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Carriage of *bla*$_{NDM-1}$ in *Pseudomonas aeruginosa* through multiple Inc type plasmids in a tertiary referral hospital of northeast India

Sir,

Pseudomonas aeruginosa is known to be a predominant opportunistic pathogen and also a frequent cause of nosocomial infection in patients with compromised immune system. Treatment option becomes complicated when this type of organism harbour resistance determinants such as New Delhi metallo-β-lactamase-1 (NDM-1). The genetic vehicles carrying this gene are often responsible for their horizontal spread, dissemination and maintenance within a broad host range[1]. Knowledge about transmission dynamics of *bla*$_{NDM-1}$ is a key to succeed in the effort of infection control and slowing down the spread of multidrug resistance. This study was undertaken to characterize *bla*$_{NDM-1}$ in clinical isolates of *P. aeruginosa*, their transmission dynamics and plasmid Inc types responsible for their horizontal transfer in a tertiary referral hospital of northeast India.

The samples for the present study were collected from the patients who were admitted or attended outpatient department of Silchar Medical College and Hospital, Silchar, Assam, India from October 2012 to September 2013. The protocol was approved by Institutional Research and Ethical Committee. During this period, a total of 290 consecutive non-duplicate clinical isolates of *P. aeruginosa* were collected, of which 88 isolates were found to be non-susceptible to carbapenem by minimum inhibitory concentration (MIC). Isolates with MIC above 4, 1, 8 µg/ml for imipenem, meropenem, and ertapenem, respectively were selected as per CLSI (Clinical Laboratory Standards Institute) guidelines[2] and were subjected to modified Hodge test for detection of carbapenemase production[3] and further confirmed for the presence of metallo-β-lactamase by imipenem-EDTA disc diffusion test[4]. PCR assay was performed to characterize the *bla*$_{NDM}$ gene as well as other metallo-β-lactamase genes *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{SIM}$ and *bla*$_{SMB}$[4-7], and amplified products were sequenced to confirm the presence of resistant genes. The linkage of *bla*$_{NDM-1}$ with insertion sequence IS*Aba125* was determined by using forward primer of IS*Aba125* (5′-GAAACTGTCGCACCTCAT GTTTG-3′) and reverse primer of *bla*$_{NDM-1}$ (5′-GTAGTGCTCAGTGTCGGCAT-3′)[8]. The class of integron carried out by *bla*$_{NDM-1}$ was determined by integrase gene PCR[9]. *bla*$_{NDM-1}$ positive bacterial isolates were cultured in Luria-Bertani (LB) broth (Hi-Media, Mumbai, India) containing 0.25 µg/ml of imipenem. After overnight incubation, plasmids were extracted by QIAprep Spin Miniprep Kit (Qiagen, Germany). Plasmids of *bla*$_{NDM-1}$ were subjected to transformation by heat shock method[10] using *Escherichia coli* JM107 as recipient. Transformants were selected on LB agar with 0.25 µg/ml of imipenem, which were then confirmed both by phenotypic as well as by PCR analysis. The plasmids were classified by PCR based replicon typing, carried out for determining the incompatibility group type of the plasmid in all *bla*$_{NDM-1}$ harbouring strains. A total of 18 different replicon types such as FIA, FIB, FIC, HI1, HI2, I1/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA were targeted as described previously[11]. The antibiotic susceptibility was done by Kirby-Bauer disc-diffusion method[10] against antibiotics *viz*. piperacillin-tazobactam (100/10 µg), co-trimoxazole (25 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), polymixin B (300 units), netilmicin (30 µg), carbenicillin (100 µg) and faropenem (5 µg) (Hi-Media, Mumbai, India). MIC was performed by agar dilution method against imipenem (MSD, India), ertapenem (MSD, India), meropenem (Lupin, India) and the results were interpreted as per CLSI guidelines[2]. The clonal relatedness among the *bla*$_{NDM-1}$ producing *P. aeruginosa* isolates was determined by repetitive extragenic palindromic (REP) PCR[12].

