Introduction

Carbapenemases represent the most versatile family of beta-lactamases, with the spectrum unrivalled by the other beta-lactam-hydrolysing enzymes.[1] Although known as carbapenemases, many of these enzymes recognize almost all hydrolysable beta-lactams and are resilient against inhibition by all commercially available beta-lactamase inhibitors.[2,3] This resilience is often accompanied by resistance to other classes of antibiotics. Carbapenem-resistant Gram-negative bacilli (CRGNB) represents difficult-to-treat infections in hospitalized patients and is associated with high mortality.[4] Carbapenem antibiotics have been reserved as drugs of the last resort for salvage treatment for infections caused by multidrug-resistant Gram-negative bacteria. Thus, resistance to carbapenems becomes a real threat to the survival of seriously ill patients, with overall mortality exceeding 50%.[5] It is estimated that we are on the edge of a worldwide carbapenemase-pandemic. This is a prospective study performed on a significant number of strains to establish the prevalence of different carbapenemase genes in fermenting and nonfermenting Gram-negative bacilli isolated from patients.
admitted in wards or attending the outpatient department (OPD) of a tertiary care hospital in Northern India. We compared different methods for detection of metallo-β-lactamase (MBL) so as to establish the most specific test. Clinical characteristics and risk factors associated with patients infected with MBLs were assessed.

**Materials and Methods**

The present study was conducted in patients admitted to the wards or attending the OPD of Jawaharlal Nehru Medical College Hospital, AMU, Aligarh in the Department of Microbiology from February 2014 to December 2015. This study was done after approval from the Institutional Ethics Committee of JNMC, and the procedures followed in the study were in accordance with its guidelines. Clinical specimens including pus, urine, and fluid submitted to the bacteriology were investigated. Urinary tract infection (UTI) was defined as positive quantitative urine culture (>10^5 microorganisms/ml) with a maximum of two isolated microbial species in patients with symptoms suggestive of UTI. Wound infection was defined as per Southampton grading. Majority of the cases belonged to Grade III and IV. Fluid samples were obtained from intercostal tube drainage, peritoneal fluids, and pleural fluids. Fluid cultures were interpreted according to Bergey’s Manual of Systematic Bacteriology Vol 3, 2009. The samples were obtained with proper aseptic techniques and transported to the laboratory within 1 h of their collection. The organisms were identified on the basis of cultural characteristics, morphology, and biochemical tests (Bailey and Scotts, 2007, Mackie and McCartney, 2007[7]). Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion technique on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) 2010, M 100-S20[8] guidelines. However, certain other drugs apart from CLSI 2010 guidelines and risk factors associated with patients infected with MBLs were assessed.

### For enterobacteriaceae (pus and fluids)

- Amikacin (30 μg), gentamicin (10 μg), levofloxacin (5 μg), ofloxacin (5 μg), ceftriaxone (30 μg), cefoperazone (75 μg), cefotaxime (30 μg), cefixime (5 μg), cefepime (30 μg), ceftazidime (30 μg), cefepoxide (10 μg), cefoperazone/sublactam (75/10 μg), ceftazidime/clavulanic acid (30/10 μg), ceftriaxone/sublactam (30/15 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazidime/tazoactam (80/10 μg), pipercillin (100 μg), teicoplanin (200 μg), tobramycin (10 μg), sparfloxacin (5 μg), ertapenem (10 μg), faropenem (5 μg), and imipenem (IMP) (10 μg).

### For nonfermenting Gram-negative bacilli

- Amikacin (30 μg), gentamicin (10 μg), levofloxacin (5 μg), ceftriaxone (30 μg), cefoperazone (75 μg), cefepime (30 μg), nitrofurantoin (300 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefepoxide (10 μg), cefoperazone/sublactam (75/10 μg), ceftriaxone/sublactam (30/15 μg), ceftazidime/clavulanic acid (30/10 μg), ticarcillin (75 μg), piperacillin (100 μg), piperacillin/tazobactam (100/10 μg), tobramycin (10 μg), sparfloxacin (5 μg), IMP (10 μg), colistin (10 μg), and Polymyxin B (300 units).

