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Pseudomonas aeruginosa Clinical Isolates in Nepal Coproducing Metallo-β-Lactamases and 16S rRNA Methyltransferases

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ABSTRACT A total of 11 multidrug-resistant *Pseudomonas aeruginosa* clinical isolates were obtained in Nepal. Four of these isolates harbored genes encoding one or more carbapenemases (DIM-1, NDM-1, and/or VIM-2), and five harbored genes encoding a 16S rRNA methyltransferase (RmtB4 or RmtF2). A novel RmtF variant, RmtF2, had a substitution (K65E) compared with the same gene in RmtF. To our knowledge, this is the first report describing carbapenemase- and 16S rRNA methyltransferase-coproducing *P. aeruginosa* clinical isolates in Nepal.

KEYWORDS 16S rRNA methylase, *Pseudomonas aeruginosa*, carbapenemase, multidrug resistance

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Metallo-β-lactamases (MBLs) confer resistance to all β-lactams, except the monobactams, and are characterized by their efficient hydrolysis of carbapenems (1). The metallo-β-lactamase DIM-1 was first identified in a *Pseudomonas stutzeri* strain obtained from a Dutch patient in 2007 (2). DIM-1 hydrolyzes broad-spectrum cephalosporins and carbapenems but not monobactams. Since then, DIM-1 producers, including *P. stutzeri* and *Enterobacteriaceae* spp., have been isolated in India (3) and Sierra Leone (4), respectively.

Acquired 16S rRNA methyltransferase genes responsible for an extremely high level of resistance against various aminoglycosides are widely distributed among *Enterobacteriaceae* and glucose-nonfermentative bacteria (5). To date, 10 different 16S rRNA methyltransferases, including ArmA, RmtA, RmtB, RmtC, RmtD, RmtE, RmtF, RmtG, RmtH, and NpmA, have been found in clinical isolates (6–9). One of these, RmtB, was found to have three variants, RmtB2 (accession no. JN968578), RmtB3 (accession no. JN968579), and RmtB4 (accession no. KM999534). The 16S rRNA methyltransferase RmtF was first identified in a clinical isolate of *Klebsiella pneumoniae* on the island of Réunion in 2011 (7). Since then, RmtF-producing *Enterobacteriaceae* have been isolated in India, the United Kingdom, the United States, and Nepal (7, 10, 11).

Between 2012 and 2013, 11 multidrug-resistant *Pseudomonas aeruginosa* clinical isolates were obtained from 11 inpatients treated at a university hospital in Nepal. Multidrug-resistant *Pseudomonas aeruginosa* isolates are defined as strains showing resistance to carbapenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 32 μg/ml), and fluoroquinolone (MIC ≥ 4 μg/ml), as previously described (12). Of these isolates, 7 were from sputum, 3 from urine samples, and 1 from a pus sample. The MICs of various antibiotics were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (13). The entire genomes of these isolates

were sequenced by MiSeq (Illumina, San Diego, CA). Their genomes were searched for drug resistance genes, including genes encoding β-lactamases (carbapenemases and extended-spectrum β-lactamases), 16S rRNA methyltransferases, and aminoglycoside-acetyl/adenyltransferases, using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/). Point mutations associated with quinolone resistance were searched in *gyrA* and *parC*. Multilocus sequence type (MLST) was deduced, as described by the protocols of the PubMLST (http://pubmlst.org/paeruginosa/) databases. The complete genome of *P. aeruginosa* IOMTU133 was determined using PacBio RS II (Menlo Park), as described previously (14). The genomic environments surrounding genes encoding carbapenemases and/or 16S rRNA methyltransferases were confirmed by Sanger sequencing. DNA plugs of all isolates tested (digested with I-CeuI or S1 nuclease) were prepared and separated by pulsed-field gel electrophoresis, and Southern hybridization was performed using probes of 16S rRNA, bla_{DIM-1}, bla_{NDM-1}, bla_{VIM-2}, *rmtB4*, and *rmtF2* (15, 16).

All 11 isolates were resistant to meropenem, aztreonam, amikacin, and ciprofloxacin (Table 1), with MICs ≥16 μg/ml. Three isolates showed higher MICs to imipenem or meropenem, ≥64 μg/ml, than the other isolates. Five of the 11 isolates were extremely highly resistant to amikacin and arbekacin, with MICs >1,024 μg/ml, and to ciprofloxacin, with MICs of 32 to 256 μg/ml. All isolates were susceptible to colistin, with MICs ≤0.5 μg/ml.

