PHENOTYPIC-GENOTYPIC FEATURES OF MDR *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANNII* FROM DHAKA, BANGLADESH

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ABSTRACT

Pseudomonas aeruginosa and *Acinetobacter baumannii* are one of the most common causes of MBL mediated morbidity and mortality throughout the world. Day-by-day these isolates are showing increasing resistance trends to different antimicrobial agents. But there are few data in Bangladesh, the study was designed to observe the pattern of antimicrobial resistance, prevalence of MBL, AmpC and finally phylogenetic distributions. A total of 200 isolates were analyzed in this study, comprising of 100 MDR-carbapenem resistant *P. aeruginosa* and 100 MDR-carbapenem resistant *A. baumonnii*. Isolates were tested for antimicrobial susceptibility, AmpC test, phenotypic and genotypic detection of MBL production. Antimicrobial susceptibility tests demonstrated that out of the 100 *P. aeruginosa* isolates, 99% were resistant to ceftriaxone and cefixime followed by ciprofloxacin/amikacin/netilmicin (94%), imipenem (93%) and meropenem (91%). On the other hand, 100% of the studied *A. baumannii* isolates were resistant to ceftriaxone, co-trimoxazole, cefixime and meropenem followed by imipenem (99%), ciprofloxacin (97%), amikacin (95%) and ceftazidime (94%). *P. aeruginosa* and *A. baumannii* showed 48% and 32% carbapenemase, 34% and 25% AmpC and 92% and 35% MBL positive, respectively. Phylogenetic analysis of bla_{VIM} gene of 16 *P. aeruginosa* showed similarities with the sequences from the global origin. Phylogenetic analysis of the bla_{NDM-1} gene sequences from 5 *A. baumannii* isolates (5%) revealed that 4 sequences from strains formed distinct lineages with that of the stains from India, while only one was found to be closely related to the sequences from global sources. This study found a high percentage of MBL production in MDR-carbapenem resistant *P. aeruginosa* (92%) and *A. baumannii* (35%) isolates. These findings indicate significant concern given the predominant socio-economic factors that promote the rapid spread of antimicrobial resistance and infectious diseases in developing countries such as Bangladesh.

KEYWORDS: MDR, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*. VIM, NDM-1.

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Introduction

The serious implications of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in healthcare settings, along with the emergence of carbapenemases and their worldwide dissemination represent a great health problem that poses infection control challenges. This involves with high mortality rates and extended hospital stays. Infections with such organisms include; bloodstream, urinary tract, soft tissue, pulmonary, and device-related infections (Guan *et al.*, 2016). Carbapenemase-producing *A. baumannii* and *P. aeruginosa* are being increasingly detected globally limiting the effective use of carbapenem as last-line antibiotics. Therefore, detection and surveillance of carbapenemase-producing bacteria have become critical for selecting appropriate therapeutic regimens and for the implementation of infection control measures.

In recent years, metallo-β-lactamases (MBL) producing Gram-negative nonfermenting bacteria have shown the most widespread and significant carbapenem resistance prevalence (Molton *et al.*, 2013). Outbreaks of organisms producing MBL have occurred all over the world. The New Delhi metallo-β-lactamase-1 enzyme (NDM-1) was found on almost every continent within a year of its identification in isolates of Indian origin. MBL enzymes are encoded on plasmids that spread rapidly between bacteria, and it has been increasingly identified in nonfermentative Gram-negative bacilli predominantly in clinical *P. aeruginosa* and *A. baumonnii* worldwide. These bacteria can hydrolyze a variety of β-lactam antibiotics such as cephalosporins, penicillins, cephamycins, carbapenems, but are unable to hydrolyze monobactam such as aztreonam. Their catalytic activities are not neutralized by various β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam, (Shirani *et al.*, 2016). Zinc-ion is essential for MBLs to hydrolyse the β-lactam antibiotics and due to the dependence on zinc-ions, metal-chelating agents like EDTA inhibits the MBL mediated catalysis (Page and Badarau 2008). MBLs are encoded either chromosomally or extra-chromosomally via horizontal gene transfer (acquired MBLs) (Sheikh *et al.*, 2014). Among the seven identified MBLs, the widespread MBLs include imipenemase (bla_{IMP}), Verona integron-encoded

metallo-β-lactamase (bla_{VIM}), and New Delhi metallo-β-lactamase (bla_{NDM}) (Bora *et al.*, 2014). EDTA double-disk synergy is a simple, reliable and cost-effective test for the determination of MBLs. However, the laboratory detection of these strains is not well defined.

Antimicrobial resistance is one of the most pressing health care problems globally, particularly for low and middle-income countries like Bangladesh, which is confounded with several issues like inadequate infection control interventions, inadequate antimicrobial stewardship programs and misuse/overuse of antibiotics. Additionally, there are inadequate surveillance data on antimicrobial resistance, particularly on carbapenem resistance. Having said that, the focus of this study is to identify and perform molecular characterization of the MBL-producing clinical isolates of *P. aeruginosa* and *A. baumannii* from a tertiary-level diagnostic centre in Dhaka, Bangladesh. Understanding the epidemiology and resistance mechanisms of these two clinically relevant pathogens is pertinent, as their treatment often requires therapy with molecules other than β-lactam antibiotics.

