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Original Article

# Diversity of carbapenemases in clinical isolates: The emergence of *bla*$_{VIM-5}$ in Bangladesh☆

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ABSTRACT

Global emergence and dissemination of carbapenemases are clinically threatening, notably in countries with endemic *bla*$_{NDM}$. To analyze the extent of carbapenemases in Bangladesh, 71 isolates were collected from 7 different clinical sources: wound swab (n = 38), pus (n = 13), urine (n = 9), blood (n = 4), tracheal aspirate (n = 3), pleural fluid (n = 1) and vaginal swab (n = 3) from Dhaka Medical College Hospital, Bangladesh. Among the isolates, 25 were resistant to at least one of the three carbapenems (imipenem, meropenem and doripenem), including 15 being resistant to all. These resistant isolates were identified as *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, P. hibiscicola, Proteus mirabilis, Providencia stuartii* and *Citrobacter sedlakii.* Carbapenemase detection among these 25 isolates varied in individual phenotypic assays (83% in Modified Hodge Test, 50% in Combined Disk Test for Metallo-β-lactamase prediction) as compared with the genotypes observed (96% prevalence of various carbapenemases including *bla*$_{OXA-1,48}$, *bla*$_{NDM-1,5}$, *bla*$_{VIM-2,5}$). *bla*$_{OXA-48}$ was the most prevalent gene (84%) followed by *bla*$_{NDM}$ (72%). Coexistence of multiple gene combination such as *bla*$_{NDM}$+*bla*$_{OXA-48}$+*bla*$_{OXA-1}$ was prevalent (48%). Harborage of *bla*$_{VIM-5}$ (n = 1) was characterized for the first time, while *bla*$_{NDM-5}$ (n = 5) was reported contemporarily with a recent study in Bangladesh. Presence of plasmids (64%) and integron class 1 (100%) signifies the transferable potential of resistant traits. The emergence of such new variants along with the presence of the mobile genetic elements demands strict surveillance and combating strategies.



## 1. Introduction

The carbapenem resistance in Gram negative organisms, specially *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobater baumannii* (EPA) has become the major concern in recent years due to the lack of new alternative treatment options [1]. Among multifactorial resistance mechanisms, carbapenemases are clinically most significant [2]. Carbapenemases can hydrolyze almost all β-lactams and are capable of being transferred horizontally [2], which is remarkable for Asia, the epicenter of horizontal transfer of resistance genes between Gram negative organisms [3].

On the basis of geographical spread and hydrolytic capability, the most effective carbapenemases are IMP, NDM, VIM of Ambler molecular class B or Metallo-β-lactamases (MBLs), KPC of class A and OXA of class D [4]. Among various MBLs, *bla*$_{NDM-1}$ has received wide attention because of broad hydrolytic activity [5] and worldwide dissemination [6]. NDM-5 has greater hydrolytic activity than NDM-1 [7], and has been reported in recent time from Asian countries such as China [8], India [9], Nepal [10] etc. Another MBL being associated with clinically important pathogens, *bla*$_{VIM-2}$ is globally distributed [11], while *bla*$_{VIM-5}$ has been reported from Turkey [12], India [13] and Greece [14]. However, reports of *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ in association with *bla*$_{NDM}$ and

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*bla*$_{VIM}$ in India or Indian origin indicate the increasing resistance of carbapenems in this region [15,16].

Unfortunately, lack of implementation of national antimicrobial surveillance system with little quality data makes the evaluation of carbapenem resistance in Bangladesh difficult, although recent studies estimated high rate of resistance in *Enterobacteriaceae* and even >50% prevalence in *A. baumannii* [17–19]. In Bangladesh, *bla*$_{NDM}$ is endemic and *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48/OXA-181}$ have also been reported in 2016 [20,21]. So, this study is aimed to investigate the prevalence and dissemination potential of carbapenemases among the carbapenem (imipenem, meropenem, doripenem) resistant isolates of diverse clinical sources.

## 2. Materials and methods

### 2.1. Sample collection and isolation of bacteria

A total of 71 isolates were collected from Dhaka Medical College Hospital (DMCH), Dhaka in March 2016 of seven different clinical sources: wound swab (n = 38), pus (n = 13), urine (n = 9), blood (n = 4), tracheal aspirate (n = 3), pleural fluid (n = 1) and vaginal swab (n = 3). Isolates from respective samples were plated onto the Mueller Hinton agar (MHA) (Oxoid, England), and incubated at 37 °C for 18–24 h followed by retrieval of pure colonies.

### 2.2. Screening and identification of carbapenem resistant isolates

All the isolates were screened for carbapenem resistance using imipenem (IPM) (10 μg), meropenem (MEM) (10 μg) and doripenem (DOR) (10 μg) disks (Oxoid Limited, UK) according to the standardized disk diffusion method known as the Kirby Bauer method [22]. The inhibition zone sizes were interpreted by using the zone diameter interpretive standards from Clinical and Laboratory Standards Institute (CLSI) [23].

Only the isolates resistant to at least any one of the three carbapenems tested were subjected to presumptive identification by the conventional approaches (colony morphology and biochemical assays) according to 'Bergey's Manual of Determinative Bacteriology' [24]. Universal primers 27F and 1429R were used for the amplification of 16S rRNA gene [25], and ARDRA (Amplified ribosomal DNA restriction analysis) genotyping of the amplified products was performed. The amplicons of each ARDRA group were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sequenced using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems®, USA). Raw data were generated in ABI Genetic Analyzer (Applied Biosystems®, USA) before the assembly of data using SeqMan version 7.0 (DNASTAR, Inc., Madison, WI, USA). Afterwards, NCBI BLAST search was implemented using assembled sequences of the genes followed by submission to NCBI GenBank.