Of the 11 isolates, three had a novel *rmtF* variant, designated *rmtF2* (accession no. LC050387). Analysis of its predicted amino acid sequence revealed a substitution (K65E) compared with the sequence of RmtF. Four isolates had genes encoding one or more metallo-β-lactamases, i.e., bla_{DIM-1}, bla_{NDM-1}, and/or bla_{VIM-2}; and four had genes encoding other β-lactamases, i.e., bla_{PDCs}, bla_{PSE-2}, bla_{TEM-1}, or bla_{VEB-1a} (Table 1). In addition, 5 isolates had a 16S rRNA methyltransferase encoding gene, *rmtB4* or *rmtF2*; and nine had genes encoding an aminoglycoside acetyl- and adenylyl-transferase, including AAC(6′)-Ib, AACA7, AACC5b, and AADB (Table 1). A novel *rmtF2* gene was located in the class 1 integron (Fig. 1). All isolates except for IOMTU3 had amino acid substitution point mutations S83I in GyrA and S80L in ParC; IOMTU3 had amino acid substitution point mutations S83L and D87E in GyrA and S80L in ParC.

A total of 6 isolates were classified as ST664, two as ST235, and one each as ST244, ST654, and ST1047. The two ST235 isolates harbored bla_{NDM-1}, bla_{VIM-2}, and *rmtB4*; two of the ST664 isolates harbored *rmtF2*; the ST654 isolate harbored bla_{VIM-2}; and the ST1047 isolate harbored bla_{DIM-1} and *rmtF2*.

Because IOMTU133 belonged to ST1047 and harbored several drug resistance genes, its complete genome was sequenced and deposited in GenBank under accession no. AP017302. This isolate had no plasmids. The complete genome sequence of IOMTU133 had 283-fold coverage for one chromosome, IOMTU133, which consisted of a single circular chromosome of 6,897,018 bp with an average GC content of 65.98%. The chromosome was found to contain 6,245 protein-encoding genes, including 63 tRNA genes and one transfer messenger RNA (tmRNA) gene for all amino acids. IOMTU133 also harbored a carbapenemase-encoding gene, bla_{DIM-1}; a 16S rRNA methyltransferase encoding gene, *rmtF2*; and an aminoglycoside acetyltransferase encoding gene, *aac(6′)-Ib*. The bla_{DIM-1} gene and a novel *rmtF2* gene were located within the same integron on the chromosome (Fig. 1).

The genomic environments surrounding bla_{DIM-1}, bla_{NDM-1}, bla_{VIM-2}, *rmtB4*, and *rmtF2* are shown in Fig. 1. The genomic environments of bla_{DIM-1}, bla_{NDM-1}, and bla_{VIM-2} were *IS6-intI1-*bla_{DIM-1}*-dfr2e-aac(6′)-Ib-rmtF2-insE-cat-orf1* (gene encoding a hypothetical protein)-*IS6* (accession no. AP017302), *tnp-*bla_{NDM-1}*-orf2* (gene encoding a hypothetical protein) (accession no. LC054839), and *intI1-aadB-aacA7-*bla_{VIM-2}*-dhfrB5-aacA5-tniR-tniQ-tniB-tniA* (accession no. LC054840), respectively. The genomic environments surrounding these carbapenemase-encoding genes were unique to these isolates.

The genomic environment of *rmtB4* was *tnp-groEL-orf3* (gene encoding queuine tRNA-ribosyltransferase)-*orf4* (gene encoding a hypothetical protein)-*rmtB4-orf5* (gene encoding a putative Na^+/H^+ antiporter) (accession no. LC052325), whereas the

TABLE 1 Summary of characteristics of the 11 *Pseudomonas aeruginosa* strains, including antimicrobial resistance profiles and resistant genes

