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Molecular characterisation of carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates in Nepal

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ABSTRACT

Objectives: The emergence of carbapenem-resistant *Pseudomonas aeruginosa* has become a serious worldwide medical problem. The aim of this study was to determine the genetic and epidemiological properties of carbapenem-resistant *P. aeruginosa* strains isolated from hospitals in Nepal.

Methods: A total of 43 carbapenem-resistant *P. aeruginosa* isolates obtained from patients in two hospitals in Nepal between 2018 and 2020 were analysed. Their whole genomes were sequenced by next-generation sequencing. A phylogenetic tree was constructed from single nucleotide polymorphism (SNP) concatemers. Multilocus sequence typing (MLST) was performed and antimicrobial resistance genes were identified.

Results: Of the 43 isolates, 17 harboured genes encoding carbapenemases, including IMP-1, IMP-26, KPC-2, NDM-1, VIM-2 and VIM-5, and 12 harboured genes encoding 16S rRNA methylases, including RmtB4 and RmtF2. The carbapenem-resistant *P. aeruginosa* isolated in Nepal belonged to various sequence types (STs), including ST235 (5 isolates), ST244 (7 isolates), ST274 (1 isolate), ST357 (10 isolates), ST654 (3 isolates), ST664 (1 isolate), ST773 (1 isolate), ST823 (3 isolates), ST1047 (8 isolates), ST1203 (2 isolates) and ST3453 (2 isolates).

Conclusion: To the best of our knowledge, this is the first molecular epidemiological analysis of carbapenem-resistant *P. aeruginosa* clinical isolates from Nepal. The findings strongly suggest that *P. aeruginosa* isolates producing carbapenemases and 16S rRNA methylases have spread throughout medical settings in Nepal.

1. Introduction

The emergence of carbapenem-resistant *Pseudomonas aeruginosa* has become a serious worldwide medical problem [1]. Most carbapenem-resistant *P. aeruginosa* isolates encode carbapenem and aminoglycoside resistance factors, including metallo-β-lactamases (MBLs), aminoglycoside-modifying enzymes and 16S rRNA methylases [2,3]. Moreover, *P. aeruginosa* isolates producing GES-type carbapenemases have recently become a serious concern in medical settings in developed countries, including in Europe, Russia and Japan [2,4].

MBLs confer resistance to all β-lactams except for monobactams and are characterised by their efficient hydrolysis of carbapenems [5]. *Pseudomonas aeruginosa* isolates producing IMP- or VIM-type MBLs have been detected in various Asian countries [5]. For example, isolates producing IMP-type MBLs have been observed in China, Japan, Korea, Malaysia, Singapore, Thailand and Vietnam, and isolates producing VIM-type MBLs have been identified in China, India, Indonesia, Korea, Malaysia, Myanmar, Saudi Arabia and Taiwan [6,7]. In addition, isolates producing an NDM-type MBL have been reported in India [8].

Acquired 16S rRNA methylase genes responsible for extremely high levels of resistance to various aminoglycosides are widely distributed among Gram-negative bacteria, including *P. aeruginosa, Acinetobacter* spp. and Enterobacteriaceae [9]. To date, ten types of 16S rRNA methylases, including ArmA, RmtA, RmtB, RmtC, RmtD, RmtE, RmtF, RmtG, RmtH and NpmA, have been found in clini-

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Table 1
Minimum inhibitory concentrations (MICs) and percentage resistance (%R) of carbapenem-resistant *Pseudomonas aeruginosa* isolates ($n = 43$).

Antimicrobial agent	Resistance breakpoint (μg/mL)	%R	MIC (μg/mL) Range	MIC_{50}	MIC_{90}
Imipenem	≥8	97.7	1 to >512	32	512
Meropenem	≥8	100	8 to >512	32	>512
Ceftazidime	≥32	86.0	16 to >512	512	>512
Cefepime	≥32	95.3	32 to >512	512	>512
Amikacin	≥64	72.1	32 to >512	64	>512
Ciprofloxacin	≥4	97.7	≤0.25 to >512	32	256
Colistin	≥4	18.6	≤0.0625 to 16	0.5	8
Aztreonam	≥32	79.1	8 to >512	128	>512

