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Microbiology Section

Emergence of NDM – 1 in the Clinical Isolates of *Pseudomonas aeruginosa* in India

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ABSTRACT

Objective: The present study was undertaken to detect the prevalence of the bla_{NDM-1} metallo beta lactamases (MBLs) in the isolates of Pseudomonas aeruginosa, which were recovered from various clinical samples from hospitalized patients in a tertiary care centre in Pune, India.

Methods: A total of 200 isolates of *P. aeruginosa* which were obtained from various clinical samples were subjected to antibiotic susceptibility testing by the disc-diffusion method and their MICs were determined by the Vitek – 2 Automated Antimicrobial Identification and Susceptibility Testing System against imipenem, meropenem, ticarcillin, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, tigecycline, trimethoprim/sulfamethoxazole, ampicillin/sulbactam, piperacillin/tazobactam, cefoperazone/sulbactam, cefepime, tetracycline, ceftazidime, ceftriaxone and colistin. Their MICs were also determined by the Etest method against imipenem, meropenem, piperacillin, tobramycin, ceftazidime, tigecycline and colistin. The presence of bla_{NDM-1} was detected by PCR and it was confirmed by sequencing the gene which was present in the isolates which exhibited carbapenem resistance. The experimental transferability of the plasmids which carried bla_{NDM-1} was determined by using *E. coli* J53 as the recipient.

Result: In the present study, four isolates of *P. aeruginosa*, which carried the bla_{NDM-1} gene, were resistant to imipenem and meropenem. These bla_{NDM-1} carrying isolates remained susceptible to colistin. The plasmid carrying bla_{NDM-1} was successfully transferred from the four isolates to *E. coli* J53 recipients.

Conclusions: We are reporting the emergence of the *P. aeruginosa* carrying NDM-1gene, which exhibited resistance to imipenem and meropenem, for the first time from India.

Key words: *P. aeruginosa*, Multidrug resistant, Carbapenems, bla_{NDM-1}

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the leading gram negative organisms which causes nosocomial infections which include blood stream infections, urinary tract infections and ventilator associated pneumonia. Carbapenem resistance has been observed frequently in *P. aeruginosa*. This resistance to the carbapenems is caused by the decreased outer membrane permeability via the loss of the OprD porin, the increase in the up-regulation of an active efflux pump system of the cytoplasmic membrane and the alteration of the penicillin binding proteins and the carbapenem hydrolyzing enzymes [1]. The acquired metallo-beta lactamases (MBL) have recently emerged as one of the resistance mechanisms in *P. aeruginosa*, owing to their capacity to hydrolyze all the beta-lactams which include the carbapenems, with the exception of the monobactams in vitro [2]. The increasing frequency of the multi-drug-resistant *Pseudomonas aeruginosa* (MDRPA) strains is of concern, as efficacious antimicrobial options are severely limited. The risk factors for the MDRPA infection include a prolonged hospitalisation, exposure to the antimicrobial therapy, and immunocompromised states [3].

P. aeruginosa is found in the hospital environment and it tends to remain viable on both animate and inanimate objects around the patients. The emergence of a MBL-positive isolate in a hospital setting poses an increased therapeutic problem. It is recognized as a serious threat for the hospital infection control, especially when the isolates are multidrug resistant. The fact that these genes are carried on highly mobile genetic elements, allows their easy dissemination to other bacteria, leaving little choice for the effective antimicrobial agents.

The presence of bla_{NDM-1} in *P. aeruginosa* has recently been reported from Serbia [4]. We undertook this study to determine the prevalence of bla_{NDM-1} in *P. aeruginosa* in the patients who were admitted to our tertiary care hospital and to observe the clinical outcome in these patients after their treatment.

