Indian J Med Res 140, October 2014, pp 546-550

Detection of New Delhi metallo beta lactamase-1 (NDM-1) carbapenemase in *Pseudomonas aeruginosa* in a single centre in southern India

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Received May 1, 2013

***Background & objectives*: New Delhi metallo β-lactamase-1 (NDM-1) producing *Pseudomonas aeruginosa* isolates are potential threat to human health. This study was conducted to detect the presence of bla_{NDM-1} in carbapenem resistant *P. aeruginosa* in a tertiary care center in southern India.**

***Methods*: Sixty one carbapenem resistant clinical isolates of a total of 212 *P. aeruginosa* isolates cultured during the study period were screened for the presence of NDM-1by PCR. Clinical characteristics of the NDM-1 positive isolates were studied and outcome of the patients was followed up.**

***Results*: Of the 61 isolates, NDM-1 was detected in four isolates only. These were isolated from patients in the intensive care units and chest medicine ward. The source specimens were pus, sputum, bronchoalveolar lavage and endotracheal aspirate. The NDM-1 producers were susceptible only to polymyxin B. Only one patient responded to polymyxin B therapy, while the others succumbed to the infection.**

***Conclusion*: These findings reveal that NDM-1 is not a major mechanism mediating carbapenem resistance in *P. aeruginosa* in this centre. However, continuous surveillance and screening are necessary to prevent their dissemination.**

Key words India - NDM-1 - *Pseudomonas aeruginosa*

New Delhi metallo beta lactamase-1 (NDM-1), the recently discovered transferable molecular class B β-lactamase is a growing threat worldwide.First reported in 2008 in Sweden from a patient previously hospitalized in India, NDM-1- producing *Enterobacteriaceae* are now the focus of attention globally[1-3]. NDM-1-producing strains exhibit multidrug resistant profile because they harbour other genes that encode for resistance to aminoglycosides and fluoroquinolones[4]. Till date, reports of NDM-1 in *Pseudomonas aeruginosa* are scarce. There are only two reports of its occurrence in patients from Serbia and none of them had a history of travel to the Indian subcontinent[5,6]. Though reports are scarce and sporadic, knowledge of its prevalence

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is essential because *P. aeruginosa* is an environmental pathogen with intense colonization capacity and ability to persist for indefinite periods in the hospital environment[7,8]. NDM-1 has not been reported in *P. aeruginosa* from any other part of the world. In the present study, we report the presence of NDM-1 in carbapenem resistant *P. aeruginosa* clinical isolates in a university teaching hospital in south India.

Material & Methods

The study was conducted in the department of Microbiology, Sri Ramachandra Medical College and Research Institute, a 1600 bedded university teaching hospital in Chennai, South India, from April to October 2010. During this period, a total of 212 clinical isolates of *P. aeruginosa* were collected, of which 61 were carbapenem resistant. All of these were from patients hospitalized for 48 h or more. These were cultured from clinical specimens such as blood (8), urine (21), exudative specimens (11) such as pus, wound swabs, body fluids including cerebrospinal fluid (CSF) and lower respiratory secretions (21) which included bronchoalveolar lavage (BAL), endotracheal aspirates (ETA) and sputum. The organisms were identified upto species level using Microscan Walk Away 96, Gram-negative panels (Siemens Healthcare Diagnostics Inc – Sacramento CA, USA). Commensals were differentiated from pathogens for isolates obtained from non-sterile sites (respiratory tract, urinary tract and wound swabs) by ascertaining their significance based on clinical history, presence of the organism in the Gram stain, presence of intracellular forms of the organism and pure growth in culture with significant colony count. The isolates from sputum and endotracheal aspirates were further verified for their clinical significance by correlation with other respiratory parameters and findings in the chest radiograph. The clinical characteristics of the study patients were noted from records and follow up was done till discharge /death.

The study protocol was approved by the institutional ethics committee.