Materials and Methods

Bacterial strains and antimicrobial susceptibility

A total of 100 *P. aeruginosa* and 100 *A. baumannii* MDR-isolates that are resistant to imipenem and/or meropenem collected and stored in the Clinical Microbiology and Immunology Laboratory of icddr,b between January 2012 to December 2015 were included in this study. These isolates were part of a routine diagnostic screening that was identified by standard microbiological procedure, biochemical tests and the species-level confirmation was done by API 20 NE (Biomeriux).

Modified Kirby-Bauer disk diffusion method was employed to examine the resistance pattern of strains using following antibiotics; amikacin, ceftazidime, cefixime, ciprofloxacin, co-trimoxazole, ceftriaxone, imipenem, meropenem, netilmicin, nitrofurantoin, polymyxin B and colistin (Oxoid Ltd. Basingstoke Hampshire, England). The inhibition zone was determined and the obtained data were interpreted based on CLSI (CLSI 2012) guidelines. The MIC (minimum inhibitory concentration) was determined by the agar dilution method. For the quality control, *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used. Those *P. aeruginosa* and *A. baumannii* showed resistance to at least three or more main classes of antibiotics (carbapenem, cephalosporine, aminoglycoside, fluoroquinolone) included in this study. Subsequently, 100 *P. aeruginosa* and 100 *A. baumannii* were randomly selected and subjected to further analysis from 2,340 *P. aeruginosa* and 1,083 *A. baumannii* strains identified at icddr,b between January 2012 to December 2015.

Detection of metallo-β-lactamase (MBLs)

a. EDTA double-disk synergy (EDDS) test

MBL production was determined by the EDTA double-disk synergy (EDDS) test. The presence of an expanded growth inhibition zone between the two disks (ceftazidime and meropenem) was interpreted as positive for MBL production (Noyal *et al.*, 2009).

b. Modified Hodge test (MHT)

MHT was used to determine carbapenemase production. The presence of a 'cloverleaf shaped' zone of inhibition due to carbapenemase production by the test strain was considered as positive, briefly, an inoculum of *E. coli* ATCC 25922 was prepared and incubated for 2 hrs and adjusted to 0.5 McFarland standard and was inoculated on an MHA plate. After drying, a 10 µg meropenem disk was placed at the centre of the plate and then streaked the test strain from the edge of the disk to the periphery of the plate in four different directions. The plates were then incubated for 24 hrs at 35-37°C (Noyal *et al.*, 2009).

AmpC test

AmpC disk test was performed on meropenem resistant strains to detect AmpC β-lactamase production. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain was interpreted as positive for the production of AmpC β-lactamase. An organism with an undistorted zone was considered negative for AmpC β-lactamase (Noyal *et al.*, 2009).

Detection of carbapenemase genes by PCR

Carbapenemase-producing isolates were screened for the following genes; bla_{IMP}, bla_{VIM} and bla_{NDM-1} using gene-specific primers listed in Table-1. The PCR amplification reactions were carried out under the following conditions: initial denaturation of 5 min at 94°C and 36 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by 7 min at 72°C for bla_{IMP} gene. For bla_{VIM}, an initial denaturation of 5 min at 94°C and 36 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 7 min at 72°C. An initial denaturation at 94°C for 5 min and 36 cycles of 1 min at 94°C, 30 sec at 55°C and 45 sec at 72°C, followed by 7 min at 72°C for bla_{NDM-1} gene (Amudhan *et al.*, 2012; Islam *et al*., 2012).

Table 1. MBL primers used in this study with product size

Primers	Nature	Primer sequence (5′→3′)	Bases	Tm value (°C)	PCR product (bp)	Source of reference
NDM-1	forward	CTTCCAACGGTTTGATCGTC	20	51.8	465	Islam *et al*., 2012
	reverse	TAGTGCTCAGTGTCGGCATC	20	53.8		
IMP	forward	GTTTATGTTCATACWTCG	18	41.2	432	Amudhan *et al*.,2012
	reverse	GGTTTAAYAAAACAACCAC	19	43.5		
VIM	forward	TTTGGTCGCATATCGCAACG	20	51.8	500	Amudhan *et al*.,2012
	reverse	CCATTCAGCCAGATCGGCAT	20	53.8		

Sequencing of bla*$_{VIM}$ and *bla*$_{NDM-1}$ genes *and phylogenetic analysis

Sequencing of PCR products was performed using the dideoxynucleotide chain termination method with the ABI PRISM BigDye terminator cycle sequencing reaction kit (Perkin-Elmer Applied Biosystem, Foster City, Calif) on an automated sequencer (ABI PRISM 310). The consensus forward and reverse primers 5′TTTGGTCGCATATCGCAACG3′ for *bla*$_{VIM}$ and 5′CTTCCAACGGTTTGATCGTC3' for *bla*$_{NDM-1}$ were used to sequence the specific gene. Phylogenetic analyses were done using the MEGA version 7.0 software package (Kumar *et al*., 2016). The dendrogram was constructed using the neighbor-joining method.