$MIC_{50/90}$, MIC required to inhibit 50% and 90% of the isolates, respectively.

cal isolates [9]. Of these, RmtB was found to have three variants, namely RmtB2 (GenBank accession no. **JN968578**), RmtB3 (GenBank accession no. **JN968579**) and RmtB4 (GenBank accession no. **KM999534**), and RmtF was found to have a variant, RmtF2 [10]. Our previous study reported a novel RmtF variant, RmtF2, characterised by an amino acid substitution (Lys65Glu), from a medical setting in Nepal [10]. To our knowledge, this is the first molecular epidemiological analysis of carbapenem-resistant clinical isolates of *P. aeruginosa* from Nepal.

2. Materials and methods

2.1. Bacterial isolates

A total of 43 carbapenem-resistant *P. aeruginosa* isolates were obtained between October 2018 and January 2020 from patients treated at two hospitals in Nepal, including 39 isolates from Hospital A and 4 from Hospital B. Carbapenem-resistant *P. aeruginosa* isolates were defined as strains resistant to carbapenems, including imipenem or meropenem, with minimum inhibitory concentrations (MICs) of ≥16 μg/mL. Bacteria were identified by biochemical methods and 16S rRNA sequencing [11]. Of the 43 isolates, 22 were obtained from the respiratory tract, 10 from urine, 5 from pus, 5 from swabs and 1 from body fluid. MICs were determined by the broth microdilution method [12].

2.2. Whole-genome sequencing

Whole genomic DNA of the 43 isolates was extracted using a DNeasy® Blood & Tissue Kit (QIAGEN, Tokyo, Japan) and was sequenced by MiSeq (Illumina Inc., San Diego, CA, USA). More than 25-fold coverage was achieved for each isolate. Raw reads of each isolate were assembled using CLC Genomic Workbench v.11.0.1, and antimicrobial resistance genes were identified using ResFinder 3.0 (https://cge.cbs.dtu.dk//services/ResFinder/). Multilocus sequence typing (MLST) was performed as described according to PubMLST protocols (http://pubmlst.org/paeruginosa/).

2.3. Phylogenetic analysis based on single nucleotide polymorphisms (SNPs)

In addition to the 43 strains, 11 strains of *P. aeruginosa* isolated from 2012–2013 in Nepal [10] were included in the phylogenetic analysis, with *P. aeruginosa* PAO1 (GenBank accession no. **AE004091**) used as the reference strain. A phylogenetic tree was constructed from the SNP concatemers using kSNP3.0 [13].

2.4. GenBank accession numbers

Raw reads of all 43 isolates were deposited at GenBank as accession numbers **DRA010736** (experiment, DRX233311 to DRX233337; run, DRR243511 to DRR243537; and BioSample SAMD00244736 to SAMD00244762) and **DRA011203** (experiment, DRX248448 to DRX248463; run, DRR258733 to DRR258748; and BioSample, SAMD00260828 to SAMD00260843). Raw read data of the 11 *P. aeruginosa* isolates obtained between 2012 and 2013 in our previous study were deposited in GenBank as accession no. **DRA011590** (experiment, DRX265126 to DRX265136; run, DRR275542 to DRR275552; and BioSample, SAMD00282185 to SAMD00282195). The BioProject of this study was deposited as **PRJDB10493**. The genomic environment surrounding genes encoding carbapenemases and 16S rRNA methylases from the assembled contigs were deposited in GenBank as accession numbers **LC633538, LC635088, LC635757, LC635758, LC635759, LC636065, LC636066, LC636067** and **LC636409**.

3. Results

3.1. Antimicrobial susceptibility and drug resistance factors

According to Clinical and Laboratory Standards Institute (CLSI) guidelines, all 43 carbapenem-resistant isolates were resistant to meropenem (Table 1). In addition, 42 isolates (97.7%) were resistant to imipenem and ciprofloxacin, 41 (95.3%) were resistant to cefepime, 37 (86.0%) were resistant to ceftazidime, 34 (79.1%) were resistant to aztreonam, 31 (72.1%) were resistant to amikacin and 8 (18.6%) were resistant to colistin.