Antimicrobial susceptibility testing: Susceptibility to various classes of antimicrobial agents was determined by disc diffusion method in accordance with Clinical Laboratory Standard Institute (CLSI) guidelines[9]. The antibiotics tested were amikacin (30 µg), ciprofloxacin (5 µg), ceftazidime (30 µg), aztreonam (30 µg), piperacillin-tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), colistin (10 µg) and polymyxin B (300 units) (Hi-media Laboratories, Mumbai, India).

Determination of minimum inhibitory concentration (MIC): Minimum inhibitory concentrations of imipenem and meropenem were determined by agar dilution method using cation adjusted Mueller Hinton Agar (MHA) (Hi-media Laboratories, India). Pure forms of imipenem and meropenem (Sigma Aldrich, India) were used.The scheme for preparing dilutions of antimicrobial agents and the methodology was according to CLSI guidelines[9].

Phenotypic tests: Carbapenemase production was screened by the Modified Hodge test (MHT) and MBL production by inhibitor potentiated disk diffusion test with ethylene diamine tetra acetic acid (EDTA)[10]. Though CLSI does not advocate the use of MHT for the detection of carbapenemases production in *P. aeruginosa*, several investigators have found this test using meropenem as a useful screening test[10,11]. For the isolates which were negative on initial testing by the above method, the test was repeated on Mueller-Hinton agar supplemented with zinc sulphate at a concentration of 70 mg/l[11].

Polymerase chain reaction PCR): All study isolates were subjected to PCR using primers targeting $bla_{\text{NDM-1}}$[1,12,13]. Co-existence of other MBL encoding genes namely bla_{VIM} and bla_{IMP} were looked for using consensus primer[14]. The primers used in the study are listed in Table I. For optimization of PCR, strains previously confirmed by PCR and gene sequencing were used as positive control and *P. aeruginosa* ATCC 27853 was used as negative control. All isolates were subjected to multiplex PCR for the detection of MBL genes: VIM, IMP, GIM, SIM and SPM[15].

DNA sequencing: PCR products of the isolates that carried the $bla_{\text{NDM-1}}$ gene were purified using the PCR DNA purification kit (QIA quick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and subjected to automated DNA sequencing (ABI 3100, Genetic Analyser, Applied Biosystems, USA). The aligned sequences were analyzed with the Bioedit sequence program and similarity searches for the nucleotide sequences were performed with the BLAST program (*http://www.ncbi.nlm.nih.gov*).

Results & Discussion

Of the 61 isolates tested, $bla_{\text{NDM-1}}$ was found in four isolates only (MS 5639, MS 5641, MS 6196 and R 1780). The PCR screening results were validated by sequencing and the sequence of the $bla_{\text{NDM-1}}$ genes showed 100 per cent identity with

Table I. Primers used in PCR and multiplex PCR for various genes in the study

Primer	Primer sequence (5'-3')	Product size (bp)
Bla $_{NDM-1}$-F	GGG CAG TCG CTT CCA ACG GT	475
Bla $_{NDM-1}$-R	GTA GTG CTC AGT GTC GGC AT	
Bla $_{VIM}$-F	TTTGGTCGCATATCGCAACG	500
Bla $_{VIM}$-R	CCATTCAGCCAGATCGGCAT	
Bla $_{IMP}$-F	GTTTATGTTCATACWTCG	432
Bla $_{IMP}$-R	GGTTTAAYAAAACAACCAC	
Primers used for multiplex PCR		
IMP family- F	GGA ATA GAG TGG CTT AAY	188
IMP family- R	TCT C CCA AAC YAC TAS GTT ATC T	
VIM family- F	GAT GGT GTT TGG TCG CAT A	390
VIM family- R	CGA ATG CGC AGC ACC AG	
GIM-1- F	TCG ACA CAC CTT GGT CTG AA	271
GIM-1- R	AAC TTC CAA CTT TGC CAT GC	
SPM-1-F	AAA ATC TGG GTA CGC AAA CG	477
SPM-1-R	ACA TTA TCC GCT GGA ACA GG	
SIM-1-F	TAC AAG GGA TTC GGC ATC G	570
SIM-1-R	TAA TGG CCT GTT CCC ATG TG	