### 2.3. Antimicrobial susceptibility tests

Resistant isolates were tested with a panel of antibiotics as recommended in CLSI document M100-S23 [23] by the Kirby Bauer method [22]. Seventeen antibiotics of 10 antibiotic groups were tested which were: Chloramphenicol (CHL) (30 μg), Nitrofurantoin (NIT) (300 μg), Ampicillin (AMP) (10 μg), Doxycycline (DOX) (30 μg), Tetracycline (TET) (30 μg), Gentamycin (GEN) (10 μg), Trimethoprim (TMP) (5 μg), Nalidixic acid (NAL) (1st generation) (30 μg), Ciprofloxacin (CIP) (2nd generation) (5 μg), Levofloxacin (LVX) (3rd generation) (5 μg), Cefalexin (LEX) (1st generation) (30 μg), Cefuroxime (CXM) (2nd generation) (30 μg), Cefotaxime (CTX) (3rd generation) (30 μg), Cefepime (FEP) (4th generation) (30 μg), Aztreonam (ATM) (30 μg), Colistin (CT) (10 μg) and Polymyxin B (PB) (10 μg).

### 2.4. Minimum inhibitory concentration (MIC) of carbapenems

Minimum inhibitory concentration (MIC) of imipenem and meropenem was determined by microdilution method with two-fold dilutions in a concentration range from 2 to 512 μg/ml according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [23]. The MICs were determined following overnight incubation of the microtiter plates at 37 °C.

### 2.5. Phenotypic detection of carbapenemase production

For detecting carbapenemase production, Modified Hodge Test (MHT) was performed using the carbapenems to which the isolates were found resistant [26]. Besides, the differentiation of metallo-β-lactamases (MBLs) and class A *Klebsiella pneumoniae* carbapenemases (KPCs) was performed by Combined disk test [27,28] using Phenylboronic acid (PBA) and EDTA (Ethylenediaminetetraacetic acid) as the inhibitor of KPC and MBL, respectively, in combination with MEM (10 μg) disks (Oxoid Limited, England). However, the meropenem susceptible isolates were investigated using the other carbapenem disks to which they were resistant. Sensitivity and specificity of these assays were determined using SPSS program version 18.0 [29].

### 2.6. Molecular characterization of carbapenem resistance genes

Resistant isolates were assayed for the harborage of carbapenemase using uniplex gene specific PCR. Under class A, *bla*$_{KPC}$ (*bla*$_{KPC1}$, *bla*$_{KPC2}$, *bla*$_{KPC3}$); class B, *bla*$_{VIM}$ (*bla*$_{VIM1}$, *bla*$_{VIM2}$); *bla*$_{DIM1}$, *bla*$_{SIM1}$, *bla*$_{NDM}$, *bla*$_{IMP}$ (*bla*$_{IMP1}$, *bla*$_{IMP2}$, *bla*$_{IMP3}$), and class D, *bla*$_{OXA}$ (*bla*$_{OXA1}$, *bla*$_{OXA48}$) were targeted, and each of the gene specific PCR was performed at optimum conditions following pre-described methods with defined annealing temperature of each primer (Table 1). We performed gel electrophoresis and purification of the PCR products before raw sequence data were processed, aligned, annotated and published into NCBI GenBank as previously described in the 'Screening and identification of carbapenem resistant isolates' subsection of the 'materials and methods' section.

### 2.7. Phylogenetic analyses

Maximum likelihood (ML) [30,31] method was implied using MEGA7 [32] for inferring the evolutionary history of both the 16S rRNA gene of 19 bacterial isolates and the carbapenemase genes identified based on the Kimura-2 parameter model [31].

### 2.8. Plasmid isolation and presence of integrons

Plasmid DNA was extracted using Wizard® Plus SV Minipreps plasmid DNA Purification kit (Promega, USA) according to the manual instruction and analyzed by electrophoresis in 0.8% agarose gel. Extracted plasmid DNA from *Esherichia coli* V517 was used as extraction and molecular weight determination control.

Integron class 1 was investigated in the resistant isolates by uniplex PCR using primers Int-1F (5'-GGGTCAAGGATCTGGATTTCG-3') and Int-1R (5'ACATGGGTGTAAATCATCGTC-3') at optimum conditions as previously described [33].

### 2.9. Accession numbers

The nucleotide sequences of 16S rRNA genes, carbapenemases and class 1 integron-integrase genes of the selected isolates were assigned the accession numbers in the NCBI GenBank: 16S rRNA (MH150788-MH150802, MH150805-MH150808), *bla*$_{NDM-5}$ (MH168506-MH168510), *bla*$_{NDM-1}$ (MH168512-MH168516),