Statistical analysis

Analyses of the data were done using the Statistical Package for Social Sciences (SPSS, version 20.0 for Windows, SPSS Inc. Chicago, IL, USA) Student's t-test was used to test the null hypothesis that there is no significant difference between each parameter measured in the control and treatment groups over time. If $p \leq 0.05$, then the difference was considered to be significant.

Results

High prevalence of resistance among P. aeruginosa and A. baumannii isolates

We cultured 29,136 samples and obtained 2,340 (8%) isolates that were identified as *Pseudomonas* spp. and around 1,073 (4%) isolates were identified as *Acinetobacter* spp. Overall about (1521/2340) 65% of isolated *Pseudomonas* spp. and (815/1073) 75% of *Acinetobacter* spp. were found to be MDR. For further analysis, we randomly selected one hundred *P. aeruginosa* and 100 *A. baumannii* isolates that showed resistance to at least three or more main classes of these antibiotics, including, carbapenems, cephalosporins, aminoglycosides, and fluoroquinolones. Particularly, of the 100 MDR *P. aeruginosa* isolates analyzed, 99% were found resistant to ceftriaxone and cefixime followed by ciprofloxacin (94%), amikacin (94%), netilmicin (94%), imipenem (93%), co-trimoxazole (92%), meropenem 91% and ceftazidime 86% (Figure-1). Only polymyxin B (4%) and colistin showed (2%) low resistance rates. On the other hand, nitrofurantoin showed 100% resistance (Figure-1). Of the hundred MDR strains of *A. baumannii*, 100% of the isolates were resistant to ceftriaxone, co-trimoxazole, cefixime and meropenem followed by imipenem (99%), ciprofloxacin (97%), amikacin (95%), ceftazidime (94%) and netilmicin (65%), whereas resistance to Polymyxin B and colistin was relatively low with a prevalence of 2% and 3%, respectively (Figure-1). Isolates of both *P. aeruginosa* and *A. baumannii* demonstrated high antimicrobial resistance rates and displayed similar antimicrobial resistance patterns for the antibiotics tested. The selected isolates of *P. aeruginosa* and *A. baumannii* showed high MIC values. Selected 16 *P. aeruginosa* showed 256 µg/L against imipenem as well as meropenem and 6 *A. baumannii* showed 256 µg/L against imipenem and 128 µg/L against meropenem.

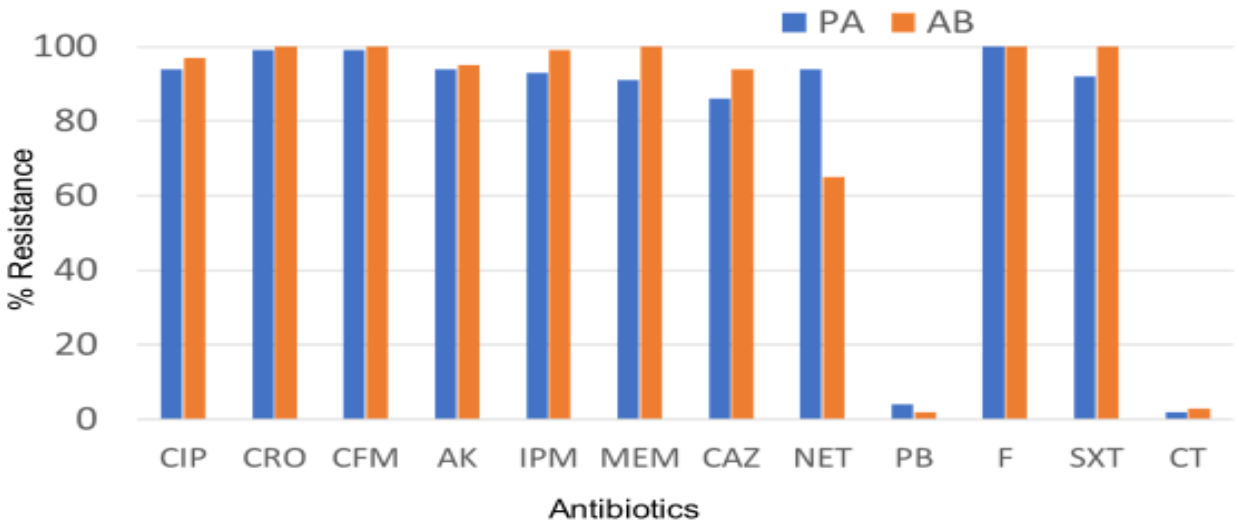


Figure 1. Antibiotic resistance pattern of MDR *P. aeruginosa* (PA) and *A. baumannii* (AB): CIP-Ciprofloxacin, CRO-Ceftriaxone, CFM-Cefixime, AK-Amikacin, IPM-Imipenem, MEM-Meropenem, CAZ-Ceftazidime, NET-Netilmicin, PB-Polymyxin B, F-Nitrofurantoin, SXT-Cotrimoxazole, CT-Colistin.

