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Phenotypic identification & molecular detection of bla_{ndm-1} gene in multidrug resistant Gram-negative bacilli in a tertiary care centre

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***Background & objectives*: Carbapenemase-producing *Enterobacteriaceae* isolates have been increasingly identified worldwide. Though molecular data regarding New Delhi metallo-beta-lactamase-1 (NDM-1) producers are available, data regarding their rate of infection in a hospital setting and percentage among different clinical isolates are scarce. Hence, this study was undertaken to determine the occurrence of bla_{NDM-1} gene among clinical isolates of multidrug resistant Gram-negative bacilli (MDRGNB) in a tertiary care centre in Bangalore, Karnataka, India.**

***Methods*: A total of 74 MDRGNB isolates were studied. These were screened for MBL production by phenotypic assays such as double disk synergy test (DDST) and Modified Hodge's test (MHT). PCR was performed for the molecular detection of the gene and antibiograms were confirmed by automated bacteriology system.**

***Results*: Of the 74 MDRGNB isolates, 34 were positive for bla_{NDM-1} gene. All isolates were resistant to aztreonam and two isolates were resistant to tigecycline. Complete resistance to the tested carbapenems was seen in 28 (82.35%) of the positive isolates whereas variable carbapenem resistance was seen in six (17.64%) of the positive clinical isolates. Of the total 34 PCR positive isolates, 33 (97.05%) NDM-1 producers were identified by DDST and 26 (76.47%) by MHT as producers of MBL.**

***Interpretation & conclusions*: A high percentage of plasmid encoded NDM was noted in MDRGNB. Phenotypic and molecular screening should be employed along with routine antimicrobial susceptibility testing to reflect the true number of metallo-beta-lactamase producers.**

Key words Double disk synergy test - Modified Hodge's test - multidrug resistant Gram-negative bacteria - New Delhi metallo β lactamase-1

In 1965, the first report of a plasmid-encoded beta-lactamase in a Gram-negative bacterium appeared from Greece[1]. Metallo-beta-lactamases are enzymes that break down beta lactam drugs[2]. New Delhi metallo β lactamase-1 is a type of carbapenemase produced by certain strains of bacteria, and is able to inactivate all β-lactams except aztreonam[3]. Most NDM-1-positive strains also express the CMY-4 and CTX-M-15 β-lactamases, which confer resistance to all β-lactams. Thus it provides resistance against all compounds that contain a beta-lactam ring such as penicillins, cephalosporins, and the carbapenems. NDM-1 enzyme has been found clinically in *Enterobacteriaceae* and *Acinetobacter baumannii*[4].

The gene that encodes for NDM-1 is called bla_{NDM-1} and has been identified on bacterial chromosomes and plasmids. Plasmids carrying the bla_{NDM-1} gene also carry a number of other genes conferring resistance to all

aminoglycosides, macrolides, and sulphamethoxazole, thus making these isolates multidrug resistant or, because of other non-plasmid-mediated resistances, resistant in some cases to all antibiotics[5]. Plasmids carrying the gene for this carbapenemase, *bla*NDM-1, can have up to 14 other antibiotic resistance determinants and can transfer this resistance to other bacteria, resulting in multidrug-resistant or extremely drug-resistant phenotypes. bla_{NDM-1} gene has been reported to be carried on plasmids ranging from 140 to 400 kb in most isolates[6].

In view of the increasing reports of NDM-1 producing strains from India and around the world[7-10], the present work was conducted to examine the occurrence of NDM-1 gene among nosocomial isolates of MDRGNB from a tertiary care centre in southern India.