Of the 88 consecutive non-repetitive carbapenems non-susceptible (showing MIC of imipenem >4 µg/ml, meropenem >1 µg/ml and ertapenem >8µg/ml) *Pseudomonas* isolates, 16 were found carrying *bla*$_{NDM-1}$ as confirmed by sequencing. The different clinical features of these 16 *bla*$_{NDM-1}$ harbouring *P. aeruginosa* are given in Table I. In contrast, no other metallo-β-lactamase genes (*bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{SIM}$ and *bla*$_{GIM}$) were detected in any of the isolates. These *bla*$_{NDM-1}$ positive isolates showed resistance towards most of the antibiotics including piperacillin/tazobactam, co-trimoxazole, faropenem, aminoglycoside and quinolone group of drugs. Seven isolates were resistant to polymixin B as well. Minimum inhibitory concentration results showed high MIC breakpoint (Table II) against all the tested antibiotics cephalosporins, monobactam and carbapenems. Integrase PCR showed that all the *bla*$_{NDM-1}$ positive isolates were harbouring class 1 integron while in four isolates both class 1 and class 2 integrons were observed (Table I). In 11 isolates, IS*Aba125* was found in the upstream region of *bla*$_{NDM-1}$. Plasmid analysis showed that *bla*$_{NDM-1}$ gene was located within the plasmid of approximately 25-40 Kb in size and in transformation assay, NDM-1 gene was found to be horizontally transferable and the resistance determinant was carried within diverse Inc group *viz*. FIA, FIC and K types. However, in six transformants, the plasmid was untypable (Table I). REP PCR results revealed that these MBL producing isolates were heterogenous (Figure) and no particular clonal strain was responsible for any epidemic spread. No association could be established between REP PCR patterns and plasmid incompatibility types.

After the first detection of NDM in *Klebsiella pneumoniae* it was also reported in *Escherichia coli*, *Citrobacter fruendii*, *Enterobacter*[13] and in *Acinetobacter* spp[14]. Due to the rapid horizontal transmission this gene was also reported in *P. aeruginosa* from different parts of the world[15,16]. In this study, we described the occurrence of *bla*$_{NDM-1}$ gene among a number of MDR *P.aeruginosa* isolates indicating the spread of this resistant gene in the northeastern part of India. Toleman *et al*[8] have described the association of insertion sequence IS*Aba125* in the upstream region of *bla*$_{NDM-1}$ harbouring *A. baumarnii* and it is also established that whole IS*Aba125* or a truncated portion of it is excised along with the resistant gene when it is horizontally transmitted among the members of *Enterobacteriaceae* family. In our study also, similar kind of association of IS*Aba125* with *bla*$_{NDM-1}$ in the upstream region was observed, which may be due to the horizontal transmission of this gene along with IS*Aba125* at interspecies level. Thus, this mobile genetic element may act as a unit of interspecies transmission in our setting. This horizontal transmission may also

Table I. Features of New Delhi metallo-β-lactamase (NDM-1) producing *Pseudomonas aeruginosa* and patients' characteristics

Patient sex & age (yr)	Clinical specimen	Hospitalization unit	ISAba125	Integron type	Plasmid Inc group
F-26	Ear swab	ENT	-	Class 1	Untypable
F-55	Pus	Surgery	-	Class 1 & 2	FIA
M-40	Pus	Orthopedics	+	Class 1	Untypable
F-40	Urine	Gynaecology	+	Class 1 & 2	FIA
F-20	Pus	FBU	+	Class 1	FIC
F-44	Pus	Surgery	-	Class 1	Untypable
F-40	Pus	Gynaecology	+	Class 1	FIB
F-12 days	Nasal secretion	Paediatrics	-	Class 1	K/B
M-40	Sputum	Medicine	+	Class 1	Untypable
F-19	Urine	Paediatrics	-	Class 1	K/B
F-20	Pus	Surgery	+	Class 1	Untypable
M-9	Urine	Paediatrics	+	Class 1	FreB
M-13	Urine	Medicine	+	Class 1 & 2	FIA & FIB
M-12	Urine	Surgery	+	Class 1	Untypable
M-53	Pus	Surgery	+	Class 1	K/B
F-18	Pus	Surgery	+	Class 1 & 2	FIA

ENT, Ear, nose and throat ward; FBU, female burn unit

Table II. Minimum inhibitory concentration (MIC) and resistance pattern of NDM-1 producing *Pseudomonas aeruginosa* isolates