Screening of MBLs and AmpC β-lactamase production

MHT revealed that out of the 100 *P. aeruginosa* isolates tested, 48 demonstrated carbapenemase production, particularly; those *P. aeruginosa* isolates that originated from the tracheal aspirate showed high (24%) positivity for carbapenemase production (Table-2). Similarly, of the 100 *A. baumannii* isolates tested by MHT 32 isolates were positive for carbapenemase production and likewise, the majority of *A. baumannii* isolates cultured from tracheal aspirate were positive for MBL production (22%) (Table-2).

Table 2. Specimen and sex wise distribution of carbapenamase (Carba) and AmpC β-lactamase in tested MDR *P. aeruginosa* and *A. baumannii*

Specimen	Female		Male		Total	
	P. aeruginosa (Carba/AmpC) %	*A. baumannii* (Carba/AmpC) %	*P. aeruginosa* (Carba/AmpC) %	*A. baumannii* (Carba/AmpC) %	*P. aeruginosa* (Carba/AmpC) %	*A. baumanni* (Carba/AmpC) %
Tracheal aspirate	8/5	8/4	16/16	14/8	24/21	22/12
Urine	2/-	1/2	7/2	1/2	9/2	2/4
Sputum	-	1/1	2/3	-/2	2/3	1/3
Blood	1/-	-/2	1/1	-	2/1	-/2
CSF	-	1/-	-	-	-	1/-
Other body fluid	3/-	1/1	8/7	5/3	11/7	6/4
Total	14/5	12/10	34/29	20/15	48/34	32/25

In contrast, EDTA Double Disk Synergy (EDDS) testing revealed that out of the 100 MDR *P. aeruginosa*, 92 isolates were metallo-β-lactamase positive. The highest prevalence (38%) however, was found in isolates cultured from tracheal aspirate followed by other body fluids (30%) and urine samples (17%) (Table-3). In case of *A. baumannii*, MBL production was detected in 35% isolates and the highest rate (21%) was found among the isolates of tracheal aspirate samples (Table-3). While resistance due to inducible AmpC β-lactamase or chromosome mediated β-lactamase was detected in 34% *P. aeruginosa* and 25% A. *baumannii* isolates, respectively.

Table 3. Summary of EDDS test for 100 MDR *P. aeruginosa* and 100 *A. baumannii*

Specimen	Type of EDTA Double Disc Synergy (EDDS) test						Total	
	CAZ (%)		CAZ+MEM (%)		MEM (%)		(%)	
	PA	AB	PA	AB	PA	AB	PA	AB
Tracheal aspirate	2	5	3	5	33	11	38	21
Urine	1	-	7	-	9	1	17	1
Sputum	-	1	2	1	2	-	4	2
Blood	1	-	1	-	1	3	3	3
CSF	-	-	-	-	-	1	-	1
Other body fluid	4	1	9	1	17	5	30	7
Total	8	7	22	7	62	21	92	35

CAZ=Ceftazidime; MEM=Meropenem; PA= *P. aeruginosa*; AB= *A. baumanni*

Molecular detection of bla*$_{VIM}$ and *bla*$_{NDM-1}$ *genes

We identified 16 *bla*$_{VIM}$ positive isolates among 100 *P. aeruginosa* but none of the *A. baumannii* isolates was positive for *bla*$_{VIM}$ gene (Figure-2A). These 16 *bla*$_{VIM}$ gene sequences together with 22 other sequences of *bla*$_{VIM}$, were obtained from the NCBI GenBank database. The obtained data were used for constructing the phylogenetic tree to detect the nearest neighbor of our study sequences. The homogeneity and genetic divergence of the sequences are observed in the phylogenetic tree (Figure-3A). The age, sex and sample distribution of these 16 isolates were described in Table-5. The *bla*$_{VIM}$ gene sequences of *P. aeruginosa* isolates in the phylogenetic tree clustered closely with *bla*$_{VIM}$ gene sequences obtained from India, Thailand, Nepal, Egypt, Turkey, UK, USA, and Tunisia. These findings suggest that the *bla*$_{VIM}$ gene detected in the studied isolates was similar to the *bla*$_{VIM}$ gene found in isolates from global sources. On the other hand, we detected six *bla*$_{NDM-1}$ positive isolates from one hundred *A. baumannii* but none from the same number of *P. aeruginosa* isolates (Figure-2B). Out of these six *bla*$_{NDM-1}$ gene products, five genes were sequenced. The age, sex and sample distribution of these 5 isolates were described in Table 6. These five DNA sequences together with seventeen other sequences of *bla*$_{NDM-1}$ sort-out from the NCBI GenBank database were used for phylogenetic tree construction. From this phylogenetic tree, the homogeneity and genetic divergence of the sequences are evident (Figure-3B). DNA sequences from our study isolates AB1, AB2, AB4 and AB6 were found to form a single clade with *bla*$_{NDM-1}$ sequences from global sources including, India, Iran, Egypt and Korea. While one strain, A5 formed a separate clade. Similar to the *bla*$_{VIM}$-based phylogeny these findings also suggest that the *bla*$_{NDM-1}$ gene detected in the studied isolates were similar to the *bla*$_{NDM-1}$ genes found in isolates from global sources. The nucleotide similarity of *bla*$_{VIM}$ gene was compared with the reference strain LC055142 retrieved from the NCBI and it was found that there were nucleotide differences in several positions for four Bangladeshi strains of *P. aeruginosa* (Figure-4A). Detailed nucleotide differences were described in Table-4. Strains p3 (MN256620), p7 (MN256624), p10 (MN256627) and p16 (MN256633) had amino acid substitution at position:

72 changed from alanine (A) to lysine (K). Alanine is a nonpolar amino acid, whereas lysine is a polar (basic) one. In position 145, tyrosine (Y) has been changed to leucine (L), here tyrosine is polar (acidic) and leucine is non-polar (Figure-5). On the other hand, in position 146, glutamic acid (E) (polar acidic) amino acid has been changed to alanine (A), which is nonpolar. In conclusion, we can say that in strains; P3 (MN256620), p7 (MN256624), p10 (MN256627) and p16 (MN256633) there were three substations. Therefore, these substitutions might cause changes in the activity of the enzyme *in vivo*. The nucleotide similarity of the bla_{NDM-1} gene was compared with the reference strain KR872624 retrieved from the NCBI and found no changes in nucleotide sequences as well as amino acid sequences (Figure 4B). Therefore, it has been concluded that our identified bla_{NDM-1} genes are clonal.

Table 4. Nucleotide changes of strains p3 (MN256620), p7 (MN256624), p10 (MN256627) and p16 (MN256633) of *P. aeruginosa* with reference strain LC055142

Nucleotide changes	Changes in positions
A to G	17, 345, 384, 430
A to C	114,437
A to T	434
G to A	36, 214, 222, 390, 407
G to C	429
C to A	215, 414
T to C	150, 266, 357, 381, 382, 408, 417, 433
T to A	402

Table 5. Age, sex and sample distribution of bla_{VIM} containing 16 strains of *P. aeruginosa*

Strain	**Age of patient (Yr)**	**Sex**	**Sample source**
p1 (MN256618)	50	M	Tracheal aspirate
p2 (MN256619)	75	M	Tracheal aspirate
p3 (MN256620)	20	M	Urine
p4 (MN256621)	52	M	Urine
p5 (MN256622)	12	M	Tracheal aspirate
p6 (MN256623)	55	M	Urine
p7 (MN256624)	55	M	Blood
p8 (MN256625)	46	M	Tracheal aspirate
p9 (MN256626)	70	M	Urine
p10 (MN256627)	54	M	Tracheal aspirate
p11(MN256628)	65	M	Tracheal aspirate
p12 (MN256629)	78	F	Urine
p13 (MN256630)	60	M	Tracheal aspirate
p14 (MN256631)	5	M	Wound swabs
p15 (MN256632)	24	M	Endo tracheal tube
p16 (MN256633)	55	M	Sputum

MN accession no.; F, Female and M, Male

Table 6. Age, sex and sample distribution of bla_{NDM-1} containing 5 strains of *A. baumanni*

Strain	**Age of patient (Yr)**	**Sex**	**Sample source**
AB1 (MN226842)	22	M	Tracheal aspirate
AB2 (MN226843)	60	F	Tracheal aspirate
AB4 (MN226844)	75	M	Tracheal aspirate
AB5 (MN226845)	40	M	Tracheal aspirate
AB6 (MN226846)	1	F	Urine

