Results

Antimicrobial susceptibility and aminoglycoside-resistant genes

The MICs at which 50% and 90% of the 101 *A. baumannii* and 15 *P. aeruginosa* isolates were inhibited (MIC50 and MIC90, respectively) were determined (Table 1). Seventy of the 101 *A. baumannii* isolates (71.3%) had MICs >1,024 mg/L to all aminoglycosides tested, including amikacin, arbekacin and gentamicin. All 70 isolates had 16S rRNA methylases, with 61 having *armA* and the remaining 9 having *rmtB* (Figure 1). The remaining 31 isolates had MICs ≤128 mg/L to amikacin, ≤32 mg/L to arbekacin and ≤128 mg/L to gentamicin and no methylase genes. Whole genome sequencing of 2 methylase-negative isolates showing relative resistance to aminoglycosides revealed that one had *aac(6')-IIb* and *aadB* and that the other had *aac(6')-IIb* and *aadA2*.

Of the 15 *P. aeruginosa* isolates, 2 had MICs >1,024 mg/L to amikacin, arbekacin and gentamicin, and harbored the 16S rRNA methylase *rmtB* (Figure 2). The 13 methylase-negative isolates had MICs <2 - 256 mg/L to amikacin (MIC_{50} 64 mg/L and MIC_{90} 128 mg/L), 1–32 mg/L to arbekacin (MIC_{50} 2 mg/L and MIC_{90} 4 mg/L), and 1–32 mg/L to <0.5 - 512 mg/L to gentamicin (MIC_{50} 256 mg/L and MIC_{90} 512 mg/L). The remaining 13 did not have any methylase genes (Figure 2).

OXAs and CTX-Ms encoding genes in 16S rRNA methylase-producing isolates

Of the 61 *A. baumannii* isolates harboring *armA*, 1 had *bla*OXA-23-like, *bla*OXA-51-like and *bla*CTX-Ms genes, 51 had *bla*OXA-23-like and *bla*OXA-51-like genes, and 8 had *bla*OXA-51-like genes. All 9 *A. baumannii* isolates harboring *rmtB* had *bla*OXA-23-like and *bla*OXA-51-like genes. In contrast, the 2 *P. aeruginosa* isolates harboring16S rRNA methylase genes had neither the *bla*OXAs nor the *bla*CTX-Ms gene.

PFGE analysis and MLST

PFGE analysis of the 101 *A. baumannii* isolates revealed 8 clusters (Figure 1). Isolates from Clusters I, III, IV, V, VI, VII, and VIII were obtained from either one or the other hospital, whereas isolates from Clusters II and III were obtained from both. These results indicate that *A. baumannii* isolates had expanded in a clonal manner in both hospitals and that some isolates may spread among hospitals in Vietnam.

The16S rRNA methylase-encoding the *rmtB* gene was detected in Cluster I *A. baumannii* isolates, whereas *armA* was present in isolates from Clusters I, III, IV, V, VI, VII, and VIII. Isolates harboring *rmtB* were obtained from one hospital and isolates harboring *armA* were from both hospitals.

The *A. baumannii* isolates producing 16S rRNA methylase belonged to ST254, ST231, ST195, ST136, ST91 and 8 new STs, ST490, ST491, ST492, ST493, ST494, ST495, ST496 and ST497 (Figure 1). Most of the *A. baumannii* isolates producing 16S rRNA methylase from hospital A in Hanoi were ST91 and ST231, whereas most from hospital B in Ho Chi Minh City were ST136, ST195 and ST254.

The two isolates harboring *rmtB* showed different patterns on PFGE, belonging to ST217 and ST313.

Genetic environment surrounding *rmtB*

The *rmtB* gene was associated with an ISCR3 mobile element upstream and a Tn3 transposon structure

Table 1 MIC50 and MIC90 values and percent antimicrobial resistance for *A. baumannii* and *P. aeruginosa* clinical isolates

	A. baumannii (n = 101)					*P. aeruginosa* (n = 15)				
Antimicrobial agent	Breakpoint forresistance[a] (mg/L)	% Resistance	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Breakpoint forresistance[a] (mg/L)	% Resistance	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Amikacin	≥64	85	<2- > 1024	>1024	>1024	≥64	60	<2- > 1024	64	>1024
Arbekacin	-	-	<2- > 1024	>1024	>1024	-	-	2- > 1024	2	>1024
Aztreonam	-	-	<2- > 256	128	>256	≥32	46	4- > 256	16	>256
Ceftazidime	≥32	55	<4- > 512	>512	>512	≥32	46	<4- > 512	16	>512
Ciprofloxacin	≥4	98	<1- > 128	128	>128	≥4	66	<1-64	8	32
Colistin	≥4	6	<0.25-4	2	2	≥8	0	<0.25-0.5	<0.25	0.5
Gentamicin	≥16	91	<2- > 1024	>1024	>1024	≥16	66	<2- > 1024	256	>1024
Imipenem	≥16	48	<4-128	16	32	≥8	53	<4-64	16	32
Meropenem	≥16	51	<4- > 256	32	64	≥8	53	<4-32	16	32
Piperacillin	≥128	100	<4- > 512	>512	>512	≥128	46	<4- > 512	32	256
Piperacillin/ Tazobactam	≥128/4	52	<4- > 512	256	512	≥128/4	13	<4-512	32	128

[a]Breakpoints for antimicrobial resistance were determined according to the guidelines of the Clinical and Laboratory Standards Institute (M07-A9).