### For urinary tract infection, the drugs used were as follows

- Amikacin (30 μg), gentamicin (10 μg), levofloxacin (5 μg), ceftriaxone (30 μg), cefoperazone (75 μg), cefoperazone/sublactam (75/10 μg), cefixime (5 μg), cefepime (30 μg), nitrofurantoin (300 μg), ceftazidime (30 μg), ceftazidime/tazoactam (80/10 μg), pipercillin (100 μg), pipercillin/tazobactam (100/10 μg), tobramycin (10 μg), sparfloxacin (5 μg), ertapenem (10 μg), faropenem (5 μg), and imipenem (IMP) (10 μg).

Consecutive, nonduplicate, and CRGNB isolates were screened phenotypically for the detection of MBL production. Confirmation of MBL production was done by polymerase chain reaction (PCR). Detailed history and investigations of all these patients were noted and followed up for outcome.

### Phenotypic detection of metallo-β-lactamase production

Modified Hodge test[9](MHT), IMP-ethylene diamine tetracetic acid-Double Disk Synergy test[10] (IMP-EDTA DDSST), and IMP-EDTA-combined disk synergy test[11] (IMP-EDTA CDST) were done for phenotypic detection of MBL.

### Genotypic detection of metallo-β-lactamase genes (BlaNDM-1, BlaVIM, BlaIMP)

Genotypic detection of MBL genes was performed only on those isolates which were phenotypically identified as MBL producers. Thus, 116 isolates were selected for detailed molecular characterization. PCR amplification was carried out using the 2x PCR master mix (Fermentas, Thermo scientific, USA) on a Gradient Thermo Cycler named Le cycler of LABNIC, USA, with primers targeting MBL genes as given below.

Molecular detection of blaNDM-1, blaVIM, and blaIMP was performed using PCR according to methods described previously (Nordmann et al., 2011[11] for blaNDM-1, Manoharan et al., 2010[12] for blaVIM and blalMP) to amplify the 621, 382, and 587 base pair fragments, respectively [Table 1]. The amplified PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

### Sequencing

DNA sequencing was performed at TRIYAT SCIENTIFIC CO.(Nagpur, India) using the ABI Prism® Big Dye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, USA), for forward and reverse primers of NDM-1 gene on 3730xl Genetic Analyzer (Applied Biosystems, USA), separately.

### Phylogenetic analysis

Phylogenetic analysis was performed using the software MEGA, version 4, after multiple alignments of data using CLUSTAL X. Pairwise evolutionary distances were estimated by Kimura’s two-parameter method, and a phylogenetic tree was constructed by the neighbor-joining method.
reliability of the topologies was estimated by performing bootstrap analysis (1000 replicates). All phylogenetic analysis was performed only for NDM-1 gene.

All CRGNB positive patients were kept isolated from the other patients, and all the hospital staff were advised to practice transmission-based precautions and other universal precautions with more conscious attention to hand hygiene.

**RESULTS**

A total of 116 consecutive, nonduplicate CRGNB were included in the study, out of which 85 (73.2%) were isolated from pus, 17 (14.6%) from urine and 14 (12.1%) from fluid. *Citrobacter* species 28 (24.1%) predominated followed by *Escherichia coli* 26 (22.4%), *Pseudomonas aeruginosa* 24 (20.6%), *Serratia* species 17 (14.6%), *Klebsiella* species 9 (7.7%), *Acinetobacter* species 5 (4.3%), *Proteus* species 4 (3.4%), *Providencia* species 2 (1.7), and *Aeromonas* species 1 (0.8%). Fractures 29 (29.5%), G. I. tract surgical site infections 27 (27.5%), and skin and soft-tissue infections 13 (13.2%) were the predominant clinical conditions associated with patients who were admitted in the hospital.