MN accession no.; F, Female and M, Male

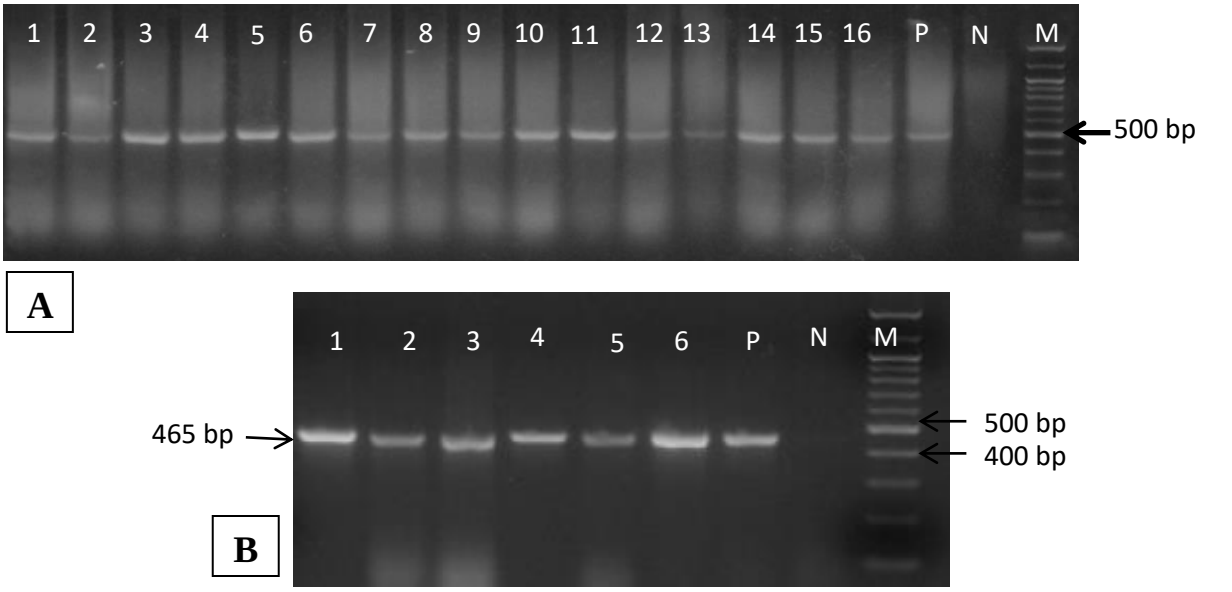
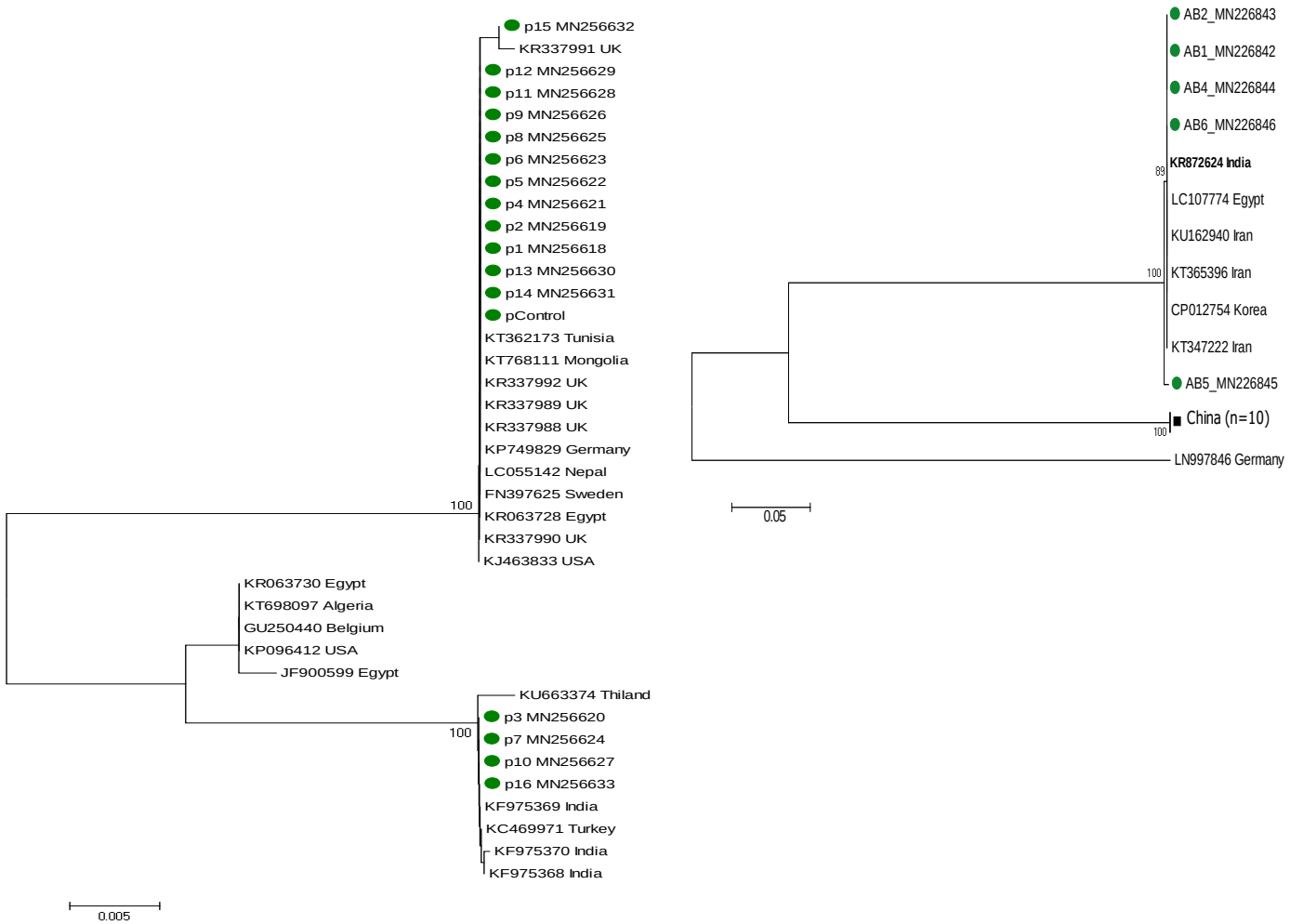


Figure 2. (**A**) PCR product of *bla*$_{VIM}$ MBL: Lane 1-16 representative test strains, M- 100bp Marker, P-Positive control, N-Negative control and (**B**) *bla*$_{NDM-1}$ MBL: Lane 1-6 representative test strains, M- 100bp Marker, P-Positive control (*K. pneumoniae*), N-Negative control



A. MDR *P. aeruginosa* **B**. MDR *A. baumannii*

Figure 3. (**A**) Neighbour-Joining Phylogenetic tree of Verona integron-encoded metallo β-lactamase (*bla*$_{VIM}$) producing MDR *P. aruginosa* of Bangladeshi strains, and (**B**) New Delhi Metallo β-lactamase-1(*bla*$_{NDM-1}$) producing MDR *A. baumannii* Bangladeshi strains