Of the 43 isolates, 17 (39.5%) harboured genes encoding carbapenemases, including IMP-1, IMP-26, KPC-2, NDM-1, VIM-2 and VIM-5, and 12 (27.9%) harboured genes encoding 16S rRNA methylases, including RmtB4 and RmtF2 (Table 2).

3.2. Multilocus sequence typing (MLST) and phylogenetic analysis

Of the 43 carbapenem-resistant *P. aeruginosa* isolates, 10 (23.3%) belonged to ST357, 8 (18.6%) to ST1047, 7 (16.3%) to ST244, 5 (11.6%) to ST235, 3 (7.0%) to ST654, 3 (7.0%) to ST823, 2 (4.7%) to ST1203, 2 (4.7%) to ST3453, and 1 (2.3%) each to ST274, ST664 and ST773. The phylogenetic tree revealed two clades, designated Clades A and B (Fig. 1). Clade A consisted of isolates belonging to ST244, ST274, ST549 (*P. aeruginosa* PAO1 reference strain), ST654 and ST664, and Clade B consisted of isolates belonging to ST235, ST357, ST773, ST823 and ST1047. Most strains isolated from 2012 to 2013 belonged to ST664 in Clade A, whereas most isolates from 2018 to 2020 belonged to ST357 and ST1047 in Clade B.

3.3. Genetic environment surrounding carbapenemase- and 16S rRNA methylase-encoding genes

The genetic environment surrounding genes encoding carbapenemases and 16S rRNA methylases was derived from each

Table 2
Characterisation of carbapenem-resistant *Pseudomonas aeruginosa* isolates (n = 43).

MLST	No. (%) of isolates	No. of strains harbouring the gene/total no. of strains for:				QRDR (no. of strains with the amino acid substitution/total no. of strains) for:	
		Carbapenemase-encoding gene(s)	16S rRNA methylases	Aminoglycoside resistance gene(s) [a]	Quinolone resistance gene(s)	GyrA	ParC
ST235	5 (11.6%)	bla_{KPC-2} (4/5), bla_{IMP-26} (1/5)	–	*aac(6′)-Ib*-cr, *aac(3)-Ic* (4/5), *aadA6* (1/5)	*crpP* (2/5)	S83I, D87N (1/5)	S87L (3/5), S80L (2/5)
ST244	7 (16.3%)	bla_{NDM-1} (2/7), bla_{IMP-1} (1/7)	–	*aac(6′)-Ib*-cr, *aadA10* (5/7)	*crpP, qnrVC1* (6/7)	S83I (7/7)	S87L (5/7), S80L (2/7)
ST274	1 (2.3%)	–	–	–	–	–	–
ST357	10 (23.3%)	bla_{VIM-5} (2/10)	–	*aac(6′)-Il, aadA1* (8/10)	*crpP* (10/10)	S83I (10/10)	S87L (1/10), S80L (9/10)
ST654	3 (7.0%)	bla_{VIM-2} (3/3)	–	–	–	S83I (3/3)	S80L (3/3)
ST664	1 (2.3%)	–	*rmtF2* (1/1)	*aac(6′)-Ib*-cr (1/1)	*crpP* (1/1)	S83I (1/1)	S80L (1/1)
ST773	1 (2.3%)	bla_{NDM-1} (1/1)	*rmtB4* (1/1)	*aadA10* (1/1)	*qnrVC1* (1/1)	S83I (1/1)	S80L (1/1)
ST823	3 (7.0%)	bla_{VIM-2} (3/3)	–	*aac(6′)-Il, aac(3)-Id* (3/3)	*crpP* (3/3)	S83I (3/3)	S80L (3/3)
ST1047	8 (18.6%)	–	*rmtF2* (8/8)	*aac(6′)-Ib*-cr (8/8)	*crpP* (8/8)	S83I (8/8)	S87L (6/8), S80L (2/8)
ST1203	2 (4.7%)	–	*rmtF2* (2/2)	*aac(6′)-Ib*-cr (2/2)	*crpP* (2/2)	S83I (2/2)	S80L (2/2)
ST3453	2 (4.7%)	–	–	*aac(6′)-Ib*-cr (2/2)	*crpP* (2/2)	S83I (2/2)	S80L (2/2)

MLST, multilocus sequence typing; ST, sequence type; QRDR, quinolone-resistance determining region.
[a] Aminoglycoside resistance genes, including those encoding aminoglycoside acetyltransferases and aminoglycoside adenylyltransferases.