Table 2 describes the risk factors associated with hospitalized patients. It was observed that 82 (70.6%) patients had indwelling devices such as intravenous catheter and urinary catheters, 60 (51.7%) patients had prolonged stay in hospital >21 days and 59 (50.8%) patients were on prior antibiotic treatment. Risk factors associated with advanced age, surgery for >2 h duration, and malnutrition were lower 18 (15.5%), 15 (12.9%) and 16 (13.7%), respectively.

In the current study, we observed that all the study isolates (urine, pus, and fluids) were resistant to amikacin, gentamicin, levoflaxacin, ceftriaxone, cefoperazone, cefepime, nitrofurantoin, ceftazidime, cefotaxime, cepodoxime, cefoperazone/sulbactam, ceftriaxone/sulbactam, ceftazidime/clavulanic acid, ticarcillin, piperacillin, tobramycin, sparfloxacin, piperacillin-tazobactam, and carbapenems (IMP, ertapenem, doripenem, and meropenem). Among higher drugs—polymyxin B (100%), ceftriaxone plus sulbactam plus EDTA (CSE) (96.5%), and colistin (90.5%) were the only drugs found to be active against all CRGNB clinical isolates. High resistance was observed to aztreonam (51.7%), tigecycline (40.1%) and fosfomycin (35.2%). Antibiotic resistance pattern of the isolates against polymyxin B, ceftriaxone plus sulbactam plus EDTA (CSE), colistin, aztreonam, tigecycline, fosfomycin among pus, urine, and fluids are depicted in Tables 3-5, respectively.

Table 2: Risk factors in hospitalized patients for acquiring carbapenem-resistant Gram-negative bacilli (n = 116)

| Risk factors                  | Number of patients (%) |
|------------------------------|------------------------|
| Obesity                      | 10 (8.62)              |
| Hypertension                 | 11 (9.48)              |
| Tuberculosis                 | 3 (2.58)               |
| Diabetes                     | 2 (1.72)               |
| Malnutrition                 | 15 (12.93)             |
| Prolonged stay >21 days      | 60 (51.72)             |
| Infection at remote site     | 2 (1.72)               |
| Presence of drain, catheter, foley’s, etc. | 82 (70.68) |
| Prior antibiotic treatment taken | 59 (50.86) |
| Duration of surgery >2 h     | 16 (13.79)             |
| Advanced age                 | 18 (15.51)             |

In the current study, we observed that all the study isolates (urine, pus, and fluids) were resistant to amikacin, gentamicin, levoflaxacin, ceftriaxone, cefoperazone, cefepime, nitrofurantoin, ceftazidime, cefotaxime, cepodoxime, cefoperazone/sulbactam, ceftriaxone/sulbactam, ceftazidime/clavulanic acid, ticarcillin, piperacillin, tobramycin, sparfloxacin, piperacillin-tazobactam, and carbapenems (IMP, ertapenem, doripenem, and meropenem). Among higher drugs—polymyxin B (100%), ceftriaxone plus sulbactam plus EDTA (CSE) (96.5%), and colistin (90.5%) were the only drugs found to be active against all CRGNB clinical isolates. High resistance was observed to aztreonam (51.7%), tigecycline (40.1%) and fosfomycin (35.2%). Antibiotic resistance pattern of the isolates against polymyxin B, ceftriaxone plus sulbactam plus EDTA (CSE), colistin, aztreonam, tigecycline, fosfomycin among pus, urine, and fluids are depicted in Tables 3-5, respectively.

Only those isolates which were screened phenotypically as MBL producers were subjected to molecular detection of MBL genes. Of the 116 isolates, MBL genes were detected in 79 (68.1%) strains. Table 2 shows the prevalence of NDM-1, VIM, and IMP-1 genes in clinical specimen. NDM-1 was found to be the most prevalent MBL gene among all specimens 66 (83.5%), followed by VIM [12 (15.1%), and IMP-1 (1 [1.2%]) [Table 6]. It was interesting to observe that all MBL producers in urine samples were NDM-1. On the other hand, maximum VIM gene positivity was seen in pus 10 (17.8%). IMP-1 was detected in ascitic fluid. Figures 1-3 show positive results of PCR for blaNDM, blalMP, and blavIM genes, respectively.