MATERIALS AND METHODS

Bacterial Isolates

From August 2011 to July 2012, this study was conducted on a total of two hundred consecutive non duplicate isolates of *P. aeruginosa* which were isolated from different clinical specimens such as urine, pus, blood and body fluids from the patients who were admitted to a 1000 bedded tertiary care hospital in Pune, India. All the specimens were collected by using strict aseptic precautions and they were immediately processed without any delay. The bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques [5]. Further identification was done by using the VITEK-2 System (bioMérieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing

The antibiotic sensitivity test was performed by the standard Kirby Bauer disc diffusion technique, using commercially available discs (Hi Media, Mumbai, India) on Mueller Hinton agar plates. The antibiotics which were tested were as follows (potency in µg/disc): Piperacillin (100µg), Ticarcillin (75µg), Piperacillin-Tazobactam (100/10µg), Ticarcillin-Clavulanic acid (75/10µg), Ceftazidime (30µg), Cefepime (30µg), Aztreonam (30µg), Imipenem (10µg), Meropenem (10µg), Colistin (10µg), Polymyxin B(300 units), Gentamicin (10µg), Tobramycin (10µg), Amikacin (30µg), Netilmicin (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Lomefloxacin (10µg) and Ofloxacin (5µg). The diameter of the zone of inhibition for each antibiotic was measured and it was interpreted as resistant, intermediate susceptible or susceptible, as per the guidelines of the Clinical Laboratory Standards Institute (CLSI) [6]. The *E. coli* ATCC 25922 and the *P. aeruginosa* ATCC 27853 strains were used for the quality control.

The minimum inhibitory concentrations (MICs) of the antibiotics were determined using VITEK-2 (bioMérieux, Marcy l'Etoile, France) and the results are given in [Table/Fig- 1].

Screening for the Carbapenemase Production

By disc diffusion, all the *P. aeruginosa* isolates with reduced susceptibilities to meropenem and imipenem (the diameters of the zones of inhibition were ≤ 15mm) were screened for the production of carbapenemase. The phenotypic detection of the carbapenemase production was performed by the modified Hodge test by using a meropenem disc (10 µg) as per the CLSI guidelines [6]. The screening for the metallo-beta-lactamase production was performed by the combined – disc test, by using two imipenem discs (10 µg), one containing 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO), which were placed 25 mm apart on a Mueller Hinton agar plate. An increase in the zone diameter of >4 mm around the imipenem-EDTA disc as compared to that around the imipenem disc alone was considered as positive for the metallo-β-lactamase production. The MBL production of the isolates was detected by the MBL (IP/IPI) E-test method (AB Biodisk, Solna, Sweden) as per the manufacturer's instructions.

Molecular Detection of The MBL Genes

DNA was extracted by using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per the manufacturer's instructions. A multiplex PCR assay was performed to detect five families with the acquired MBL genes (bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{GIM}, bla_{SIM}) in a single reaction. Multiplex PCR for the bla_{OXA-23} and the bla_{OXA-24} genes and simple PCR for bla_{KPC} were carried out on the isolates by using the Gene Amp 9700 PCR System (Applied Biosystems, Singapore). The primers and the cycling conditions for the PCR were as has been described earlier [7-9]. PCR for the detection of bla_{NDM-1} was carried out by using primers, as has been reported previously [10]. Briefly, the program consisted of an initial denaturation step at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30s, primer annealing at 49°C for 30s, and extension at 72°C for 30s; followed by a final extension for 10 min at 72°C, which resulted in a 813 bp product. The amplicons were purified by using the QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany) and they were sequenced by using the ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing results were analyzed by using the Blast software which is there on the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), which showed the presence of the bla_{NDM-1} gene. The plasmid DNA was extracted by using the QIAGEN plasmid Maxi kit as per the manufacturer's instructions and the bla_{NDM-1} gene was detected in the plasmid by PCR analysis, as has been mentioned above.

Transfer of the resistance determinants and the plasmid analysis

Transfer of the resistance genes by conjugation was assayed by doing mating experiments in Luria–Bertani broth by using the *P. aeruginosa* isolates as the donors and an azide-resistant *E. coli* J53 strain as the recipient strain. The transconjugants were selected on Luria–Bertani agar, with the selection being based on the growth on the agar, in the presence of ceftazidime (30 mg/L) and sodium azide (100 mg/L). The plasmid DNA was extracted from the *E. coli* J53 recipient strain by using the QIAGEN plasmid Maxi kit (QIAGEN GmbH, Hilden, Germany) as per the manufacturer's instructions and the bla_{NDM-1} gene was detected by PCR, as has been mentioned above. The phenotypic drug resistance assay which was done on the *E. coli* J53 transconjugants revealed resistance to the same carbapenems as the donor strain.