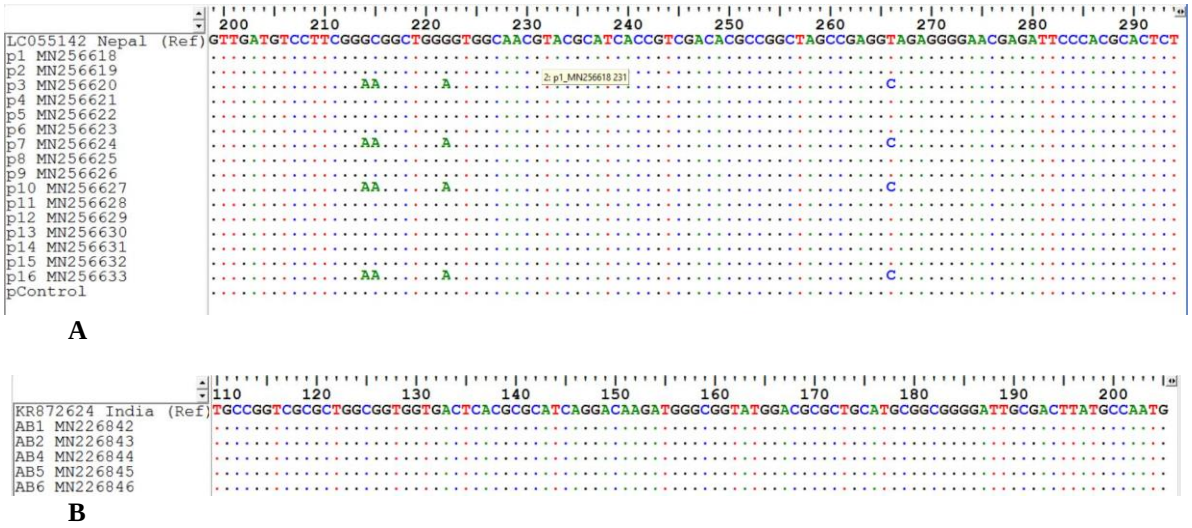


Figure 4. (**A**) Nucleotide sequence similarity percentage of *bla*$_{VIM}$ producing *P. aeruginosa* of Bangladeshi strains and (**B**) *bla*$_{NDM-1}$ producing *A. baumannii* of Bangladeshi strain

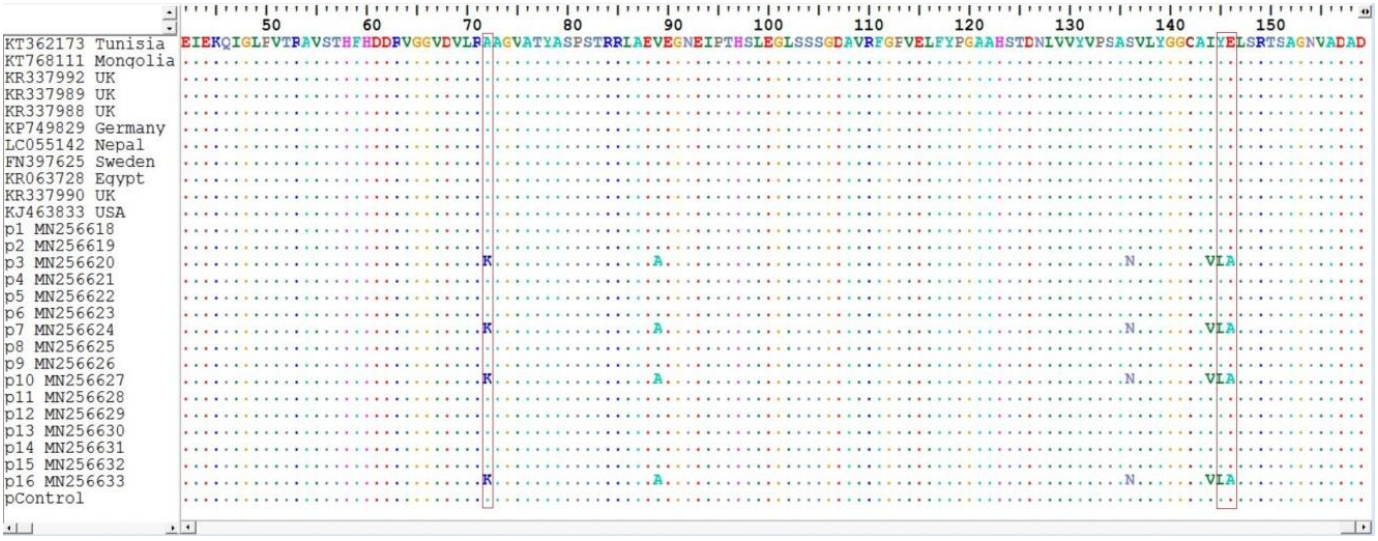


Figure 5. Amino acid sequence changes in 16 Bangladeshi strains of *P. aeruginosa*

Discussion

Gram-negative bacilli such as Pseudomonads and *Acinetobacter* spp. have been increasingly identified in recent decades as significant opportunistic hospital-acquired infections, including in Bangladesh (Anwar, *et al.*, 2010). Management of infections due to these pathogens is a challenge for physicians, as the increasing resistance to high order antibiotics often leads to clinical failure. Appropriate guidelines for the initial choice of antibiotics can help physicians make therapeutic decisions. However, these guidelines require constant updating based on the local pattern of antimicrobial susceptibilities.