Strain	MLST	MICs (μg/ml) for[a]:								β-Lactamase(s)	16S rRNA methylase	Aminoglycoside acetyl/adenylyltransferase(s)	Mutation(s) in DNA gyrase	
		IPM	MEM	ATM	CAZ	AMK	ABK	CIP	CST				GyrA	ParC
IOMTU 3	654	128	32	16	128	128	1	256	≤0.5	VIM-2, PDC-58		AADB	S83L, D87E	S80L
IOMTU 7	244	16	32	>1024	>1024	32	16	32	≤0.5	VEB-la, PDC-61		AAC(6′)-Ib	S83I	S80L
IOMTU 9	235	512	>1024	32	>1024	>1024	>1024	64	≤0.5	NDM-1, VIM-2, PDC-35	RmtB4	AACA7, AACC5b	S83I	S80L
IOMTU 133	1047	32	64	16	256	>1024	>1024	32	≤0.5	DIM-1, PDC-32	RmtF2	AAC(6′)-Ib	S83I	S80L
IOMTU 143	664	1	4	16	8	128	1	32	≤0.5	PDC-98		AAC(6′)-Ib	S83I	S80L
IOMTU 155	664	1	4	32	8	128	1	32	≤0.5	PDC-98		AAC(6′)-Ib	S83I	S80L
IOMTU 161	664	1	4	16	8	128	1	32	≤0.5	PCD-98		AAC(6′)-Ib	S83I	S80L
IOMTU 179	664	1	4	32	4	128	1	32	≤0.5	TEM-1, PDC-98		AAC(6′)-Ib	S83I	S80L
IOMTU 184	664	8	32	128	>1024	>1024	>1024	64	≤0.5	PSE-2, PDC-98	RmtF2	AAC(6′)-Ib	S83I	S80L
IOMTU 304	235	512	>1024	32	>1024	>1024	>1024	64	≤0.5	NDM-1, VIM-2, PDC-35	RmtB4	AACA7, AACC5b	S83I	S80L
IOMTU 487	664	2	32	128	512	>1024	>1024	32	≤0.5	PSE-2, PDC-98	RmtF2	AADB	S83I	S80L

[a]IPM, imipenem; MEM, meropenem; ATM, aztreonam; CAZ, ceftazidime; AMK, amikacin; ABK, arbekacin; CIP, ciprofloxacin; CST, colistin.