**Table 1**
Primers used for carbapenemase detection and corresponding annealing time and temperature used for PCR.

| Target gene | Primers | Sequence (5′→ 3′) | Size (bp) | Annealing temp (Annealing time) | Ref |
|---|---|---|---|---|---|
| [a]$bla_{KPC-1}$ | KPC5F | TGTCACTGTATCGCCGTC | 900 | 58 | [57] |
| | KPC10R | CTCAGTGCTCTACAGAAAACC | | (30sec) | |
| [a]$bla_{KPC-2}$ | KPCIF | CGGAACCTGCGGAGTGTATG | 802 | 56 | [58] |
| | KPCJR | CAGCAGTTCAGGCCAACACC | | (45sec) | |
| [a]$bla_{KPC-3}$ | KPC3F | ATGTCACTGTATCGCCGTCT | 911 | 55 | [59] |
| | KPC3R | TTTTCAGAGCCTTACTGCCC | | (45sec) | |
| [b]$bla_{VIM-1}$ | VIM1F | TTATGGAGCAGCAACCGATGT | 920 | 60 | [60] |
| | VIM1R | CAAAAGTCCCGCTCCAACGA | | (45sec) | |
| [b]$bla_{VIM-2}$ | VIM2F | ATTGGTCTATTTGACCGCGTC | 748 | 56 | [61] |
| | VIM2R | TGCTACTCAACGACTGAGCG | | (30sec) | |
| [b]$bla_{DIM-1}$ | DIM-1A | TCTATTCAGCTTGTCTTCGC | 688 | 53 | [62] |
| | DIM-1B | TGTTAGAGGCTGTCTCAGCC | | (45sec) | |
| [b]$bla_{SIM-1}$ | SIM1-F | TACAAGGGATTCGGCATCG | 551 | 56 | [63] |
| | SIM1-R | TAATGGCCTGTTCCCATGTG | | (30sec) | |
| [b]$bla_{NDM}$ | pre-A | CACCTCATGTTTGAATTCGCC | 984 | 52 | [64] |
| | pre-B | CTCTGTCACATCGAAATCGC | | (45sec) | |
| [b]$bla_{NDM}$ | ISAba125 ext | GCTTACACCATTAGAGAAATTTGC | 1055 | 54 | [65,66] |
| | $ble_{MBL}$-iR | CGCATAAAACGCCTCTGTCA | | (45sec) | |
| [b]$bla_{IMP-1}$ | IMP-1F | CTACCGCAGCGAGTCTTTG | 587 | 56 | [67] |
| | IMP-1R | AACCAGTTTTGCCTTACCAT | | (45sec) | |
| [b]$bla_{IMP-3}$ | IMP3F | CGGATGAAGGCACGAAC | 741 | 51 | [68] |
| | IMP3R | AAGCAGACTTGACCTGA | | (45sec) | |
| [b]$bla_{IMP-4}$ | IMP4F | ATGAGCAAGTTATCTGTATTCT | 474 | 58 | [69] |
| | IMP4R | AGTGTGTCCCGGGCCACC | | (30sec) | |
| [d]$bla_{OXA-1}$ | OXA1F | AGCCGTTAATTAAGCCC | 882 | 56 | [70] |
| | OXA1R | CTTGATTGAAGGGTTGGGCG | | (30s) | |
| [d]$bla_{OXA-48}$ | OXA-F | GCGTGGTTAAGGATGAACAC | 438 | 54 | [71] |
| | OXA-R | CATCAAGTTCAACCCAACCG | | (30 s) | |

([a] Class A carbapenemase, [b] Class B carbapenemase, [d] Class D carbapenemase).
Ref = references.

$bla_{OXA-1}$ (MH168519), $bla_{OXA-48}$ (MH168522-MH168523), $bla_{VIM-5}$ (MH168524) and class 1 integron-integrase (MH168525).

## 3. Results

### 3.1. Prevalence of carbapenem resistance in clinical isolates

Twenty five out of 71 clinical isolates (35.21%) were resistant to at least one of the three carbapenems tested. Among these 25 isolates, 15 isolates (60%) were resistant to all three carbapenems. Varying resistance to individual carbapenems such as 84% (21 out of 25) to imipenem, 76% (19 out of 25) to meropenem and 64% (16 out of 25) to doripenem was observed. ARDRA with AluI enzyme differentiated these 25 isolates into 5 different groups (I–V). Group III and IV were further differentiated as IIIa, IIIb and IVa, IVb using a 2nd restriction enzyme, HaeIII and PstI respectively (Fig. S1), which was consistent with the findings of the conventional approaches. The isolates belonging to ARDRA groups I, II, IIIa, IIIb, IVa, IVb and V were identified as *P. aeruginosa* (n = 10), *P. hibiscicola* (n = 2), *A. baumannii* (n = 4), *Providencia stuartii* (n = 1), *Klebsiella pneumoniae* (n = 6), *Citrobacter sedlakii* (n = 1) and *Proteus mirabilis* (n = 1) respectively (Fig. 1).

### 3.2. $MIC_{90}$ of imipenem and meropenem

Broth microdilution of MIC in the range of 2–512 μg/ml revealed that 5 isolates belonging to *P. aeruginosa* (n = 2) and *K. pneumoniae* (n = 3) showed MIC of >512 μg/mL in case of both imipenem and meropenem (Table 2). Besides, 14 out of 21 imipenem resistant isolates (66.67%) showed high MIC of imipenem at the level of ≥256 μg/mL including 6 isolates having MIC of >512 μg/mL. On the other hand, 16 out of 19 meropenem resistant isolates (84.21%) showed high MIC of meropenem at the level of ≥256 μg/mL including 11 isolates having MIC of >512 μg/mL (Table 2).