Material & Methods

Bacterial isolates: The total number of patients in this study was 61. In some cases more than one type of clinical sample (blood, urine, tracheal aspirate) was drawn from the same patient, thereby, giving a total of 70 clinical samples. The repeat samples from the same patient giving the same culture were counted for only once and the repeat samples of the same type from the same patient giving different cultures were counted individually as this indicated newly acquired infections. The 70 clinical samples on culture yielded 74 MDRGNB, due to the growth of two different isolates each from four of the clinical samples. The 74 MDRGNB were isolated based on conventional laboratory diagnostic techniques during September 2011 to February 2012 in the Neuromicrobiology department, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India. The Gram-negative isolates resistant to 1st and 2nd line antibiotics by standard disk diffusion test[11] were considered multidrug resistant, stocked in semisolid agar tubes and used for further characterization. The following antibiotics (concentration/disk in µg) were considered; ampicillin (10), amikacin (30), gentamycin (10), ciprofloxacin (5), ofloxacin (5), cefotaxime (30), ceftriaxone (30), ceftazidime (30), imipenem (10), cefoperazone+sulbactam (75/10), piperacillin+tazobactam (100/19), aztreonam (30), cefipime (30), tobramycin (19) (Hi-Media, Mumbai, India). The isolates were further identified at the species level and antibiograms generated by Vitek 2 Compact 60 (bioMerieux, Germany) automated bacteriology system.

Phenotypic assay: Modified Hodge's test (MHT) was performed according to the standard Clinical and Laboratory Standards Institute (CLSI) guidelines for the detection of carbapenemase in *Enterobacteriaceae*[11]; 0.5 McFarland of negative control *E.coli* ATCC 25922 was uniformly swabbed onto Muller Hinton Agar (MHA) and test isolate was streaked as a straight line from the edge of the imipenem (Ipm) disk (10 µg), to the edge of the plate. An indentation in the growth of the negative control towards the imipenem disk on either side of the test isolate was considered as positive for the production of beta lactamase (BL+ve) by the test isolate. *Klebsiella pneumoniae* BAA 2156 was used as positive control.

Double disk synergy test (DDST) was performed using two Ipm disks (10 µg), one containing 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO). Disks were placed 25 mm apart and an increase in zone diameter of >4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for MBL production[12]. These isolates were considered to be of the MBL+ve phenotype.

Detection of NDM-1 gene: Plasmid DNA was extracted from all 74 isolates by alkaline lysis method and 1µl of the isolated DNA was subjected to PCR with specific primers; NDM - Forward (5'-GGGCAGTCGCTTCCAACGGT) and NDM-Reverse (5'-GTAGTGCTCAGTGTCGGCAT) that amplified 475bp internal fragment of the gene[13]. *K. pneumoniae* ATCC BAA2156 was used as a positive control. DNA fragments were visualized by electrophoresis on 2 per cent agarose gel at 100 V for 1 h in 1X Tris acetate EDTA (TAE) containing 0.05 mg/l ethidium bromide. The samples were run alongside a 100 bp ladder that served as a molecular weight marker and an amplified product corresponding to 475 bp was considered positive (Figure A & B).The PCR amplicons including that of the positive control strain were sent for sequencing to Eurofins Genomics Pvt. Ltd., Bangalore, India.

The study protocol was approved by the ethics committee of NIMHANS, Bangalore.

Statistical analysis: All data were analyzed using SPSS 16.0 statistical software (SPSS Inc., Chicago, Illinois, USA). Chi-square test was done and $P<0.05$ was considered significant.

Results

During the study period, 4976 samples were screened and of these 74 (1.48%) were MDRGNB.