Source: Refs 1, 12-15

previously reported genes (Figure). These four NDM-1 positive *P. aeruginosa* were isolated from patients in multidisciplinary intensive care unit (2), neurosurgical intensive care unit (1) and chest medical ward (1). Both the phenotypic tests employed were positive in the NDM-1 producers. The source specimens were BAL, ETA, sputum and pus from post-operative laparotomy wound site. The isolate from pus also carried the bla_{VIM}.

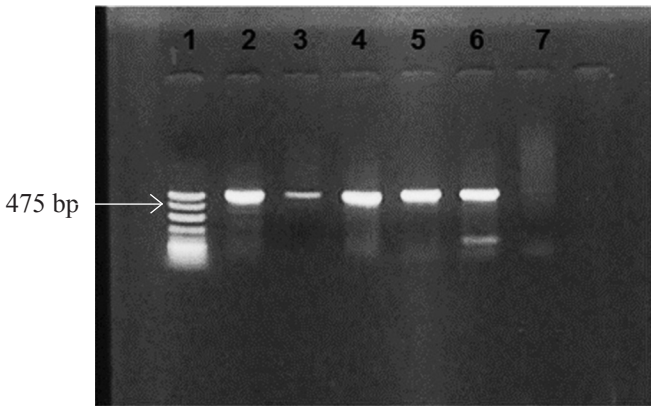

Fig. PCR for detection of bla_{NDM-1}. Lane 1: molecular mass marker (100bp DNA ladder); Lane 2: positive control; Lanes 3-6: bla_{NDM-1} positive isolates (amplicon size- 475bp); Lane 7: negative control.

bla_{NDM-1} was the only gene detected in the other three isolates. The NDM-1 producers were susceptible only to colistin and polymyxin B and resistant to amikacin, ciprofloxacin, aztreonam, piperacillin-tazobactam and ceftazidime. The clinical characteristics of the four patients are depicted in Table II.

Overall, among the study isolates, bla_{NDM-1} alone was detected in three, *bla* $_{NDM-1}$ coexisted with bla_{VIM} in one isolate, bla_{VIM} alone was detected in 33 isolates and two isolates carried the *bla* $_{IMP}$ gene alone. Further, bla_{SIM}, bla_{SPM} and bla_{GIM} were not detected in any of the study isolates. In 22 isolates none of the genes looked for, were detected.

NDM-1 was first identified in *Enterobacteriaceae* and recently reported in *Acinetobacter baumannii* also. Variants of NDM-1 namely NDM-2 to NDM-7 differing in single aminoacid sequences have also been described[7,14]. Identification of bla_{NDM-1} in many genera and species of Gram-negative bacteria indicate that this gene can spread at a high rate[2,17]. The genes encoding NDM are heterogeneous on the basis of molecular size and location. In *Enterobacteriaceae*, it is plasmid-borne, while in *A. baumannii* both chromosomal and plasmid locations have been confirmed[7,17]. At the Military Medical Academy in Serbia, routine analysis of carbapenemase producing bacterial isolates revealed NDM-1 in seven clinical isolates of *P. aeruginosa*. The source patients were hospitalized in Serbia and had no history of travel to any other country[5]. Subsequently, in 2012 recurrent pyelonephritis due to NDM-1 producing *P. aeruginosa* was reported from France. This patient had history of prior hospitalization in Serbia and hence it was hypothesised that the Balkan States may be endemic for NDM-1[6].