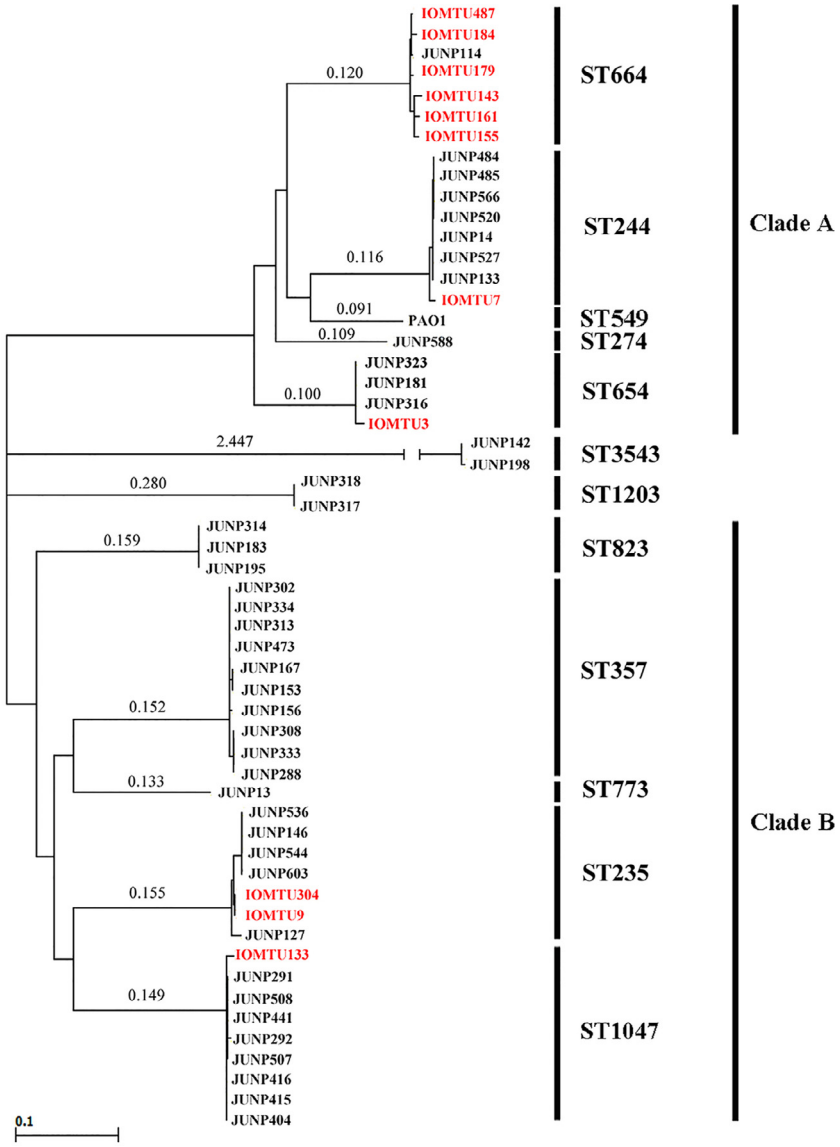

Fig. 1. Molecular phylogeny of 54 *Pseudomonas aeruginosa* strains, including 43 isolates obtained from 2018–2020 and 11 isolates obtained from 2012–2013. A maximum-likelihood phylogenetic tree was constructed from the genomes of the 54 carbapenem-resistant isolates. The IOMTU strains from 2012–2013 are indicated in red. The phylogenetic tree revealed two clades, designated A and B. Clade A consisted of isolates belonging to ST244, ST274, ST549 (*P. aeruginosa* PAO1 reference strain), ST654 and ST664. Clade B consisted of the isolates belonging to ST235, ST357, ST773, ST823 and ST1047. Isolates belonging to ST1203 and ST3543 were strains that branched out of clades A and B. ST, sequence type.