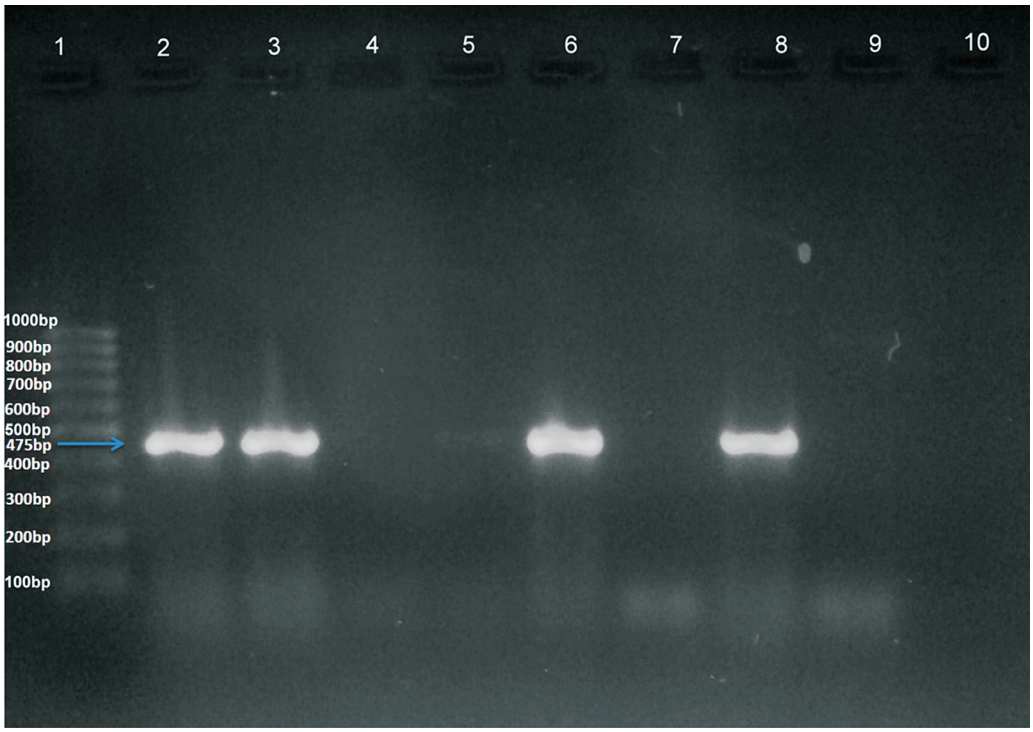

Fig. A. Amplified DNA after PCR with specific primers. Lane 1: 100 bp ladder. Lane 2: Positive control (*Klebsiella pneumoniae* BAA2156). Lanes 3, 6 and 8: Clinical isolates showing positive result. Lanes 4, 5, 7 and 9: Clinical isolates showing negative result. Lane 10: negative control (*Escherichia coili* 25922).