Table 7 shows the distribution of NDM-1, VIM, and IMP genes among various MBL producers. All *E. coli* 26 (100%) were positive for NDM-1 gene, followed by *Klebsiella* species 7 (77.7%), *Citrobacter* species 14 (50%), and *Serratia* species 8 (47%). Majority of VIM was observed in *P. aeruginosa* 7 (29.1%) followed by *Citrobacter* species 3 (10.7%). IMP was detected only in one isolate of *P. aeruginosa*.

Results of blaNDM-1 gene were further confirmed by DNA sequencing for all the 11 representative strains (DNA extraction No. 90, 97, 103, 110, 111, 115, 116, 119,139, 159, and 176). After analyzing the nucleotide sequences of our study strains for NDM-1 gene, we prepared a phylogenetic tree with the help of MEGA 4 software. We observed that most of the
The predominant CRGNB in ward patients were Citrobacter species (25.2%), E. coli (24.2%), and P. aeruginosa (18.2%) followed by Serratia species (13.1%) and Klebsiella species (8.1%), whereas in outpatients, the predominant CRGNB were P. aeruginosa (35.2%), Serratia species (23.5%), Citrobacter species (17.6%) and followed by E. coli (11.7%). Overwhelmingly, most isolates were from inpatients. Wankhede et al., 2011[13] reported that P. aeruginosa (23.7%), Acinetobacter spp. (18.4%), K. pneumoniae (8.3%), and E. coli (5%) were the most common MBL producers.

This study highlights that the drugs of choice for treating CRGNB are polymyxin B which was active against all isolates, ceftriazone -sulbactam plus EDTA, and colistin with 96.56% and 90.5% sensitivity, respectively. Unfortunately, high resistance was observed for aztreonam,
tigecycline (40.1%), and fosfomycin. Thus, in our center, use of the latter drugs is discouraged. Behera et al., 2008[14] reported that MBL-positive isolates are usually resistant to all β-lactam antibiotics, aminoglycosides, tetracycline, and fluoroquinolones. However, they remain sensitive to polymyxin B. Nordmann et al., 2011[11] reported that therapeutic options against serious infections due to NDM-1 producers are limited to tigecycline and polymyxins although the former may not reach desired serum levels to treat systemic infections.

**Table 5: Antimicrobial resistance pattern of carbapenem resistant Gram-negative bacilli among fluid samples**

| Pathogen        | Total sample (n=116) | Total pus sample (n=14) | Tgc, n (%) | At, n (%) | Cse, n (%) | Cl, n (%) | PB, n (%) | Fos (in urine), n (%) |
|-----------------|----------------------|-------------------------|------------|-----------|------------|-----------|-----------|----------------------|
| Citrobacter     | 28                   | 5                       | 2 (7.1)    | 3 (10.7)  | 1 (3.5)    | 1 (3.5)   | 0         | -                    |
| Escherichia coli| 26                   | 2                       | 1 (3.8)    | 2 (7.6)   | 0          | 1 (3.8)   | 0         | -                    |
| Pseudomonas     | 24                   | 4                       | 3 (12.50)  | 4 (16.66) | 1 (4.1)    | 1 (4.1)   | 0         | -                    |
| Serratia        | 17                   | 1                       | 1 (5.8)    | 1 (5.8)   | 0          | 1 (5.8)   | 0         | -                    |
| Klebsiella      | 9                    | 1                       | 1 (11.11)  | 1 (11.11) | 0          | 1 (11.11) | 0         | 1                    |
| Acinetobacter   | 5                    | 1                       | 1 (20.0)   | 1 (20.0)  | 0          | 0         | 0         | 1                    |
| Proteus species | 4                    | 0                       | 0          | 0         | 0          | 0         | 0         | -                    |
| Providencia     | 2                    | 0                       | 0          | 0         | 0          | 0         | 0         | -                    |