### 3.3. Antibiotic susceptibility of carbapenem resistant isolates

According to the antibiogram pattern, all the isolates were Multi-drug resistant (MDR) (Table S1). 100% resistance to Ampicillin, 1st generation (Cephalexin) and 2nd generation (Cefuroxime) Cephalosporin was observed. The *Enterobacteriaceae* isolates were also resistant to 3rd generations of Cephalosporins (Cefotaxime). However, *Pseudomonas* spp. and *A. baumannii* isolates were sensitive to Colistin and Polymyxin B except *A. baumannii* strain DMC-15a being Colistin resistant according to disk diffusion assay.

### 3.4. Genotypes of carbapenemases

Gene specific PCR followed by sequencing and phylogenetic analysis revealed the presence of 6 different carbapenemase genes: $bla_{VIM-2}$, $bla_{VIM-5}$, $bla_{NDM-1}$, $bla_{NDM-5}$, $bla_{OXA-1}$ and $bla_{OXA-48}$ in 24 out of 25 resistant isolates (96%) (Table 2). 21 out of these 24 isolates (87.5%) harboured multiple carbapenemase genes rather than a single carbapenemase, and the most prevalent combination detected was $bla_{NDM}$ + $bla_{OXA-1}$+$bla_{OXA-48}$, while $bla_{NDM}$ included either $bla_{NDM-1}$ or $bla_{NDM-5}$ (Table 2). The most prevalent carbapenemase detected was $bla_{OXA-48}$ (21 out of 24 isolates, 87.5%) followed by $bla_{NDM}$ (18 out of 24 isolates, 75%), and the least prevalent one was $bla_{VIM-5}$ with only one incidence. Sequencing of 10 out of 18 $bla_{NDM}$ genes confirmed that 5 isolates contained $bla_{NDM-5}$ while the rest 5 isolates harbored $bla_{NDM-1}$ (Fig. 2).

### 3.5. Correlation between carbapenemase phenotypes and genotypes

Among 25 carbapenem resistant isolates, 19 isolates were phenotypically positive for carbapenemases in MHT using meropenem or other carbapenems. In contrast to MHT results, molecular analysis yielded 83% sensitivity (SE) and 100% specificity (SP).

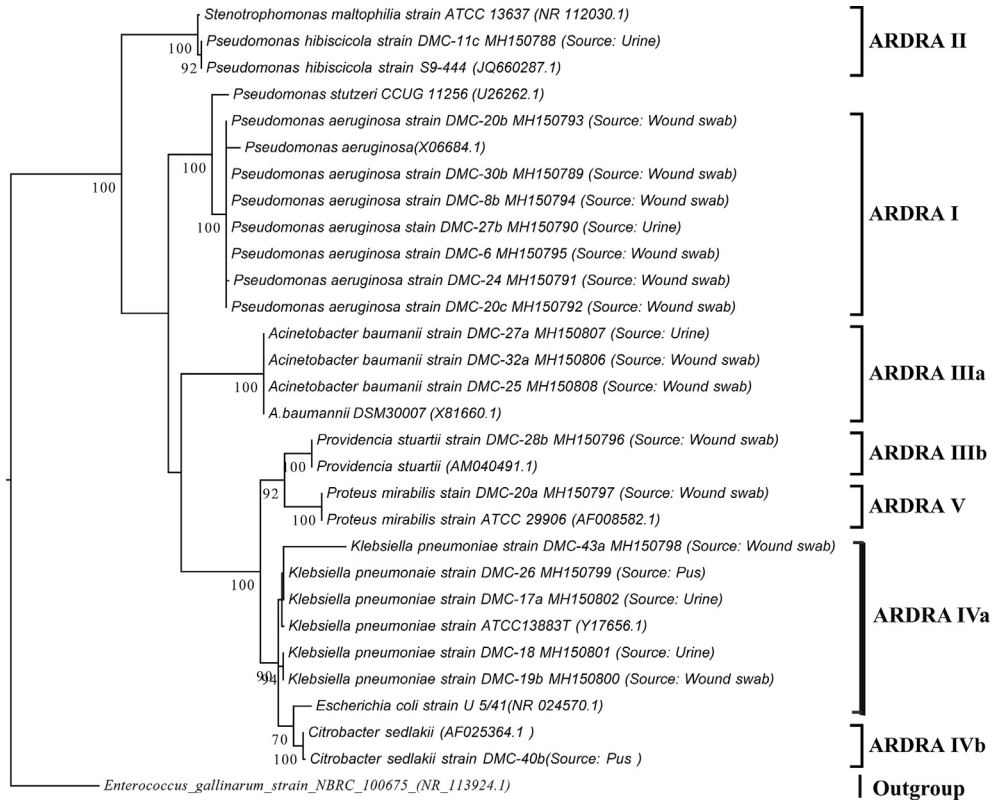


**Fig. 1.** Phylogenetic representation of 16S rRNA genes: Molecular phylogenetic tree of 16S rRNA gene sequences from the isolated bacteria from each ARDRA group generated by ML method based on Kimura-2 parameter model. The ARDRA groups each having the reference sequences of isolates and the outgroup containing *Enterococcus gallinarum* strain NBRC 100675 (NR 113924.1) have been annotated in the tree. The sources of the bacterial isolates are shown along with accession numbers in the tree.