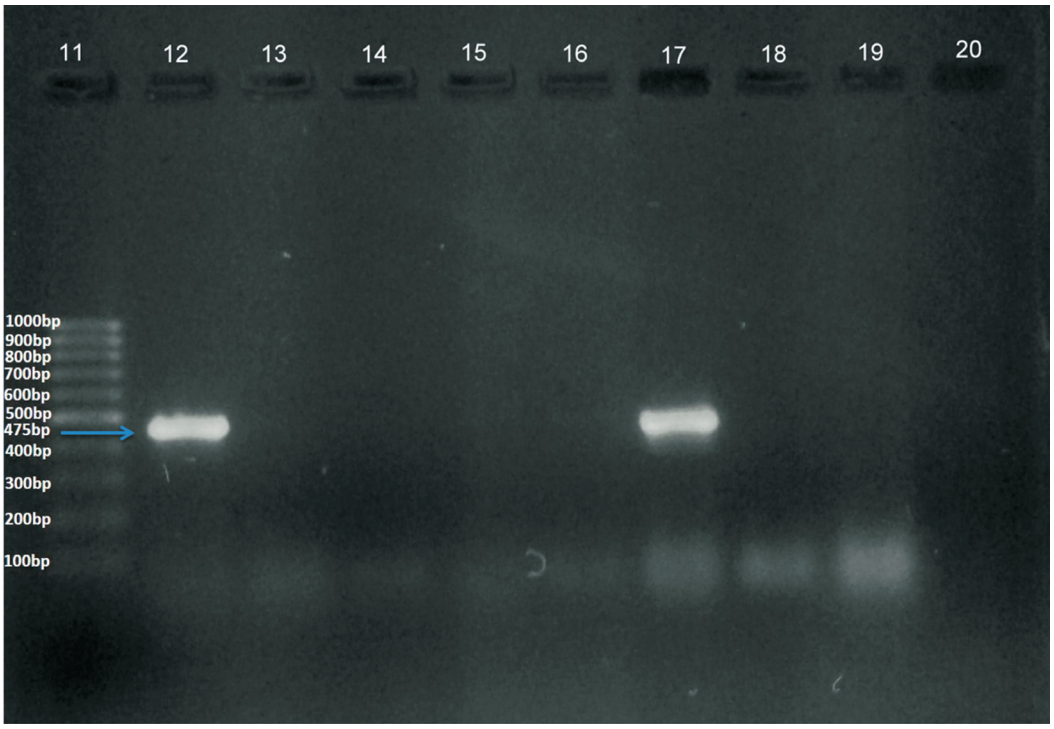

Fig. B. Amplified DNA after PCR with specific primers. Lane 11: 100 bp ladder. Lane 12: Positive control (*Klebsiella pneumoniae* BAA2156). Lanes 13 to 16, 18, 19: Clinical isolates showing negative result. Lanes 17: Clinical isolates showing positive band. Lane 20: (*Escherichia coili* 25922).

Of the 74 MDRGNB isolates, 34 were positive for bla_{NDM-1} gene by PCR. Sequencing of all 34 PCR amplicons showed that it was part of the bla_{NDM-1}gene. Phenotypically MBL production was seen in 69 of 74 (93.24%) of the MDR isolates. Of the 34 PCR positive isolates, 33 (97.05%) of NDM-1 producers were identified by DDST, 26 (76.47%) by MHT and all (100%) were flagged by Vitek 2 Compact 60 as producers of MBL (Table I).

All 34 PCR positive isolates were resistant to aztreonam among which two isolates were also resistant to tigecycline. The number of positive isolates completely resistant to the tested carbapenems was 28 of 34 (82.35%) whereas six (17.64%) isolates showing variable carbapenem resistance. Respiratory tract infection (RTI) and urinary tract infection (UTI) were the two main types of infections observed in the study.NDM positive MDRGNB caused 33.33 per cent (13/39) of the total RTIs and 60 per cent (9/15) of the total UTIs.

Of the 70 clinical samples, 32 grew Gram-negative bacteria positive for the gene. The different clinical samples were tracheal aspirate (n=39), urine (n=15), blood (n=2), central line tips (n=3), pus (n=2), wound swabs (n=4), CSF (n=3) and subgaleal collection (n=2). The different clinical isolates cultured from these samples were *Acinetobacter baumannii* (n=20), *Proteus vulgaris* (n=2), *Pseudomonas aeruginosa* (n=4), *Alcaligenes fecalis* (n=1), *Citrobacter freundii* (n=1), *Enterobacter cloacae* (n=4), *Roultella ornitholytica* (n=1), *Pseudomonas putida* (n=1), *Providencia rettgeri* (n=19), *Escherichia coli* (n=10), *Klebsiella pneumoniae* (n=10) and *Burkholderia cepacia* (n=1) (Table II).

The blood samples (n=2) yielded two NDM positive *P. rettgeri*. The subgaleal collection (n=2) yielded *A. baumannii* negative for NDM-1 and *K. pneumoniae* positive for the gene. The pus samples (n=2) yielded NDM positive *K. pneumoniae* and *E. coli.*

Table I. Comparison of conventional and automated phenotypic assay

Method used for phenotypic assay	No. of isolates
Total number of isolates positive for $bla_{NDM\ 1}$ gene	34
Number of isolates detected by DDST	33
Number of isolates detected by MHT	26
Number of isolates detected by Vitek 2 C 60	34

The mean age of the 61 patients was 32.40 yr (3-75yr) with 39 (63.93%) males and 22 (36%) females. The rate of infection was 16/22 (72.7%) in females, which was significantly (P<0.05) higher than males 15/39 (38.46%).

