sequencing has emerged as a crucial tool in addressing the challenges posed by MDR (Multidrug-Resistant) XDR (Extensively Drug-Resistant) and PDR (Pandrug-Resistant) bacteria in the healthcare sector. By utilizing sequencing technologies in detected resistance genes, researchers can uncover a wealth of information, including finding mutation points and evaluation of genetic diversity. Geographical distribution patterns and so on. This comprehensive knowledge is essential for molecular surveillance and in formulating effective strategies to prevent the devastating effects of these formidable organisms. Very few genomic studies of this pathogen are available from Bangladesh,especially from Chattogram. The unaware inattention toward the genomic study of this infectious opportunistic pathogen roused the importance and urgency to study this pathogen in Bangladesh,particularly in hospitalized patients.

Materials and methods

This was a cross-sectional type of study conducted in the Department of Microbiology of Chittagong Medical College, Chattogram in collaboration with different disciplines of Chattogram Medical College Hospital (CMCH) from July 2022 to June 2023. A total of 54 non-duplicate Pseudomonas spp. was isolated from urine, wound swab, sputum, blood and endotracheal aspirate culture after taking prior consent using predesigned data sheets from admitted patients with suspected urinary tract infection, respiratory tract infection, wound infection, infected burn wound, febrile illness, severely ill patients of NICU, PICU and patients who were in mechanical ventilation in ICU irrespective of age, gender and antibiotic intake.

The study received ethical approval from the Institutional Review Board of Chittagong Medical College (CMC) Chattogram, Bangladesh.

Identification of Pseudomonas spp.

Pseudomonas spp. was identified by observing Gram staining character, colony morphology, hemolytic criteria on blood agar media, pigment production, pale colonies on MacConkey media, relevant biochemical tests such as oxidase (Positive) catalase (Positive) and other biochemical tests after inoculation in TSI (Alkaline slant, alkaline butt, no gas and no H_2S production) MIU (Motile, indole and urease negative) Simmon's citrate media (Utilize citrate).[22]

Methods of antimicrobial susceptibility test

Modified Kirby – Bauer disc diffusion method was used to determine the susceptibility of isolated bacteria. For Pseudomonas following antibiotic discs were used, amikacin (30µg) aztreonam (30µg) cefepime (30µg) ceftazidime (30µg) ciprofloxacin (5µg) gentamicin (10µg) imipenem (10µg) levofloxacin (5µg) meropenem(10µg) piperacillin-tazobactam (100/10µg). The zone of inhibition was interpreted according to CLSI guidelines, M100 2022. Discs from each batch were first standardized by testing against reference strains of Pseudomonas aeruginosa ATCC-27853 and zone of inhibition was compared with the standard value.

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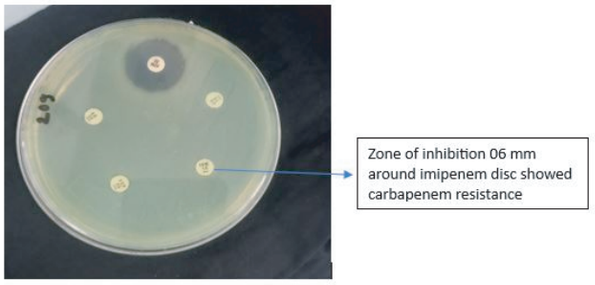

Figure 1 Antimicrobial susceptibility test by modified Kirby Bauer disc diffusion method showing carbapenem resistance

Modified Carbapenem Inactivation Method (mCIM) for detection of carbapenemase-producingPseudomonas spp.[23]

A 10µL loopful of test bacteria from an overnight agar plate was transferred to a tube containing 2 ml of Trypticase Soy Broth (TSB) and the suspension is vortexed for 15 seconds. A standard 10µg meropenem disc was added to the suspension. The TSB-disc suspension was incubated for 4 hours at 35 in ambient air. Just before the completion of the 4-hour incubation cycle, a 0.5 McFarland suspension of E. coli ATCC 25922 was prepared and inoculated onto an MHA plate following the routine disk diffusion procedure. The meropenem disk was removed from the TSB suspension using a 10µL loop, taking care to remove excess liquid from the disc. Then the disc was placed on an MHA plate that had been inoculated with E coli ATCC 25922. The plate was incubated at 37 in ambient air. Following overnight incubation, the zone of inhibition around the meropenem disc was measured. 6-15 mm zone of inhibition or presence of colonies within a 16-18 mm zone were considered carbapenemase positive. Zone diameter ≥19 mm was considered carbapenemase negative and zone 16-18 mm was considered indeterminate. Quality control of the mCIM test was done by testing simultaneously with the quality control strain of mCIM positive K.pneumoniae ATCC BAA -1705 strain.