This study was conducted to detect for the presence of bla_{NDM-1} in carbapenem resistant *P. aeruginosa.* Considering that of the 61 carbapenem resistant *P. aeruginosa* isolates tested, only four were found to harbour NDM-1, it can be reasonably assumed that NDM-1 is not a major mechanism mediating carbapenem resistance in *P. aeruginosa* in this hospital. This being a single centre report, further studies are required at national or regional levels to understand the magnitude and prevalence of NDM-1 in *P. aeruginosa*. Additionally, co-existence with other carbapenemase encoding genes was evident in a single isolate. On receipt of the microbiology culture report, all patients were initiated on polymyxin B therapy.

Table II. Clinical characteristics of the patients infected with NDM-1 producing *P. aeruginosa*

Characteristics	Patient -1	Patient -2	Patient -3	Patient -4
Isolate Number	MS 5639	MS 5641	MS 6196	R 1780
Age (yr)/sex	47, male	63, Female	51, Female	58, Male
Hospital location	ICU	ICU	ICU	ward
Admitting unit	Neurosurgery	Neurology	General surgery	Chest medicine
Date of isolation	28/9/2010	28/9/2010	31/10/2010	30/10/2010
Underlying disease/diagnosis	Road traffic accident, ventilator associated pneumonia	Meningo encephalitis, ventilator associated pneumonia	Intestinal tuberculosis with obstruction and peritonitis, surgical site infection	Chronic obstructive pulmonary diseases, hospital acquired pneumonia
Co-morbid conditions	None	Diabetis mellitus, hypothyroidism	None	Corticosteroid therapy
Days in hospital	31 days	23 days	70 days	16 days
Surgical procedures, if any	Craniotomy, VP shunt	None	Laprotomy	None
Antimicrobials used prior to detection of NDM-1	Amikacin, metronidazole, pipercillin-tazobactam, imipenem, vancomycin	Ceftriaxone, cefoperazone -sulbactam, amikacin	Amikacin, metronidazole, pipercillin- tazobactam, imipenem, vancomycin, fluconazole,	Amoxicillin clavulanic acid
Indwelling devices	Central line, peripheral venous line, urinary catheter	Peripheral venous line, urinary catheter	Peripheral venous line, drain from surgical site, urinary catheter	Peripheral venous line
Mechanical ventilation	Intubated	Intubated	Intubated	Nil
Outcome	Expired	Expired	Expired	Improved
Source specimen	Endotracheal secretion	Broncho alveolar lavage	Pus from post-operative laparotomy wound site	Sputum
MIC-IMP (mg/l)	32	32	32	128
MIC-MEM (mg/l)	64	64	32	128
MHT	Positive	Positive	Positive	Positive
MBL screen test	Positive	Positive	Positive	Positive
Carbapenemase	NDM-1	NDM-1	NDM-1 & VIM	NDM-1

MIC, minimum inhibitory concentration; IMP, imipenem; MEM, meropenem

While one patient responded, the others succumbed to the infection. The lone survivor was followed up till discharge after recovery. Of the previously reported Serbian patients infected with NDM-1 *P. aeruginosa*, two expired and the one with pyelonephritis recovered on treatment with colistin[5,6].

None of the MBL encoding genes looked for were detected in 22 isolates. The possible operative mechanisms in these isolates may be hyperproduction of Amp C or other beta lactamases, porin defect and /or upregulation of efflux pumps[8]. In this study, however, the presence of these mechanisms was not evaluated.

The ability of NDM-1 producing *P. aeruginosa* to survive under a wide range of environmental conditions and potential to spread in hospital settings make them unique. Though not as prevalent as other MBLs such as IMP and VIM, strict vigilance and continuous surveillance of NDM-1 is essential considering the difficulties in therapeutic management and control. In the present study, though NDM-1 was detected in only four of 61 clinical isolates of *P.aeruginosa* tested, these were associated with a high degree of mortality. Therefore, their identification is crucial for appropriate and early initiation of treatment and also to implement infection prevention measures directed to curtail their dissemination.

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