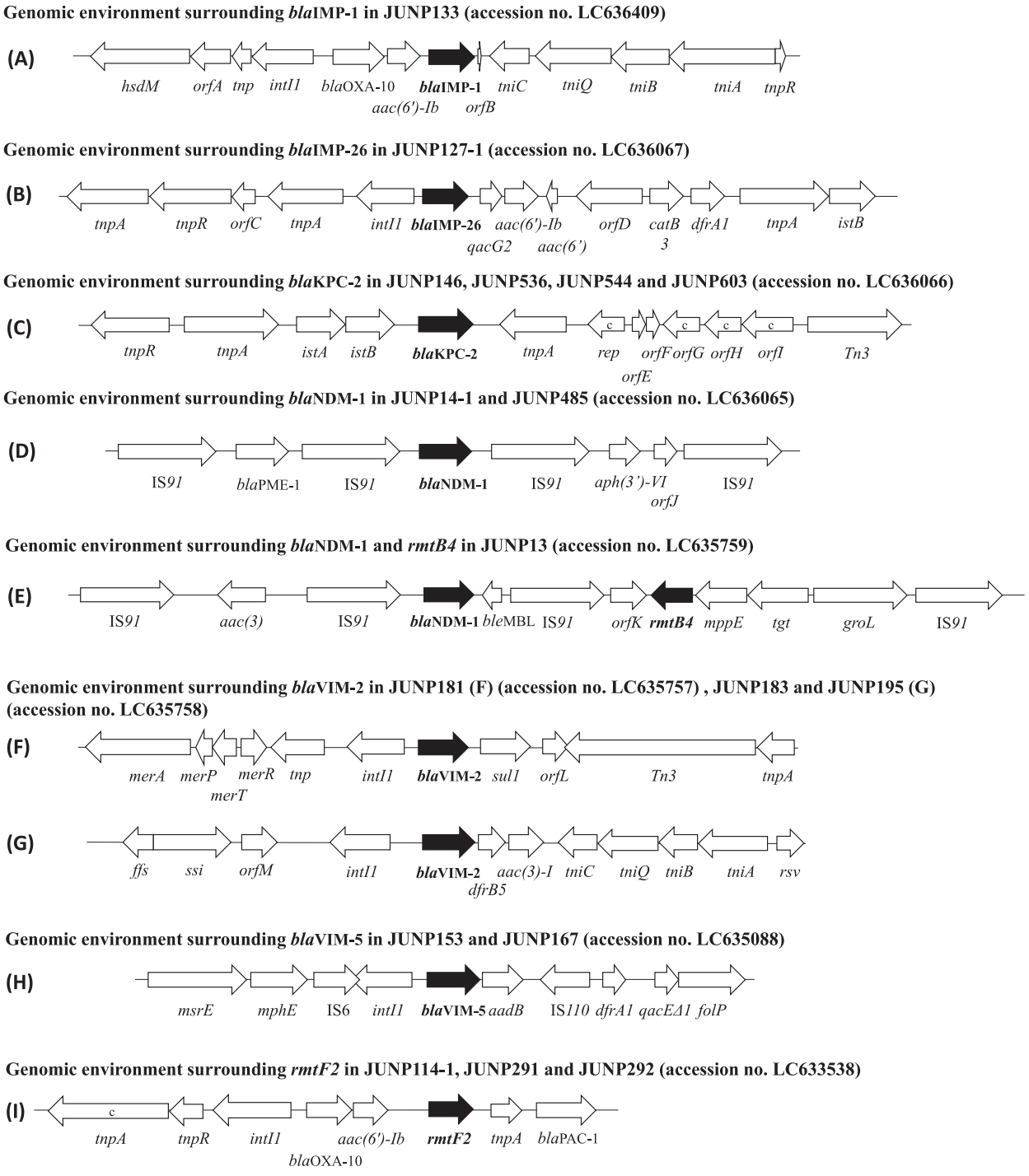

Fig. 2. Structures of the variable regions surrounding carbapenemase-encoding genes, including (A) bla_{IMP-1}, (B) bla_{IMP-26}, (C) bla_{KPC-2}, (D,E) bla_{NDM-1}, (F,G) bla_{VIM-2} and (H) bla_{VIM-5}, and 16S rRNA methylase genes, including (E) *rmtB4* and (I) *rmtF2*. *orfA*, type I restriction–modification system subunit M gene; *orfB, orfD, orfG* to *orfJ, orfL* and *orfM*, hypothetical protein-encoding genes; *orfC, orfE* and *orfF*, recombinase family protein genes; and *orfK*, putative Na^+/H^+ antiporter gene.