The infection due to MDRGNB resolved in 58 of the 61 patients. The remaining three patients succumbed to death.

Discussion

Non lactose fermenting Gram-negative bacteria such as *Providencia rettgeri* and *Acinetobacter baumannii*, widely associated with long term care facilities, were the most common organisms isolated in this study. Respiratory tract infections were the most common infections observed being caused by multidrug resistant *P. rettgeri* and *A. baumannii*. Urinary tract infections formed 21.42 per cent of the infections observed in the study. Multidrug resistant *E. coli* was the major causative organism of UTIs followed by *A. baumannii* 3/15 (20%) and *K. pneumonia*. An isolate of *K. pneumoniae* co-producing NDM-1 with *Klebsiella pneumonia* carbapenemase-2 (KPC-2), showing wide-spectrum resistance to β-lactams, aminoglycosides, fluoroquinolones, co-trimoxazole, nitrofurantoin and tigecycline has been reported in a hospital in Chennai[14]. All *K. pneumoniae* isolates and 60 per cent of the *E. coli* isolates causing UTI harboured the gene. However, the gene was absent in the *A. baumannii* isolates causing UTIs. Patients with long term indwelling catheters such as in the ICUs often have been reported to have UTIs caused by *E. coli, P. aeruginosa, Proteus mirabilis, Providencia stuartii, Morganella morganii* and *A. baumannii*[15].

Of the three CSF samples, two that showed the growth of MDRGNB were positive for the gene. A MDR *R. ornitholytica* isolate from tracheal aspirate and a *B. cepacia* isolate from urine sample were positive for the gene. *Pseudomonas putida* harbouring the gene has been obtained from sewage sample[5]. However, in our study a MDR *P. putida* isolate with NDM-1 gene was isolated from tracheal aspirate.

In the present study, 48.57 per cent isolates were positive for the gene by PCR, of which 11.4 per cent were *P. rettgeri* and *K. pneumoniae* each. In a study[4] conducted in India, the percentage of *Enterobacteriaceae* harbouring the gene was 30 per cent in Chennai, 13 per cent in Haryana and 44 per cent in the UK with *E. coli* and *K. pneumoniae* being

Table II: Type and percentage occurrence of different multidrug resistant strains isolated with the bla_{NDM-1} gene

Clinical samples	Tracheal aspirate n=39		Urine n=15		Wound swab n=4		Tips n=3		CSF n=3		Others n=6		Total n=70	
Clinical isolates	Total	PCR + ve	Total	PCR + ve	Total	PCR + ve	Total	PCR + ve	Total	PCR + ve	Total	PCR + ve	Total	PCR + ve
Acinetobacter baumannii	14(35.89)	4(10.25)	3(20)	0	1(25)	0	-	-	1(33.33)	0	1(16.66)	0	20(28.57)	4(5.7)
Escherichia coli	4(10.25)	1(2.5)	5(33.33)	3(20)	-	-	-	-	-	-	1(16.66)	1(16.66)	10(14.28)	5(7.1)
Klebsiella pneumoniae	2(5.1)	0	3(20)	3(20)	1(25)	1(25)	2(66.66)	2(66.66)	-	-	2(33.33)	2(33.33)	10(14.28)	8(11.4)
Providencia rettgeri	13(33.33)	3(7.69)	2(13.33)	2(13.3)	1(25)	0	-	-	1(33.33)	1(33.33)	2(33.33)	2(33.33)	19(27.14)	8(11.4)
Enterobacter cloacae	3(7.69)	3(7.69)	-	-	1(25)	1(25)	-	-	-	-	-	-	4(5.7)	4(5.7)
Alcaligenes fecalis	1(2.5)	0	-	-	-	-	-	-	-	-	-	-	1(1.42)	0
Pseudomonas aeruginosa	4(10.25)	0	-	-	-	-	-	-	-	-	-	-	4(5.7)	0
Proteus vulgaris	-	-	-	-	-	-	1(33.33)	1(33.33)	1(33.33)	1(33.33)	-	-	2(2.8)	2(2.8)
Burkholderia cepacia	1(2.5)	1(2.5)	1(6.6)	1(6.6)	-	-	-	-	-	-	-	-	1(1.42)	1(1.42)
Roultella ornitholytica	1(2.5)	1(2.5)	-	-	-	-	-	-	-	-	-	-	1(1.42)	1(1.42)
Pseudomonas putida	1(2.5)	1(2.5)	-	-	-	-	-	-	-	-	-	-	1(1.42)	1(1.42)
Citrobacter freundii	1(2.5)	0	-	-	-	-	-	-	-	-	-	-	1(1.42)	0
Total	45(115.38)	14(35.89)	14(93.33)	9(60)	4(100)	2(50)	3(100)	3(100)	3(100)	2(66.66)	6(100)	5(83.33)	74(105)	34(48.57)