Serial no.	MIC (µg/ml)					Co-resistances
	IMP	ERT	MER	CEF	AZT	
1	16	16	16	16	32	CXT, TGC, FAR
2	>256	>256	64	>256	>256	PIT, CXT, AMK, GEM, CIP, PB, NET, CB, FAR
3	64	>256	64	128	128	PIT, CXT, AMK, GEM, CIP, TGC, NET, CB, FAR
4	64	128	32	>256	>256	PIT, CXT, AMK, GEM, CIP, PB, NET, CB, FAR
5	>256	>256	64	>256	>256	PIT, CXT, AMK, GEM, CIP, PB, TGC, NET, CB, FAR
6	>256	>256	>256	128	>256	PIT, CXT, AMK, GEM, CIP, NET, CB, FAR
7	>256	>256	256	>256	>256	CXT, AMK, GEM, CIP, TGC, NET, CB, FAR
8	>256	>256	256	>256	>256	PIT, CXT, AMK, GEM, CIP, PB, NET, CB, FAR
9	32	32	16	32	64	CXT, TGC, FAR
10	16	32	16	32	32	CXT, PB, CB, FAR
11	128	256	64	128	128	PIT, CXT, AMK, GEM, CIP, NET, CB, FAR
12	32	64	32	>256	>256	CXT, CIP, PB, CB, FAR
13	>256	>256	>256	>256	256	PIT, CXT, AMK, GEM, CIP, PB, TGC, NET, CB, FAR
14	32	64	32	>256	>256	CXT, CB, FAR
15	64	64	32	>256	>256	CXT, CB, FAR
16	>256	>256	128	>256	>256	PIT, CXT, AMK, GEM, CIP, TGC, CB, FAR

CXT, co-trimoxazole; TGC, tigecycline; FAR, faropenem; PIT, piperacillin-tazobactam; GEM, gentamicin; CIP, ciprofloxacin; AMK, amikacin; CB, carbenicillin; NET, neticillin; PB, polymyxin B, IMP, imipenem ; ERT, ertapenem; MER, meropenem; CEF, cefepime; AZT, aztreonam

be facilitated due to the association with gene capture mechanism as it is known to be an important mean of spreading resistance in clinical isolates of Gram-negative bacilli[17]. An earlier study revealed that class 1 type of integron was mostly associated with clinical pathogens[9], as also supported by our study where all the isolates were found carrying class 1 integron. It is observed that different incompatibility types of plasmid act as a genetic vehicle for transmission of this resistance gene, which reflects acquisition of bla_{NDM-1} harbouring plasmid from different sources. But in our study, in case of some transformants, we

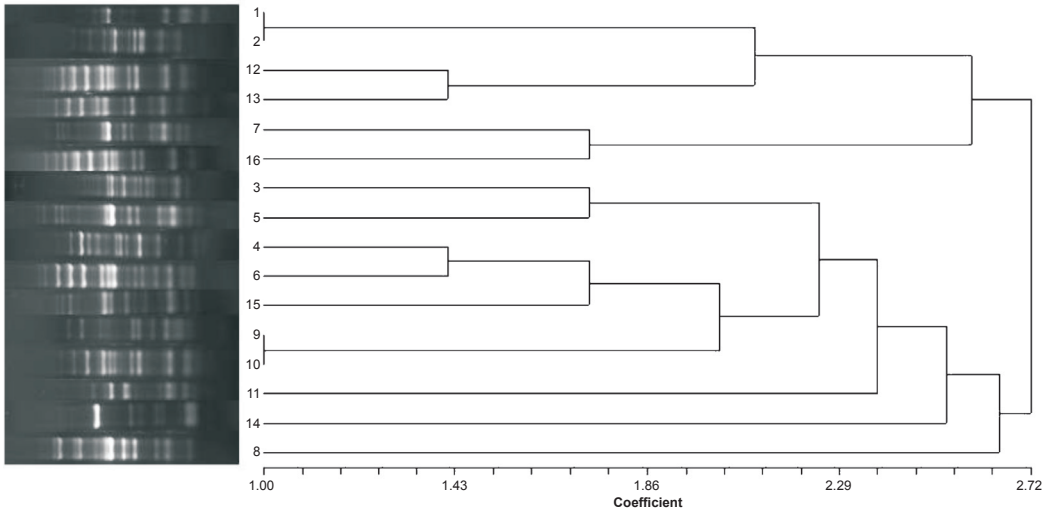

Figure. Dendogram showing the clonal relatedness of 16 New Delhi metallo-β-lactamase producing *Pseudomonas aeruginosa* isolates based on REP-PCR band patterns.

were unable to determine the incompatibility group of that plasmids. This may be due to the presence of any new incompatible type of plasmid that could not be detected by our target primers. Presence of new Inc type plasmids encoding *bla*$_{NDM-1}$ corresponds their transplasmid expansion and diverse source of carriage. In an earlier study, it was reported that NDM-1 producing isolates were resistant to nearly all classes of antimicrobial agents except polymyxins and tigecycline[13], but in our study the *bla*$_{NDM-1}$ harbouring isolates showed resistance to all the antibiotics tested including polymixin B and tigecycline.

In conclusion, carriage of *bla*$_{NDM-1}$ in different Inc type plasmids within a single hospital setting and their expansion may be a serious matter of concern in combating the carbapenem resistance.

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