contig after assembling the raw data, and comparative genomics revealed eight types of genetic structures surrounding carbapenemase-encoding genes, including bla_{IMP-1} (Fig. 2A), bla_{IMP-26} (Fig. 2B), bla_{KPC-2} (Fig. 2C), bla_{NDM-1} (Fig. 2D,E), bla_{VIM-2} (Fig. 2F,G) and bla_{VIM-5} (Fig. 2H), and two types of genetic structures surrounding 16S rRNA methylase-encoding genes, including, *rmtB4* (Fig. 2E) and *rmtF2* (Figure 2I).

The bla_{IMP} genes, including bla_{IMP-1} and bla_{IMP-26}, were located in class 1 integrons (Fig. 2A,B). Part of the allelic profile surrounding bla_{IMP-1}, consisting of *intI1*–bla_{OXA-10}–*aac(6′)-Ib*–bla_{IMP-1} (Fig. 2A), was identical to that in the chromosome of *P. aeruginosa* strain 97 identified in 2015 in Ghana (GenBank accession no. **CP031449**). The genetic structure surrounding bla_{IMP-26}, consisting of *intI1*–bla_{IMP-26}–*qacG2*–*aac(6′)-Ib* (Fig. 2B), had 100% sequence identity to that in the chromosome of *P. aeruginosa* NCGM 2991 identified in 2015 in Vietnam (GenBank accession no. **LC075717**). The genetic structure surrounding bla_{KPC-2}, consisting of *tnpR*–*tnpA*–*istA*–*istB*–bla_{KPC-2}–*tnpA*–*rep* (Fig. 2C), had 99.9% sequence identity to that of a plasmid of *P. aeruginosa* 164130 identified in 2016 in France (GenBank accession no. **MN336501**).

Two types of genetic environments were found surrounding bla_{NDM-1}. One structure, consisting of IS*91*–bla_{PME-1}–IS*91*–bla_{NDM-1}–IS*91*–*aph(3′)-VI*–*orfJ*–IS*91* (Fig. 2D), was a unique gene cassette array, with the structure IS*91*–bla_{NDM-1}–IS*91* having 97.6% sequence identity to that in the chromosome of *P. aeruginosa* N15-01092 isolated in Canada in 2015 (GenBank accession no. **CP012901**). The other structure (Fig. 2E) had 99.3% sequence identity to that in the chromosome of *P. aeruginosa* PSE6684 isolated in South Korea in 2019 (accession no. **CP053917**) and to *P. aeruginosa* ST773 isolated in the USA in 2017 (accession no. **CP041945**). The genetic environment surrounding bla_{VIM-2} consisted of two types of genetic structures. One structure consisted of a unique integron, *intI1*–bla_{VIM-2}–*sul1* (Fig. 2F), with the bla_{VIM-2} downstream region, including *sul1*–*orfL*–Tn*3*–*tnpA*, identical to the chromosomal sequences in *P. aeruginosa* strain PA7 isolated in Argentina (accession no. **CP000744**) (100% sequence identity) and similar to *P. aeruginosa* OS-210 isolated in Sweden in 2007 (accession no. **FN397628**) (99.9% sequence identity). The second also included a class 1 integron, consisting of *intI1*–bla_{VIM-2}–*dfrB5*–*aac(3)-I*–*tniC*–*tniQ*–*tniB*–*tniA* (Fig. 2G), with 100% sequence identity to that of *P. aeruginosa* strain PA83 isolated in Germany in 2013 (accession no. **CP017293**). The genetic environment surrounding bla_{VIM-5}, consisting of IS*6*–*intI1*–bla_{VIM-5}–*aadB*–IS*110* (Fig. 2H), had 99.9% sequence identity to that in a chromosome of *P. aeruginosa* PA99 isolated in Thailand in 2016 (GenBank accession no. **CP042967**).