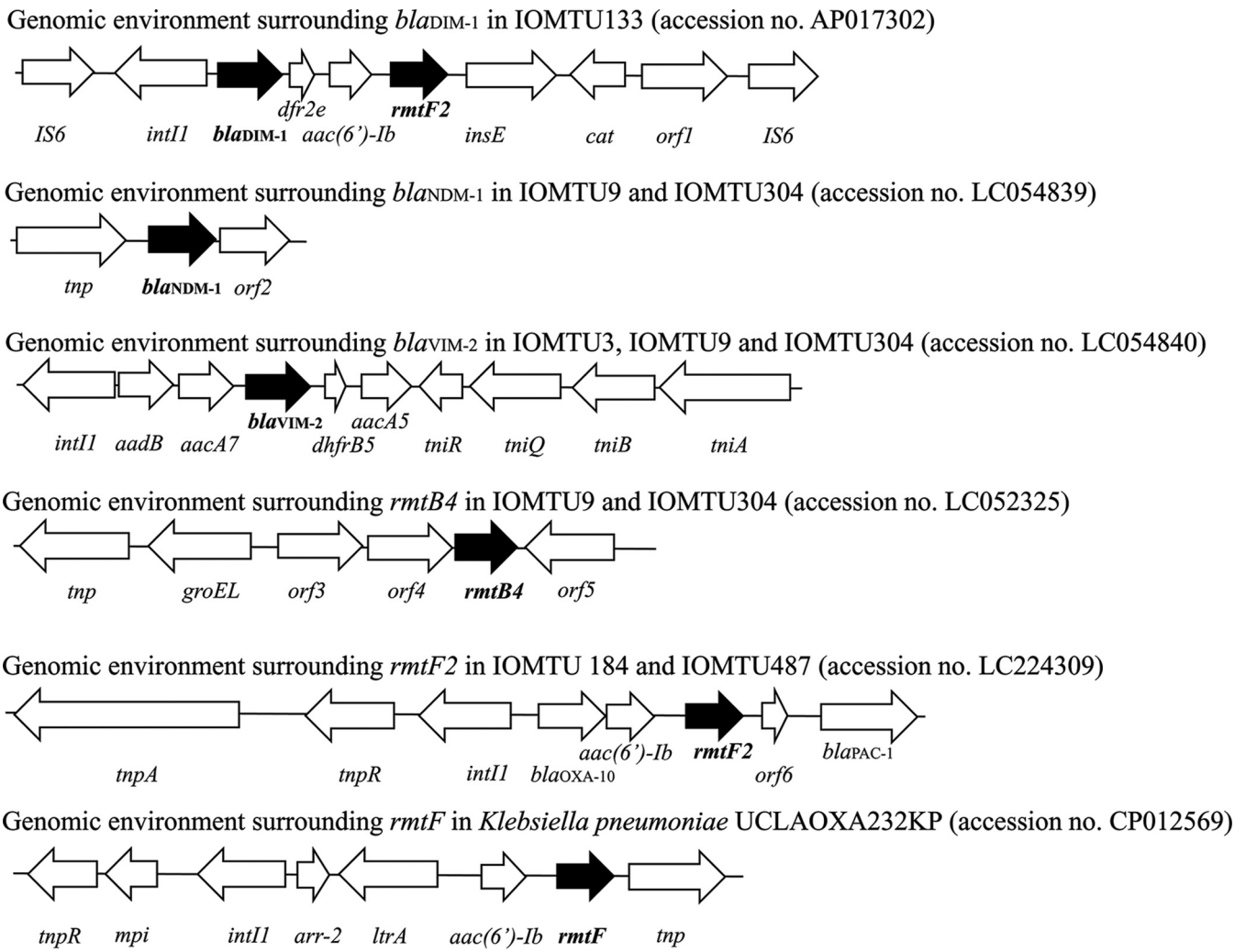

FIG 1 Genomic environment surrounding bla_{DIM-1} in IOMTU133 (accession no. AP017302); bla_{NDM-1} in IOMTU9 and IOMTU304 (accession no. LC054839); bla_{VIM-2} in IOMTU3, IOMTU9 and IOMTU304 (accession no. LC054840); *rmtB4* in IOMTU9 and IOMTU304 (accession no. LC052325); *rmtF2* in IOMTU184 and IOMTU487 (accession no. LC224309); and *rmtF* in *Klebsiella pneumoniae* UCLAOXA232KP (accession no. CP012569).

genomic environment of *rmtF2* was *tnpA-tnpR-intI1-*bla_{OXA-10}*-aac(6')-Ib-rmtF2-orf6* (gene encoding a hypothetical protein)-bla_{PAC-1} (accession no. LC224309). The *rmtF2* in IOMTU133 was located in the same integron as bla_{DIM-1} (Fig. 1). Compared to the genomic environment surrounding *rmtF* in *K. pneumoniae* UCLAOXA232KP plasmid pUCLAOXA232-3.X (accession no. CP012569), both *rmtF* and *rmtF2* were located in class I integron, which contained *aac(6')-Ib* in the upstream regions of *rmtF* and *rmtF2*; however, the other allelic profiles in each integron were different (Fig. 1). The genomic environments surrounding *rmtB4* and *rmtF2* were unique to these isolates.

Of all the isolates tested, only IOMTU487 had a 120-kbp plasmid, but the plasmid did not harbor the bla_{DIM-1}, bla_{NDM-1}, bla_{VIM-2}, *rmtB4*, or *rmtF2* genes (see Fig. S1 in the supplemental material). All of these genes were located in the chromosomes (see Fig. S2 in the supplemental material).

The findings of this study indicate that ST664 *P. aeruginosa* clinical isolates spread in a medical setting in Nepal, because the majority of *P. aeruginosa* isolates obtained in Nepal were classified as ST664. To date, seven ST664 isolates (PubMLST no. 3401, 3707, 4018, 4033, 4052, 4060, and 4787) have been registered on the PubMLST website (https://pubmlst.org/paeruginosa/). Of these, PubMLST no. 4787 (*Pseudomonas aeruginosa* VRFPA06) was isolated from human blood in 2012 in India (17), although the details of others were not reported. Of our 11 isolates, only two were classified as ST235,

which has been recognized as one of three high-risk clones, i.e., ST235, ST111, and ST175 (18). A *P. aeruginosa* strain belonging to ST1047, which was originally obtained in Norway and found to produce VIM-type MBLs, was first registered on the PubMLST website in 2011 (PubMLST no. 746).

This is the first report describing carbapenemase- and 16S rRNA methyltransferase-coproducing *P. aeruginosa* clinical isolates in Nepal. Carbapenemase- and 16S rRNA methyltransferase-coproducing *P. aeruginosa* was reported in 2007 in Brazil (19) and in 2015 in northeast India (20), which is bordered by Nepal. It is therefore necessary to survey multidrug-resistant *P. aeruginosa* in medical settings in Nepal.

Accession number(s). The sequences described were submitted to GenBank under the accession numbers LC050387, LC052325, LC054839, LC054840, LC224309, and AP017302.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00694-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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