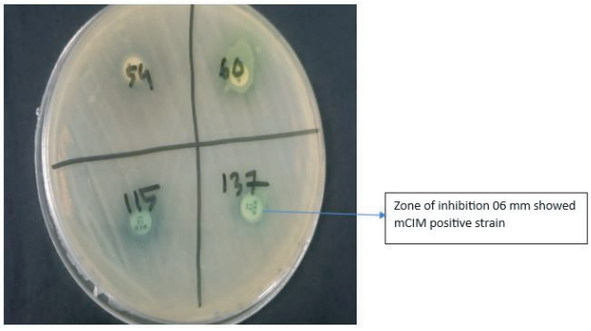

Figure 2 Modified Carbapenemase Inactivation Method (mCIM) showed positive zone for carbapenemase producers

Molecular methods

Molecular analysis of carbapenem-resistant gene blaNDM-1 in isolated Pseudomonas spp. was done by conventional PCR. Bacterial pellets were formed and DNA extraction was done by spin column with Monarch Genomic DNA Purification Kit according to the manufacturer's instructions. Mixing of master mix and primer with template DNA for each sample, a total of 25µl of the mixture was prepared by mixing 12.5µl of master mix composed of PCR buffer, MgCl2, deoxy nucleoside triphosphate / dNTP, Taq polymerase (Thermo Fisher Scientific, USA) and 2µl of forward primer, 2µl of reverse primer, 2µl of DNA template and 6.5µl nuclease-free water were added. The primer design sequence from 3'-5'for blaNDM-1(F-GCATTAGCCGCTGCATT, R- GATCGCCAAACCGT TGG) was selected in Primer-BLAST (Accession no: LC810945:1)with GC% (forward -52.94% and reverse-58.82%) and T_m (F- 56.00, R- 56.30). Amplification (PCR) was performed in a thermocycler (Eppendorf-Germany) and a total of 36 cycles were run with initial denaturation at 95° C for 3 min followed by final denaturation at 95° C for 1 min, annealing at 56° C for 40 sec and extension at 72°C for 1 min with final extension at 72°C for 10 min. Then, 2% agarose gel was prepared and the electrophoresis ran at 100 v and 90 mA for 30 minutes. After electrophoresis, the gel was photographed by a digital camera and the amplicon size was determined by comparing it with a 100 bp DNA ladder.

The procedure of DNA sequencing

Unpurified PCR products of blaNDM-1 genes were sent to Macrogen (Korea) to get the DNA sequence by the automated Sanger sequencing method (Capillary method).The sequencing step was carried out in an automated ABI 3730xl genetic analyzer (AppliedBiosystems, Foster City, USA). The forward primers of multiplex PCR were used separately to amplify and sequence.

Analysis of nucleotide sequence

The chromatogram sequences were inspected with Bioedit (Hall, 1999), version 7.1.3, and manually edited here. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Sinauer Associates, Inc). version XI software package.[24] Genetic distances were calculated using the Kimura-2-Parameter method.[25] The dendrogram was constructed using the neighbor-joining method.[26] To identify the similarity of sequences, sequences were submitted to the online BLAST (Basic Local Alignment Search Tool) program at the National Centre for Biotechnology Information Website (Available at: http://www.ncbi.nlm.gov/BLAST/) and

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ClustalW. All the reference sequences used in Multiple sequence alignment and Phylogenetic tree were retrieved from GenBank of NCBI database (National Centre for Biotechnology Information, National Institute of Health, Bethesda, MD) (Available at: http://www.ncbi.nlm.gov/BLAST/).The reliability of the NJ trees was statistically evaluated by bootstrap analysis with 1000 replicates.[27]

Safety precautions

Standard safety precaution was followed during laboratory procedures.

Data were recorded in a pre-designed data sheet. The result of the experiments was recorded systematically and statistical analysis was done by standard statistical procedure by using SPSS (Version 23.0) and Microsoft Excel.

Results

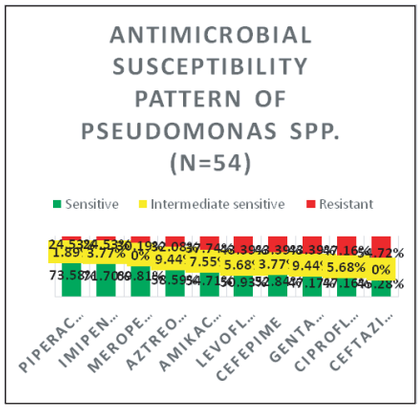

Figure 3 Antimicrobial susceptibility pattern of Pseudomonas spp (n=54)

Among 54 isolated Pseudomonas spp.carbapenem resistance was 18(33.33%) where individually meropenem and imipenem resistance were found 16(30.19%) and 13(24.53%) respectively. While highest resistance in Ceftazidime 29(54.72%) followed by Ciprofloxacin 25(47.16%) Gentamicin 23(43.39%), Cefepime 23(43.39%) Levofloxacin 23(43.39%) were observed. The lowest resistance rate other than carbapenem was observed in Piperacillin-tazobactam 13(24.53%) followed by Aztreonam 17(32.08%) and Amikacin 20(37.74%), respectively.