**Table 2**
Phenotypic and genotypic analyses of carbapenemase genes of the Carbapenem resistant isolates.

| Organisms | Isolate ID | MIC | | MHT | | | CDT | Genotype | IntI |
|---|---|---|---|---|---|---|---|---|---|
| | | IPM | MEM | IPM | MEM | DOR | | | |
| *P. aerugionsa* | [w]DMC-6 | 8 | NT | + | − | − | KPC | $bla_{NDM-5+}bla_{OXA48}$ | + |
| | [p]DMC-7 | 512 | NT | + | − | − | KPC | $bla_{VIM2}+bla_{OXA48}$ | + |
| | [w]DMC-8b | NT | 8 | NT | + | NT | MBL | $bla_{OXA48}$ | + |
| | [w]DMC-20b | NT | 256 | NT | − | NT | KPC | $bla_{VIM2}+bla_{OXA48}$ | + |
| | [w]DMC-20c | 16 | >512 | − | + | NT | KPC | $bla_{VIM2}+bla_{OXA48}$ | + |
| | [t]DMC-23b | 16 | NT | − | NT | NT | - [#] | $bla_{OXA48}$ | + |
| | [w]DMC-24 | NT | 32 | NT | − | NT | Non MBL\KPC | No carbapenemase gene detected in this study | + |
| | [u]DMC-27b | >512 | >512 | + | + | + | MBL | $bla_{VIM5}+bla_{OXA48}$ | + |
| | [w]DMC-30b | >512 | >512 | + | + | − | KPC | $bla_{NDM+}bla_{OXA48}$ | + |
| | [p]DMC44 | 16 | 256 | + | NT | + | MBL | $bla_{NDM-1}+bla_{OXA1}$ | + |
| *P. hibiscicola* | [t]DMC-10 | 256 | 256 | − | − | − | - [#] | $bla_{NDM}$ + $bla_{OXA1}+bla_{OXA48}$ | + |
| | [u]DMC-11c | 256 | >512 | − | − | − | MBL | $bla_{NDM}$ | + |
| *A. baumannii* | [b]DMC-15a | 128 | 64 | + | + | + | KPC | $bla_{NDM}$ + $bla_{OXA1}+bla_{OXA48}$ | + |
| | [w]DMC-25 | 256 | >512 | + | + | + | MBL + KPC | $bla_{NDM-1+}bla_{OXA48}$ | + |
| | [u]DMC-27a | 128 | >512 | + | + | + | KPC | $bla_{NDM+}bla_{OXA1}$ | + |
| | [w]DMC-32a | 512 | >512 | + | + | + | KPC | $bla_{NDM-1}+bla_{OXA1}+bla_{OXA48}$ | + |
| *P. stuartii* | [w]DMC-28b | >512 | NT | + | + | + | MBL | $bla_{NDM-1}+bla_{OXA1}+bla_{OXA48}$ | + |
| *K. pneumoniae* | [u]DMC-17a | >512 | >512 | + | + | + | MBL | $bla_{NDM-1}+bla_{OXA1}+bla_{OXA48}$ | + |
| | [u]DMC-18 | >512 | >512 | + | + | + | MBL + KPC | $bla_{NDM-5}+bla_{OXA1}+bla_{OXA48}$ | + |
| | [w]DMC-19b | >512 | >512 | + | + | − | MBL + KPC | $bla_{NDM-5}+bla_{OXA1}+bla_{OXA48}$ | + |
| | [t]DMC-23a | 256 | NT | + | NT | − | Non MBL\KPC | $bla_{NDM}$ + $bla_{OXA1}+bla_{OXA48}$ | + |
| | [p]DMC-26 | 512 | 256 | + | + | + | MBL | $bla_{NDM-5}+bla_{OXA1}+bla_{OXA48}$ | + |
| | [w]DMC-43a | 256 | 4 | + | + | + | MBL | $bla_{NDM-5}+bla_{OXA1}+bla_{OXA48}$ | + |
| *C. sedlakii* | [p]DMC-40b | 128 | 512 | + | NT | + | MBL | $bla_{NDM}$ + $bla_{OXA1}+bla_{OXA48}$ | + |
| *P. mirabilis* | [w]DMC-20a | 8 | >512 | NT | − | NT | Non MBL/KPC | $bla_{NDM}$ + $bla_{OXA1}+bla_{OXA48}$ | + |

Here, * represents moderate resistance, # represents discrepant result in CDT, + = Positive result, - = Negative result, NT= Not tested, IPM = imipenem, MEM = meropenem, DOR = doripenem, R = resistance, MIC = Minimum inhibitory concentration, MHT = Modified Hodge test, CDT=Combined Disk Test, IntI = class 1 integron-integrase. Sources of the isolates are represented as: Wound Swab ([w]), Pus ([p]), Tracheal Aspirate ([t]), Blood ([b]), Urine ([u]).
$bla_{NDM}$ indicates presence of either variant $bla_{NDM-1}$ or variant $bla_{NDM-5}$.
MIC of IPM and/or MEM were determined only for the isolates, those were resistant to that antibiotic in disk diffusion assay.