Molecular typing of the strains

Repetitive element based PCR (REP – PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC – PCR) assays were performed as have been described, to rapidly characterize the bla_{NDM-1} positive *P. aeruginosa* strains which were recovered from the patients [11].

RESULTS

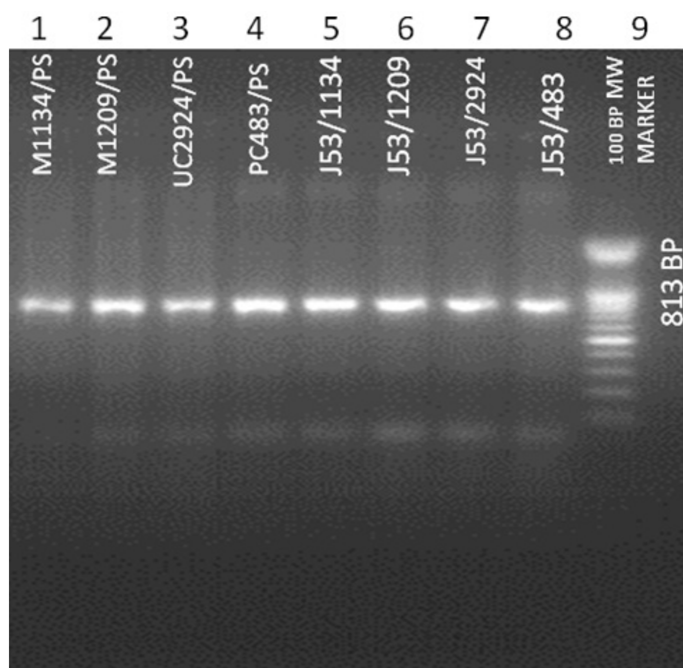
Out of the 200 clinical isolates of *P. aeruginosa*, 40 were found to be carbapenem resistant. The screening for MBL production was carried out on these 40 isolates, and 20 of them found to be positive. Among these 20 isolates, sixteen were found to be positive for bla_{VIM} and four were found to be positive for bla_{NDM-1}. The PCR results were validated by sequencing and the sequence of the bla_{NDM-1} gene showed a 100% identity with the previously reported genes [Table/Fig- 1]. The MIC values of these four isolates have been shown in [Table/Fig- 2].

Isolate 1 and Isolate 2 were from central venous catheter tip cultures. Isolate 1 was recovered from a 66 year old male patient with a necrotizing soft tissue infection (NSTI) in the left lower limb, with septicaemia. Isolate 2 was recovered from a 63 year old male patient who had developed septicaemia after undergoing cholecystectomy. Both the patients were successfully treated with colistin.

Isolate 3 was recovered from the urine sample of a 56-year old male who had pyelonephritis with pyuria. He had been treated with meropenem during his previous hospital admissions. He was successfully treated with colistin and amikacin.

Isolate 4 was recovered from the pus sample of a male patient, who was a case of a road traffic accident with a compound comminuted fracture of the tibia (left), and had developed a surgical site infection.

NDM-1 was also detected by PCR amongst the transconjugant *E. coli* J 53 strains, which indicated that the bla_{NDM-1} gene was located on a conjugative plasmid. Gel electrophoresis of the plasmid DNA



[Table/Fig- 1]: 813 bp bla_{NDM-1} gene in *P. aeruginosa* isolates and their transconjugants *E. coli* J 53.Lanes: 1, *P. aeruginosa* M1134; 2, *P. aeruginosa* M1209; 3, *P. aeruginosa* UC2924; 4, *P. aeruginosa* PC483;5, *E. coli* J53 M1134; 6, *E. coli* J53 M1209; 7, *E. coli* J53 UC2924; 8, *E. coli* J53 PC483; 9, 100 bp Ladder