Table I Distribution of carbapenem-resistant Pseudomonas spp. along with blaNDM-1 (n=54)

Specimen type	Number of carbapenem resistant isolate (%)	Number of blaNDM-1 gene (%)
Urine	7(12.96%)	6(11.11%)
Wound swab	2(3.70%)	4(7.41%)
Sputum	3(5.56%)	3(5.56%)
Blood	1(1.85%)	2(3.70%)
Endotracheal aspirate	4(7.41%)	5(9.26%)
Total	18(33.33%)	20(37.04%)

Distribution of carbapenem-resistant Pseudomonas spp. along with the blaNDM-1 gene in different clinical specimens revealed the highest 7(12.96%), carbapenem-resistant Pseudomonas isolates were found in urine samples followed by ETA 4(7.41%) and sputum 3(5.56%). Wound swabs and blood samples yielded 2(3.70%) and 1(1.85%) respectively. A total of 20(37.04%) blaNDM-1 was harbored in different clinical specimen, highest in urine 6(11.11%) followed by ETA 5(9.26%), wound swab 4(7.41%), sputum 3(5.56%) and blood 2(3.70%) respectively.

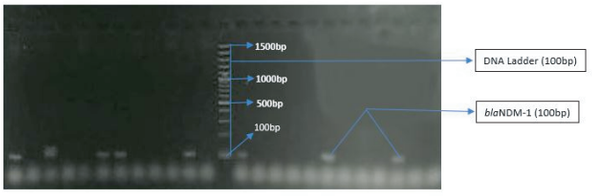

Figure 4 Agarose gel electrophoresis under UV illumination showing the 100bp amplified gene of blaNDM-1

Table II Association between phonotypic test (mCIM) and PCR (blaNDM-1) gene in Pseudomonas spp (n=54)

mCIM	PCR (blaNDM-1) gene (%)			Interference
	Positive (%)	Negative (%)	Total	of p value
Positive	12(22.22%)	02(3.70%)	14(29.17%)	χ^2 =16.49
Negative	08(14.81%)	32(59.26%)	40(74.07%)	df=1
Total	20(41.67%)	34(62.96%)	54(100%)	0.000489

N.B. Chi-square test done (Level of significance ≤ 0.05)

PCR of carbapenemase encoding genes in isolated Pseudomonas spp. showed,out of 14(29.17%) modified carbapenem inactivation method (mCIM) positive Pseudomonas spp.12(22.22%) were positive for blaNDM-1 gene and in 2(3.70%) no blaNDM-1 gene was found. And, 8(14.81%) blaNDM-1 gene were found in 40(74.07%)mCIM negative Pseudomonas spp, Chi-square test was done and p-value is <0.05 which denotes a statistically significant association.

Figure V Multiple sequence alignment (MSA) of blaNDM-1 gene aligned in ClustalW

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Multiple Sequence Alignment (MSA) was done in ClustalW to see the mutation and percentage identities among the sequences of this study (Randomly selected ID 17, 160 and 209)and blaNDM-1 genes available in the NCBI Genbank from Bangladesh in different studies. Mutation in different positions was observed in blaNDM-1(Similarities expressed by * marks) .

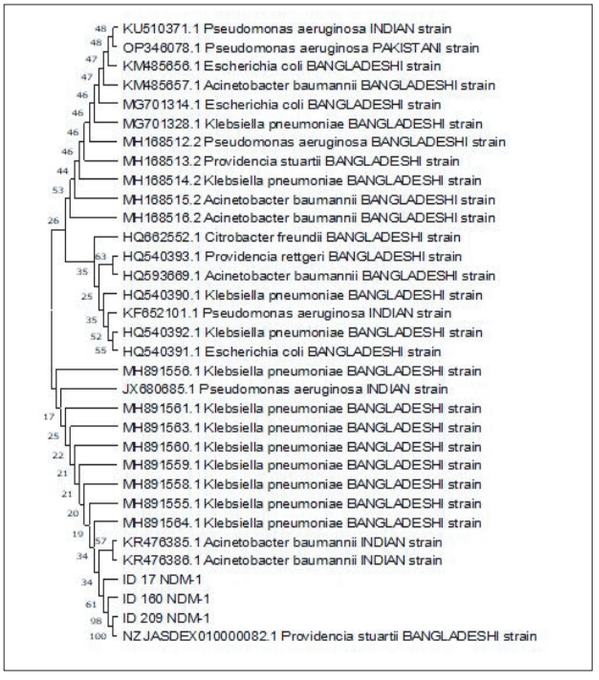

Figure 6 The neighbor-joining dendrogram (Bootstrap: 1000) of three blaNDM-1 harboring Pseudomonas spp