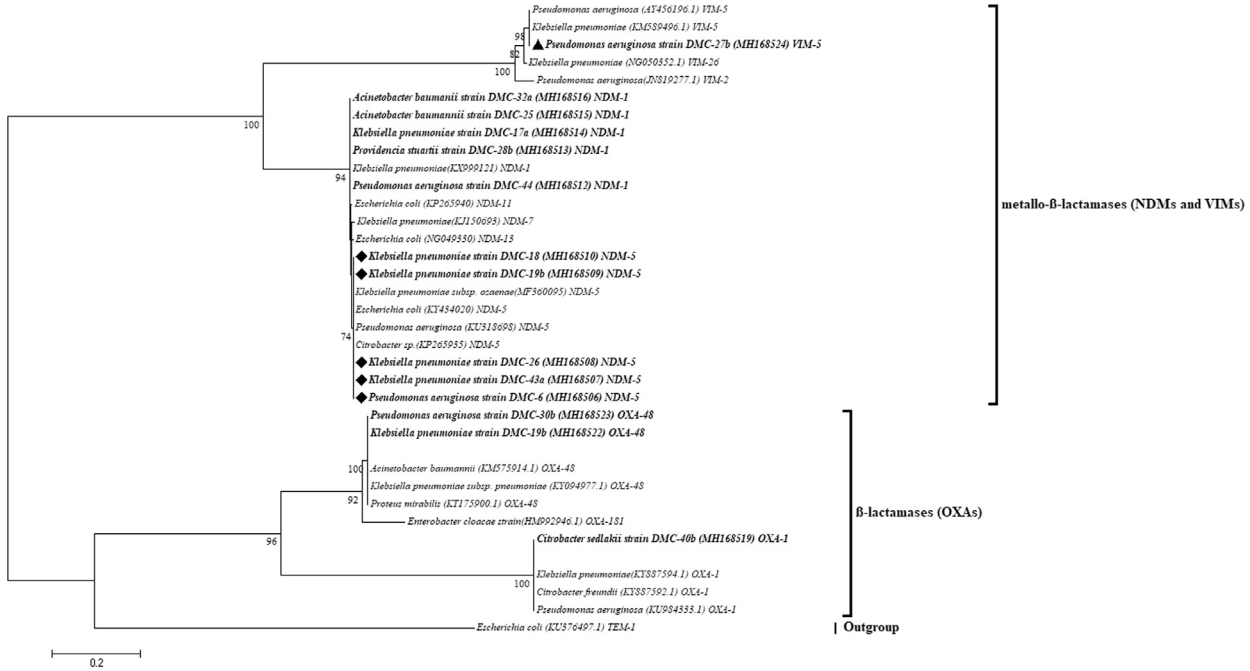


**Fig. 2.** Phylogenetic inference of the carbapenemase genes ($bla_{NDM}$, $bla_{VIM}$ and $bla_{OXA}$ carbapenemases): The three different carbapenemases formed three distinct clusters. The gene sequences of the collected sample isolates from this study have been represented in bold letters. The outgroup contains TEM-1 gene sequence of *Escherichia coli* (Accession no. KU376497.1). The emerged carbapenemases of Bangladesh which were characterized in this study: carbapenemase $bla_{VIM-5}$ and $bla_{NDM-5}$ have been marked with ▲ (filled triangle) and ◆ (filled diamond) symbols respectively.

However, overall sensitivity of MHT was better with imipenem, although meropenem showed better SE (63%) than imipenem (56%) in case of *Pseudomonas* spp. isolates (Table 3). CDT showed poor specificity (50%) and sensitivity (50%) in case of MBL prediction. KPC phenotype predicted by CDT was not supported by PCR of $bla_{KPC}$ genes (Table 2).

### 3.6. Plasmid profile and presence of integrons

Plasmid profile analysis revealed that under experimental condition, 16 out of 25 carbapenem resistant isolates harboured one or more plasmids of different sizes. No plasmid could be extracted from the rest of the resistant isolates under the same experimental conditions. Plasmids of the same size were observed among both the intra-species and the interspecies isolates (Fig. 3). Identical plasmid profile was shared between intraspecies isolates from two different sources belonging to *K. pneumoniae* strain DMC-26 (pus) and strain DMC-43a (wound swab) and also between *K. pneumoniae* strain DMC-18 (urine) and DMC-19b (wound swab) as well as between isolates of same source belonging to *P. aeruginosa* strain DMC-20b and DMC-20c (wound swab) (Fig. 3). All the isolates were positive for Integron class 1 (Table 2).

**Table 3**
Evaluation of phenotypic assays: Sensitivity (SE) and Specificity (SP) of phenotypic test, Modified Hodge test (MHT) and Combined Disk Test (CDT) for MBL prediction.

| Criteria | Categories | | MHT | CDT for MBL |
|---|---|---|---|---|
| | | | SE (SP) | SE (SP) |
| Disk used | [a] | | 0.83 (1) | 0.5 (0.5) |
| | IPM (n = 21) | | 0.81 | |
| | MEM (n = 21) | | 0.70 | |
| | DOR (n = 19) | | 0.63 | |
| Genotype | $bla_{NDM}$ + $bla_{OXA-1}$+$bla_{OXA-48}$ | | 0.92 | 0.58 |
| | $bla_{NDM}$ + $bla_{OXA}$ | | 0.94 | 0.53 |
| | $bla_{VIM}$ + $bla_{OXA}$ | | 0.8 | 1 |
| | $bla_{NDM}$ (irrespective of the presence of $bla_{OXA}$) | | | 0.56 |
| Organism | *Pseudomonas* spp. | [a] | | 0.33 |
| | | IPM | 0.56 | |
| | | MEM | 0.63 | |
| | | DOR | 0.29 | |
| | *Acinetobacter* sp. | [a] | | 0.25 |
| | | IPM | 1 | |
| | | MEM | 1 | |
| | | DOR | 1 | |
| | *Enterobacteriaceae* | [a] | | 0.75 |
| | | IPM | 1 | |
| | | MEM | 0.86 | |
| | | DOR | 0.71 | |

[a] Test result was considered positive if it yielded positive result with any of the three carbapenem disks used.