Antibiotics	PS M1134	PS M1209	PS UC2924	PS PC483
IPM	32	32	32	32
MEM	32	32	32	32
AMK	128	128	128	128
TIC	128	128	128	128
GEN	32	32	32	32
TOB	32	32	32	32
CIP	8	8	8	8
LVX	16	16	16	16
MXF	16	16	16	16
TGC	16	16	16	16
SXT	320	320	320	320
SAM	64	64	64	64
TZP	128	128	128	128
FEP	128	128	128	128
SFP	128	128	128	128
CRO	128	128	128	128
CAZ	128	128	128	128
TET	32	32	32	32
CST	<0.5	<0.5	<0.5	<0.5

[Table/Fig- 2]: Antibiotic susceptibilities of bla_{NDM-1}-positive *Pseudomonas aeruginosa* (mg/L)
IPM, imipenem; MEM, meropenem; TIC,ticarcillin ;AMK, amikacin; GEN, gentamicin ;TOB, tobramycin; CIP,ciprofloxacin; LVX,levofloxacin; MXF,moxifloxacin; TGC,tigecycline; SXT,trimethoprim/sulfamethoxazole; SAM, ampicillin/sulbactam; TZP,piperacillin/tazobactam; SFP cefoperazone/sulbactam; FEP, cefepime; TET,tetracycline; CAZ, ceftazidime; CRO,ceftriaxone; CST, colistin

which was extracted from the donor and the recipient strains demonstrated that the bla_{NDM-1} gene was present on a 50 kb transferable plasmid. The clonality of *P. aeruginosa* was confirmed by REP – PCR and ERIC – PCR and they confirmed that the isolate, 1, 2 and 4 were clonally similar and that isolate 3 was clonally different, as per the banding pattern.

DISCUSSION

With the widespread use of the carbapenems, the emergence of NDM-1 was first detected in a *Klebsiella pneumoniae* isolate in 2008, in a Swedish patient of Indian origin; it has since been reported in increasing numbers of infections in patients from all over world. Nagarajan et al., have reported a high prevalence of bla_{NDM-1} in carbapenem resistant *K. pneumoniae* (75% of the isolates) and *E. coli* (66%), in a study which was done in the southern part of India [12]. In a similar study which was done at a tertiary care hospital in the northeastern region of India, all the isolates of *K. pneumoniae* which were carbapenem resistant were found to harbour the bla_{NDM-1} gene [13]. We have also reported the first instance of the detection of the bla_{NDM-1} gene in a clinical isolate of *Raoultella ornitholytica* [14]. Previous studies have revealed that the genes which encoded NDM-1 were mostly located on plasmids and these studies had focused on the *Enterobacteriaceae* [15]. In the present study, we confirmed that the bla_{NDM-1} genes of each of the four NDM-positive *P. aeruginosa* isolates were located on plasmids which were 50 kb in size. As all the four isolates of *P. aeruginosa* which carried bla_{NDM-1} were obtained from hospital acquired infection cases, the ability of bla_{NDM-1} to be transferred from *P. aeruginosa* to other *Enterobacteriaceae* and vice versa assumed significance, since this could result in the spread of multidrug resistant pathogens which caused untreatable infections in the hospital environment at an unprecedented rate.

CONCLUSION

P. aeruginosa has been a pathogen which is known to be notorious for the antibiotic resistance in hospital acquired infections, it causes severe infections in cases of burns, respiratory tract infections in patients who are on ventilators, urinary tract infections in catheterised patients and soft tissue infections which include surgical site infections. The carbapenems are the most frequently used antibiotics for the treatment of these infections. Resistance to the carbapenems requires the use of drugs like Colistin, which are more toxic to the patients. The accurate identification and the reporting of the NDM-1 producing *P. aeruginosa* will aid in the infection control management, for preventing its spread, especially when the isolates are multidrug resistant. The NDM-1 gene has been reported in *Enterobacteriaceae*. This is the first report on the detection of MBL NDM-1 in the clinical isolates of *Pseudomonas aeruginosa* in India.

The nucleotide sequence data of the bla_{NDM-1} gene from the four isolates of *P. aeruginosa* which have been reported in the present study have been assigned the GenBank nucleotide numbers, JX680682, JX680683, JX680684 and JX680685, respectively.

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