Phylogenetic tree was constructed in MEGA software to determine the evolutionary relatedness of the blaNDM-1 gene of this study strains with other globally circulating strains. Out of three sequenced blaNDM-1 genes in this study, all had seemed to be related to all the Bangladeshi strains available in NCBIGenBank on 24.02.2024 and 5 Indian strains KU510371.1, KF652101.1, JX680685.1, KR476385.1, KR476386.1 and one Pakistani strain OP346078.1 (Figure 6)

Discussion

The most serious Gram-negative infections, which are frequently brought on by P. aeruginosa, happen in hospital settings. Because infected patients frequently need care in the Intensive Care Unit (ICU) and are at a high risk of morbidity and mortality, these bacteria are of major clinical importance in hospitals.[28,29]

In this study, Pseudomonas spp. was 21.86%, which is supported by Ferdous et al. and Kawsar et al.also observed isolated Pseudomonas spp. was 23.46% and 23.18% in Bangladesh.[30,31] The carbapenem resistance rate was 33.33%, where individually meropenem and imipenem resistance was found at 30.19% and 24.53% respectively. which is similar to Ferdous et al. and Akhter etal. in Bangladesh and Verma et al. in India where carbapenem resistance rates were 26.83%, 37.83% and 18.24% respectively.[30-33] According to the CDC's 2019 Antibiotic Resistance Threats Report, Carbapenem-Resistant Pseudomonas Aeruginosa (CRPA) is listed as an urgent threat.[34] The detection rates of various resistance and virulence genes were high and the coexistence phenomenon was serious.[35] It has been discovered that bacteria exhibit extreme resistance and are frequently Multidrug-Resistant (MDR) and because of their potent antibacterial activity, broad antibacterial range and quick action, carbapenems are nowadays commonly the antibiotics of choice for the final treatment of P. aeruginosa infections in clinical practice. However, the length of hospital stays, treatment expenses and incidence and fatality rates are all increased when Carbapenem-Resistant Pseudomonas Aeruginosa (CRPA) isolates are present.[36]

Phenotypic carbapenamase producers were identified by the mCIM method among isolated Pseudomonas spp. in this study and about 29.17% were positive for mCIM which is similar to Akhter et al. and Pragasam et al. in Bangladesh and India are 21.63% and 40% respectively.[32,37] The comparison between mCIM andPCR for the blaNDM-1 gene revealed, among isolated Pseudomonas spp. 29.17% were detected as Carbapenemase producers by mCIM and 37.04% harbored the blaNDM-1 gene, which was statistically highly significant. This might have happened as a result of phenotypic tests' incapacity to distinguish between genes that encode chromosomes and those that encode plasmids.[38] Therefore, using molecular techniques like PCR to precisely identify the genes linked to carbapenemase synthesis overcomes the limitations of phenotypic approaches.[39]

The base sequence of the PCR product of the blaNDM-1 gene in ID-17,160 and 209 is mostly identical to three Bangladeshi strains accession no: MH168512.2, MH168515.2 and MH168512.2 avaiable in NCBI Genbank and they have 100%, 91% and 89% similarities respectively.blaNDM-1 genes of the present study show 63% to 94% similarities with themselves in Multiple sequence alignment done in ClustalW.

A phylogenetic tree was constructed to determine the evolutionary relatedness of the blaNDM-1 gene of this study strains with other globally circulating strains. Out

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of three sequenced blaNDM-1 genes in this study, all seemed to be related to all the Bangladeshi strains available in NCBI GenBank on 24.02.2024 and 5 Indian strains KU510371.1, KF652101.1, JX680685.1, KR476385.1, KR476386.1 and one Pakistani strain OP346078.1. All three study blaNDM-1 were positioned on different nodes closely related to each other, though there were genetic distances. ID 209 strain indicated that the most related blaNDM-1 sequences were from Bangladesh (NZJASDEX-010000082.1). However, ID 17, 160 and 209 strains were mostly related to blaNDM-1sequences Indian strains KR476385.1, KR476386.1 (Figure 6).

Limitations

Due to resource constrain, DNA sequencing of all detected blaNDM-1 genes could not be done.

Conclusion

Significant associations were observed between phenotypic tests and molecular detection. So, to prevent therapeutic failure of wrongly chosen antibiotics as per in-vitro phenotypic tests, molecular methods may play a great role. Regular surveillance of carbapenem susceptibility patterns should be undertaken to recognize the accurate prevalence of carbapenem-resistant organisms and treat them accordingly.

Recommendation

All carbapenem-resistant Pseudomonas spp. should be confirmed by both phenotypic test and PCR where facilities are available.

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Disclosure

The authors declare no conflict of interest.

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