**Table 6: Prevalence of MBL genes in clinical specimen**

| MBL genes | Pus (n=56; 70.88%) | Urine (n=10; 12.65%) | Fluid (n=13; 16.45%) | Positivity of MBL genes (n=79) |
|-----------|--------------------|----------------------|----------------------|--------------------------------|
| NDM-1     | 46 (82.14)         | 10 (100)             | 10 (76.92)           | 66 (83.54)                      |
| VIM       | 10 (17.85)         | 0                    | 2 (15.38)            | 12 (15.18)                      |
| IMP       | 0                  | 0                    | 1 (7.69)             | 1 (1.26)                        |

**Table 7: Prevalence of NDM-1, VIM and IMP genes in various MBL producers**

| MBL producers | NDM-1 (n=66; 83.54%) | VIM (n=12; 15.18%) | IMP-1 (n=1; 1.26%) | Total positive (n=79; 68.10%) | Other mechanisms (n=37; 31.8%) |
|---------------|----------------------|--------------------|-------------------|-------------------------------|--------------------------------|
| Citrobacter   | 14 (50.0)            | 3 (10.71)          | 0                 | 17 (60.71)                     | 11 (39.28)                     |
| Escherichia   | 26 (100.0)           | 0                  | 0                 | 26 (100)                       | 0                              |
| Pseudomonas   | 7 (29.16)            | 7 (29.16)          | 1 (4.16)          | 15 (62.50)                     | 9 (37.5)                       |
| Serratia      | 8 (47.05)            | 1 (5.88)           | 0                 | 9 (52.94)                      | 8 (47.05)                      |
| Klebsiella    | 7 (77.77)            | 0                  | 0                 | 7 (77.77)                      | 2 (22.22)                      |
| Acinetobacter | 2 (40.0)             | 0                  | 0                 | 2 (40.0)                       | 3 (60.0)                       |
| Proteus       | 2 (50.0)             | 0                  | 0                 | 2 (50.0)                       | 2 (50.0)                       |
| Providencia   | 0                    | 1 (50.0)           | 0                 | 1 (50)                         | 1 (50)                         |

**Figure 3:** Two percent agarose gel electrophoresis showing results of polymerase chain reaction for the detection of blaIMP gene

**Figure 4:** Phylogenetic analysis of NDM-1 strains
In this study, the 116 CRGNB isolates were subjected to phenotypic MBL detection. It was observed that 90.5% isolates were MBL positive by MHT. According to Cury et al., 2012, [15] MHT was positive in 35.5% of Enterobacteriaceae isolates that were not susceptible to ertapenem 71% of those isolated showed true-positive results, and 29% had inconclusive results. Phenotypic detection by IPM-EDTA-DDST showed that 75% isolates were MBL positive. Similar results were observed in a study from Vellore. [16] Phenotypic detection by IMP-EDTA DDDST showed that 81% isolates were MBL positive. From our study, it was observed that among these standard phenotypic detection tests the least sensitive was CDST (75%), and MHT was the most sensitive (90.5%).

The high prevalence of CRE phenotype of Citrobacter and Serratia spp. may be due to hyperproduction of AmpC. This suggests that hyperproduction of AmpC results in a positive MHT. We suggest that in case of Citrobacter and Serratia spp., a positive phenotypic test need not suggest MBL production. They should be further subjected to genotypic detection.