The genetic environment surrounding *rmtB4* (Fig. 2E) had 99.3% sequence identity to that of *P. aeruginosa* PSE6684 isolated in South Korea in 2019 (accession no. **CP053917**) and *P. aeruginosa* ST773 isolated in the USA in 2017 (accession no. **CP041945**). The genetic environment surrounding *rmtF2*, consisting of *tnpA*–*tnpR*–*intI1*–bla_{OXA-10}–*aac(6′)-Ib*–*rmtF2*–*tnpA*–bla_{PAC-1} (Fig. 2I), had 100% sequence identity to that in *P. aeruginosa* 174313 isolated in France in 2017 (accession no. **MK534438**) and IOMTU487 isolated in Nepal in 2017 (accession no. **LC224309**) [10].

4. Discussion

The findings of this study indicate that *P. aeruginosa* strains highly resistant to carbapenems and/or aminoglycosides are spreading in medical settings in Nepal. Of the carbapenem-resistant *P. aeruginosa* isolates from Nepal, 39.5% possessed genes encoding carbapenemases, including IMP-1, IMP-26, KPC-2, NDM-1, VIM-2 and VIM-5, and 27.9% possessed genes encoding 16S rRNA methylases, including RmtB4 and RmtF2. These genes were likely transmitted from *P. aeruginosa* isolates from other Asian and European countries by tourists (https://www.ceicdata.com/en/indicator/nepal/visitor-arrivals) and contributed to the high resistance of *P. aeruginosa* isolates in Nepal to carbapenems and aminoglycosides.

To the best of our knowledge, this is the first report describing the molecular epidemiology of carbapenem-resistant *P. aeruginosa* clinical isolates in Nepal. Our previous study of 11 *P. aeruginosa* isolates obtained from a medical setting in Nepal in 2012 and 2013 found that these isolates produced carbapenemases (DIM-1, NDM-1 and/or VIM-2) and 16S rRNA methyltransferases (RmtB4 or RmtF2) [10]. Of these, clinical isolates of *P. aeruginosa* ST664 appear to be spreading in Nepal. Whole-genome sequencing showed that the genetic backgrounds of the isolates obtained between 2018 and 2020 differed from those obtained between 2012 and 2013. The recent isolates were found to belong to specific STs, such as ST274, ST357, ST773, ST823, ST1203 and ST3543, whereas the number belonging to ST664 had significantly decreased over time.

High-risk isolates of *P. aeruginosa*, including ST235, ST244, ST357 and ST654, have increased in medical settings in Nepal from 2012 to 2020. Of the 25 isolates of high-risk clones, 10 belonged to ST357. *Pseudomonas aeruginosa* ST357 was first reported in Singapore in 2008 [14]. Since then, ST357 isolates producing IMP-type MBLs, including IMP-1, -6, -7, -10, -11 and -41, have been detected in 2009 in Japan, in 2010 in the Czech Republic, in 2011 in Poland, and in 2015 in Denmark and Japan [2,15]. In contrast, ST357 isolates producing VIM-type MBLs, including VIM-2 and VIM-5, have been detected in 2013 in the Czech Republic and in 2015 in India, Korea and the UK [2]. Moreover, *P. aeruginosa* ST357 producing IMP- and VIM-type MBLs were reported in 2017 to have spread in medical settings in the Czech Republic [16].

Our results strongly suggest that various types of internationally spreading high-risk clones of *P. aeruginosa*, including ST235, ST244, ST357 and ST654, have spread throughout medical settings in Nepal. These findings emphasise the necessity of surveying multidrug-resistant *P. aeruginosa* isolates in medical settings in Nepal.

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Competing interests

None declared.

Ethical approval

This study was approved by the Ethical Committee of the Institute of Medicine, Tribhuvan University [approval no. 219 (6-11-E)^2/075/076] and the Biosafety Committee, Juntendo University [approval no. BSL2/29-1].

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