In this study, using MHT, 48% of *P. aeruginosa* isolates were shown carbapenemase positive, whereas 32% *A. baumannii* isolates were carbapenemase positive. The AmpC β-lactamase test showed 34% isolates positive among *P. aeruginosa* and 25% in *A. baumannii* isolates. Yum *et al.*, (2002) also described in their study that 50% of the MDR *A. baumannii* produces carbapenemase.

The MBLs hydrolyze all β-lactam groups of antibiotics except aztreonam. We detected 92% of clinical isolates of *P. aeruginosa* and 35% of *A. baumannii* as MBL producers when tested by EDTA Double Disc Synergy (EDDS) test and this was high when compared with a study from Karachi, Pakistan (Irfan *et al*., 2008). The results of our study were comparable with the study conducted by Aktaş, *et al*., (2008), who found 78.5% of *P. aeruginosa* and 63.6% of *A. baumanni* isolates positive for MBL in the DDST with imipenem and 0.5 M EDTA disks. Another study conducted by Siarkou *et al.* (2009) detected 50% MBL production in *P. aeruginosa* isolates. We observed that all MBL producing strains in our study were susceptible to potentially toxic antibiotics such as colistin, and polymyxin-B, this was in line with other studies (Castanheira *et al*., 2008).

In this study, we explored the molecular determinants of carbapenem resistance in *P. aeruginosa* and *A. baumannii* isolates recovered from clinical specimens that were processed at the tertiary-level referral diagnostic centre of icddrb, Dhaka, Bangladesh. All MBL positive *P. aeruginosa* detected by

EDTA double disk synergy test harbored the gene bla_{VIM}, this observation was similar to a previous report from the Mediterranean basin, which concluded that the main MBL produced by *P. aeruginosa* was bla_{VIM-2} type (Sefraoui *et al*., 2014). Recently, Al Bayssari *et al*. 2014 described the emergence of bla_{VIM-2} in clinical isolates of carbapenem-resistant *P. aeruginosa*. Additionally, Hammami *et al*. (2010) showed the presence of the bla_{VIM-2} in the gene cassette of class 1 integron in clinical *P. aeruginosa* isolates. Similarly, Farzana *et. al*. (2013) reported a higher percentage of different molecular determinants of MBL; 22.86% for bla_{NDM-1}, 37.15% for bla_{VIM-1}, 60.00% for bla_{VIM-2}, 51.43% for bla_{IMP-1}, and 25.71% for bla_{IMP-2} (Farzana *et al.*, 2013). This observation may consider the widespread prevalence of bla_{VIM} in clinically relevant *P. aeruginosa* isolates.

A number of recent studies in Bangladesh have also shown an increasing prevalence of MBL production particularly, in *P. aeruginosa* and *A. baumannii* and *Enterobacteriaceae* in general (Anwar, *et al.*, 2010, Hasan and Shamsuzzaman 2017). The phylogenetic tree generated from bla_{VIM} gene sequences of *P. aeruginosa* isolates (Figure-3A) demonstrated similarities with bla_{VIM} genes from India, Thailand, Nepal, Egypt, Turkey, UK, USA, and Tunisia. These findings suggest that the bla_{VIM} observed in the studied isolates shared similarities with the bla_{VIM} gene that is prevalent globally.

Although the study has provided valuable insights into the carbapenem resistance scenario, there were certain limitations in this study. We screened isolates for MBL production and MBL genes among carbapenem resistant-MDR isolates, this could have provided biased prevalence results and much of the data cannot be compared with the results of other studies wherein they identified the prevalence of MBL among overall isolates irrespective of MDR or carbapenem status. The second limitation was the screening of MBL production using the EDDS method instead of the broth microdilution or the E-test methods. Moreover, only three major carbapenem genes including bla_{VIM}, bla_{IMP} and bla_{NDM-1} were screened and no comprehensive molecular determinants were identified. Further studies should take these, into consideration in order to enhance their detection accuracy of carbapenem-resistant *A. baumanni* and *P. aeruginosa* isolates.

The expeditious spread of MBL producing *P. aeruginosa* and *A. baumanni* constitute a serious threat to public health, worldwide. The high incidence of MBLs in our study among imipenem and/ meropenem resistant MDR *P. aeruginosa* and *A. baumannii* isolates highlight the prevalent therapeutic challenges in Bangladesh. Only colistin and polymixin B demonstrated strong activity against carbapenem resistant *A. baumannii* and *P. aeruginosa* isolates. As the current antibiotic regimen offers little in the short term, our prime target should be curbing the spread of antibiotic-resistant organisms by accelerating microbiological diagnosis, intensified infection control programs, antimicrobial surveillance networks, antimicrobial stewardship and a strong focus on the antibiotic development pipeline.

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Competing Interests

The authors have declared that no competing interest exists.

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