The present study demonstrates that blaNDM-1 (56.8%) was the most prevalent MBL gene followed by blaVIM 10.3% and blaIMP (4.1%). Surprisingly, all MBL producers in urine samples were blaNDM-1. In our study, all E. coli (100%) were blaNDM-1 producers followed by Klebsiella species (77.7%) and Citrobacter species (50%). Amudhan et al., 2011 [17] too reported that blaNDM-1 was the most prevalent MBL (57.6%) gene in their study. In other Indian studies, the prevalence of blaNDM-1 producers among carbapenem resistant Enterobacteriaceae ranged between 31.2% and 91.6%. [12,18] Maximum blaVIM gene positivity was seen in pus 17.8% while blaIMP was found in ascitic fluid. Common blaVIM producing bacterial isolates in pus were P. aeruginosa 100% and Citrobacter species 66.6%. However, in fluids, Serratia species (100%) predominated followed by Citrobacter species (33.3%). BlaVIM was not detected in E. coli and Klebsiella species. However, 1 out of 2 isolates of Providencia species was positive for blaVIM. Further, spread of carbapenemase-producing P. aeruginosa strains would represent a significant threat for the future of β-lactams. Confirmation of blaIMP gene among our clinical isolates showed that only one P. aeruginosa species (4.16%) give positive result. The prevalence of MBL genotypes varies from one country to another. Two genotypes, namely, VIM and IMP are most common in Asian countries.

On comparing phenotypic and genotypic detection methods for MBL, only 68.1% isolates were confirmed as MBL producers by PCR. In our study, IPM-EDTA CDST correlated best with PCR results and thus is the most specific phenotypic test for MBL detection. However, an interspecies variation was also observed. For Citrobacter species, IPM-EDTA DDST with 78.5% correlation most closely matched with PCR 60.7% while in case of Escherichia coli, MHT (96.1%) was almost equivalent to PCR (100%). However, for Pseudomonas species, Serratia species and Klebsiella species, IPM-EDTA DST most closely mirrored with PCR results.

We suggest that a combination of MHT and IPM-EDTA DDST should be put up for more specific identification of MBL. Debasrita et al., 2011 [19] too compared phenotypic and genotypic methods. They observed that 26% bacteria were false positive by phenotypic method. They suggested that three phenotypic methods should be positive before labeling bacteria MBL producing. However, gold standard remains PCR. Thus, amplification clearly highlighted that phenotypic tests wrongly identify some CRGNB as MBL producers.

Thus, 47.1% strains of Serratia species, 39.29% of Citrobacter species, and 37.5% of Pseudomonas species were not MBL producers. In all probability, these strains were AmpC hyper-producers which can be effectively treated with higher doses of IMP or doripenem. Other causes may be hyperproduction of CTX-M or presence of other MBLs such as KPC or OXA. Porin defects or overexpression of efflux pumps also may play a significant role. [11] Manoharan et al., 2015 [12] reported that there are other resistance mechanisms involved apart from MBLs such as permeability mutations through the loss of porins or the upregulation of efflux systems, which may be missed by the E test or the MBL PCR.

On analysis of the phylogenetic tree, NDM gene sequences from our patients were found to be evolutionarily close to NDM-1 reference sequences. Most of the study subjects were found to be infected with NDM-1. As per our finding, other studies also support the dominance of NDM-1 strains in India. [20,21]

In our study, 77.2% patients improved clinically and discharged, 7.1% did not show any improvement while 4.04% died. As the majority of patients recovered (P < 0.001), it appears that MBL carrying bacteria may have low virulence, or on the other hand, the patients are immunocompetent as the cases were drawn from wards and not Intensive Care Units (ICUs). Since this was a study in patients admitted in wards (not seriously ill patients), their robust immune response may also have played a significant role in recovery. However, recovery did take time as is manifested by the increased duration of hospital stay (P < 0.001). Thus, in a non ICU patient, morbidity due to MBL infection increased; however, mortality was significantly low. It may be premature to think that NDM-1 is a resistant but a less virulent pathogen, although some clinical facts point toward this possibility. [22,23]

**Conclusion**

Development of quick, effective molecular diagnostic techniques for identification and epidemiological surveillance of resistance genes can significantly improve treatment protocols, which are currently guideline based. Such improved strategies can effectively intervene to prescribe on a case-to-case basis, in other words tailoring it to the requirements of the individual patient.
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Conflicts of interest
There are no conflicts of interest.

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