## 4. Discussion

Endemicity of $bla_{NDM}$ along with sporadic reports of $bla_{KPC}$ and recent reports of several carbapenemases indicates high prevalence of carbapenemases in Bangladesh [20,21]. In this study, 35.21% (25 out of 71) of the clinical isolates collected from the DMCH was resistant to carbapenems and all of them were MDR. These MDR isolates included the members of the ESKAPE pathogens [34]. In fact, 80% isolates (20 out of 25) belonged to *P. aeruginosa, A. baumannii* and *Enterobacteriaceae*, the WHO (2017) enlisted Priority 1: Critical organisms [35]. The prevalent group was *Pseudomonas* spp. including *P. aeruginosa* isolates (n = 10), the second most common cause of nosocomial infections in South-east Asia [36]. Although only four isolates were *A. baumannii,* all of them were retrieved from intensive care unit (ICU) including one from high dependency unit (strain DMC-32a). *A. baumannii* is the most common cause of nosocomial infection in Southeast Asia causing high rate of mortality and morbidity, particularly in ICU [36]. Even *A. baumannii* strain DMC-15a was colistin resistant in disk diffusion test indicating the potentiality to emerge as Pandrug resistant.

Carbapenemase specific PCR revealed that 24 out of 25 isolates were positive for MBL ($bla_{NDM}$ or $bla_{VIM}$) or/and $bla_{OXA}$ ($bla_{OXA-1}$ or $bla_{OXA-48}$) and 21 isolates harboured multiple carbapenemases rather than a single carbapenemase. All but one MBL producer

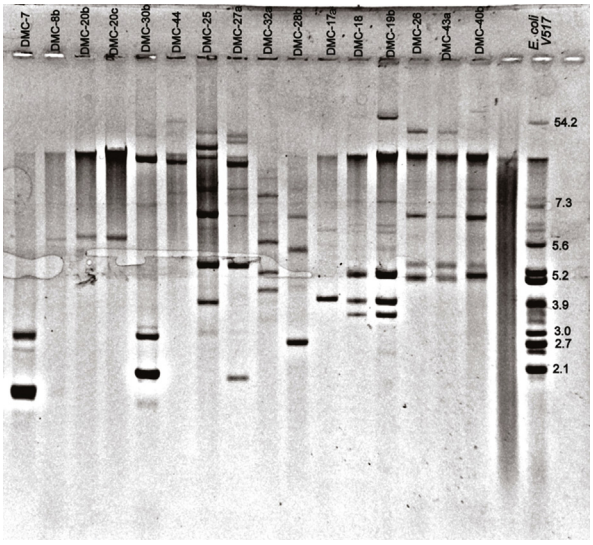


**Fig. 3.** Plasmid profile of the carbapenem resistant isolates: Agarose gel electrophoretogram (0.8% gel) of plasmid DNA of the carbapenem resistant isolates: *P. aeruginosa* strain DMC-7, DMC-8b, DMC-20b, DMC-20c, DMC-30b, DMC-44 (from lane 1 to lane lane 6), *A. baumannii* strain DMC-25, DMC-27a, DMC-32a (lane 7 to lane 9), *P. stuartii* strain DMC-28b (lane 10), *K. pneumoniae* strain DMC-17, DMC-18, DMC-19b, DMC-26, DMC-43a (from lane 11 to lane 15) and *C. sedlakii* strain DMC-40b (lane 16). *E. coli* V517 in lane 18 was used as the marker and its plasmids of different sizes (kb) have been denoted in the figure. Identical plasmid profile of the intra-species isolates were observed belonging to *K. pneumoniae* strain DMC-18 and strain DMC-19b (lane 12 & 13), *K. pneumoniae* strain DMC-26 and strain DMC-43a (lane 14 & 15) and *P. aeruginosa* strain DMC-8b, DMC-20b and DMC-20c (lane 2, 3 and 4). Plasmid bands at the same position was observed in intraspecies (*P. aeruginosa* strain DMC-30b and strain DMC-7 at the position between 2.7 kb and 3.0 kb; *A. baumannnii* strain DMC-25 and DMC-27a at the positions between 5.2 and 5.6 kb, 5.6 kb to 7.3 k and 7.3 kb) and interspecies (*K. pneumoniae* strain DMC-19b, DMC-26, DMC-43a and *C. sedlakii* strain DMC-40 b at the position of about 5.2 kb). Large plasmids at common positions were observed in interspecies *P. aeruginosa* strain DMC-30b and *A. baumannii* strain DMC-25, intraspecies *K. penumoniae* strain DMC-26 and DMC-43a; *K. penumoniae* strain DMC-18 and DMC-19b.