in majority[3]. In our study, the 14.7 per cent of *E. coli* isolates were NDM-1 positive. While the MHT has been regarded non specific for the detection of metallo-beta-lactamases[16], in the present study MHT detected 26 of the 34 NDM-1 positive isolates. The DDST detected 33 of the 34 isolates that were positive for the gene. However, the isolate that was missed by DDST was detected as a producer of beta-lactamase by MHT and Vitek 2 C 60. Therefore, both the phenotypic assays bear significance in the detection of MBLs and help in the preliminary screening of the NDM-1 positive genotypes.

Variable carbapenem resistance was seen in 17.64 per cent of the NDM-1 positive isolates, being susceptible to one of the carbapenems tested or of the intermediate susceptibility type. However, 82.35 per cent were completely resistant to the tested carbapenems. In a study conducted from four local general hospitals in Singapore, the isolates were completely resistant to the second- and third-generation cephalosporins tested as well as carbapenems[17]. Most of the NDM-1 producers have been reported to remain susceptible only to colistin and tigecycline. However, a high tigecycline resistance of 43.18 per cent has also been reported[4]. In our study, two isolates were resistant to tigecycline and all 34 were resistant to aztreonam indicating the presence of other resistance mechanisms.

The 61 subjects were on combination drugs and in 58 the infection resolved eventually. The repeated culture of their clinical samples after treatment yielded no growth. This study had certain limitations. The presence of only plasmid encoded MBL was studied. Other metallo-beta-lactamases such as VIM1/VIM2 and carbapenemases such as KPCs were not targeted in the study. Further sequencing of complete plasmids and pulse field gel electrophoresis (PFGE) profiling may be required to better understand the genetic relatedness and molecular epidemiology of these isolates. The accumulation of a number of critically ill patients within a relatively small area and use of sophisticated invasive machinery such as ventilators and catheters increase the proportion of patients who are unusually susceptible to infection and who become reservoirs for their spread. Rapid spread of carbapenem-resistant enterobacterial species in a hospital in Mumbai has been reported[18].

The NDM-1 gene spreading in *Enterobacteriaceae* is an alarming risk because these novel multidrug-resistant bacteria could disseminate worldwide very quickly thus putting an end to our current pharmacopoeia. Early identification of cases of NDM-related infections and prevention of their spread by implementing screening, hygiene measures and isolation of the carriers are needed. Therefore, their detection along with routine antimicrobial susceptibility test (AST) should be performed. Phenotypic tests though specific, do not differentiate between chromosomal and plasmid encoded genes and hence genotypic characterization should be considered.

***Conflict of interest*:** None.

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