(n = 21) co-harboured $bla_{OXA}$. Even co-existence of both $bla_{OXA-48}$ and $bla_{OXA-1}$ with $bla_{NDM}$ was frequently encountered (n = 12) and this was the prevalent genotype. Although co-existence of $bla_{NDM}$ with either $bla_{OXA-48}$ or $bla_{OXA-1}$ is well reported, co-existence of all three carbapenemases in a single isolate is less frequently reported [37]. However, $bla_{OXA-48}$ was the most prevalent gene detected, and $bla_{OXA-48}$ has already been reported from *Enterobacteriaceae* as well as nonfermenters worldwide including from Bangladesh [4]. But this high prevalence of occurrence is worthy of clinical concern. Even this concern is also valid for $bla_{NDM}$, the 2nd most prevalent gene. Both $bla_{NDM-5}$ and endemic $bla_{NDM-1}$ were detected in this study. For reference, $bla_{NDM-5}$ differed from $bla_{NDM-1}$ by two amino acid substitutions at the positions 88 (Val → Leu) and 154 (Met → Leu) due to two different single neucleotide substitutions at the positions 262 (G → T) and 460 (A →C), respectively [38]. Notably, $bla_{NDM-5}$ possesses greater hydrolytic activity towards carbapenems than $bla_{NDM-1}$ [7]. This report of $bla_{NDM-5}$ is contemporary with the report of $bla_{NDM-5}$ in Bangladesh, which has been published in 2018 [39]. However, at first the $bla_{NDM-5}$ was reported from Japan within *E. coli* TK1044, and that was also collected from the fecal sample of a traveler from Bangladesh [40]. This genotype is increasingly reported from South Asian countries in recent years [40]. Therefore, it can be assumed that $bla_{NDM-5}$ was circulatory in Bangladesh along with $bla_{NDM-1}$, but remained undetected until recently. More interestingly, each $bla_{NDM-5}$ was found in combination with other carbapenemases in the study isolates.

Apart from $bla_{NDM}$, another MBL, $bla_{VIM-2}$ was found in only *P. aeruginosa* strain DMC-7, DMC-20b and DMC-20c. $bla_{VIM-2}$ is the most-reported MBL worldwide [41] with the endemic spread in Southeast Asia [42] and was previously reported in Bangladesh [21].

Importantly, the least prevalent gene found was $bla_{VIM-5}$ in *P. aeruginosa* strain DMC-27b which has never been reported before from Bangladesh. $bla_{VIM-5}$ is endemic in Turkey with limited reports from different European countries [12,43] and India [13] indicating the possible transmission of $bla_{VIM-5}$ from this neighbouring country. However, lack of detailed medical and travel history of the patients made it impossible to investigate the source of transmission.

Investigation on mobile genetic elements revealed the presence of plasmids in 64% (16 out of 25) isolates. Fourteen out of these 16 isolates carried $bla_{OXA-48}$, among which *K. pneumoniae* strain DMC-18 and DMC-19b, *P. aeruginosa* strain DMC-44 and *C. sedlakii* strain DMC-40b harboured plasmids of >58.2 kb, while the acquisition of $bla_{OXA-48}$ is widely found in association with plasmids, specially 62 kb IncL/M plasmids [44,45]. Chromosomally encoded $bla_{OXA-48}$ has also been reported in *E. coli* [46]. So the rest of the isolates having $bla_{OXA-48}$ with no plasmid (n = 6) might have this gene on chromosome, though none of them were *E. coli* isolates. Besides, both $bla_{NDM-5}$ and $bla_{NDM-1}$ have been reported to be associated with plasmids [40,47]. $bla_{NDM-1}$ was frequently reported to be associated with large plasmids of about 50 kb [48], while 36% (9 out of 25) isolates harboured plasmids larger than chromosomal size including the isolates, *P. aeruginosa* strain DMC-44 and *A. baumannii* strain DMC-25 carrying $bla_{NDM-1}$. Besides, same or similar plasmid profile observed among the inter- or intra-species isolates implicating the possible dissemination of resistance markers through plasmids in the hospital setting.

Another mobile genetic element, integron I was present in all the isolates. This high prevalence of integrons indicates the chance of rapid dissemination of these carbapenemases as gene cassette(s), as integron I has been reported to be associated with $bla_{VIM-2}$ [49], $bla_{VIM-5}$ [43], $bla_{NDM-1}$ [50], $bla_{OXA-1}$ [51] and $bla_{OXA-48}$ [51].

Interestingly, along with isolates carrying multiple carbapenemases, presence of multiple carbapenemases producing organisms (CPOs) in a single patient was notable in this study. Three out of 42 patients from whom these isolates were collected was carrying more than one CPO. Even co-carriage of carbapenem resistant *P. aeruginosa* with inherently colistin resistant *Proteus mirabilis* was noticed, which portrayed the worst concern of clinical relevance with very little treatment option left.

Investigation of carbapenemases performed by phenotypic techniques revealed poor sensitivity and specificity. MHT showed better performance with imipenem which was reported previously [52] and also performed better with *Enterobacteriaceae* and *A. baumannii* compared to *P. aeruginosa* isolates, that was in support of reports of difficulty in carbapenemase prediction in *Pseudomonas* isolates [53]. On the other hand, KPC phenotype predicted by CDT was not supported by PCR assay which might be due to the production of AmpC-type β-lactamases (cephalosporinases) or due to the presence of the rest 16 variants of KPC not investigated by PCR [54]. However, ESBLs coexisting with porin loss in association with AmpC mimicking carbapenemase activity might be responsible for low sensitivity and specificity of CDT [55,56].

To conclude, diverse carbapenemases including $bla_{NDM-5}$ along with the emergence of $bla_{VIM-5}$ suggest rapid dissemination of carbapenemases in Bangladesh. Besides, co-existence of multiple carbapenemases and their association with mobile genetic elements demand an effective strategy to combat the arising public health crisis.

## Ethical approval

The study was approved by the University Research Ethics Committee, University of Dhaka and carried out under the direct

supervision of the laboratory biosafety officer. As the samples were collected from Dhaka Medical College Hospital, we did not handle the human subjects directly.

## Conflicts of interest

The authors declare no conflict of interests.

## Authorship statement

All the authors meet the ICMJE authorship criteria.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.01.010.

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