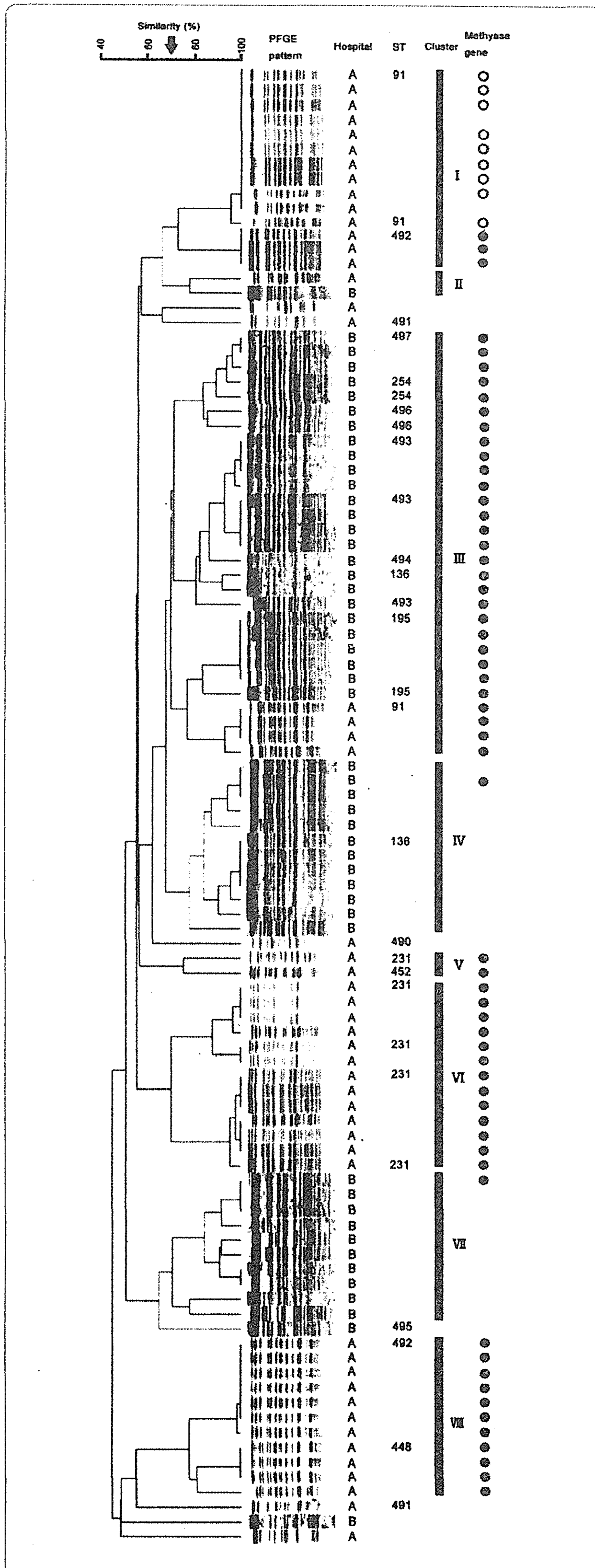

Figure 1 PFGE pattern and MLST analysis of 101 *Acinetobacter baumannii* isolates. Eight clusters (I-VIII) with more than 70% similarity were identified. Isolates harboring *armA* (●) and *rmtB* (○) are shown in the column on the right.

*bla*TEM-1-*tnpR-tnpA* downstream (data not shown). The genetic environment of *rmtB* had more than 99.9% nucleotide sequence identity, from nucleotide 1 to 8,337, to plasmid pXD2 (Gen bank accession no. JN315966) in *E. coli*, which causes bovine milk mastitis in China [12]. NCGM36, which harbored *rmtB*, had the *bla*OXA-23 and *bla*OXA-68 genes, but had neither the *aac(6')-Ib-cr* nor the *bla*CTX-Ms gene.

Discussion

The high prevalence of 16S rRNA methylase producing Gram-negative bacteria in hospitals in Vietnam may have resulted from the high rate of use of aminoglycosides. It has been estimated that 67.4% of hospitalized patients in Vietnam received antibiotics, including 18.9% who received aminoglycosides, with many 30.8% of these prescriptions considered inappropriate [13]. This rate of antibiotic use was much higher than in European countries (17.8%-32.0%) [14,15]. Moreover, the rate of inappropriate indications for antibiotic prescriptions in hospitals in Vietnam was much higher than rates reported in Malaysia (4.0%) [16], Turkey (14.0%) [17], and Hong Kong (20.0%) [18].

A. baumannii isolates from the northern and southern regions of Vietnam may be of different lineages. To date, 2 strains of *A. baumannii* showing ST91 and 3 showing ST136 have been isolated in China; 6 strains showing ST195 have been isolated, 1 in Norway, 2 in Thailand, 2 in

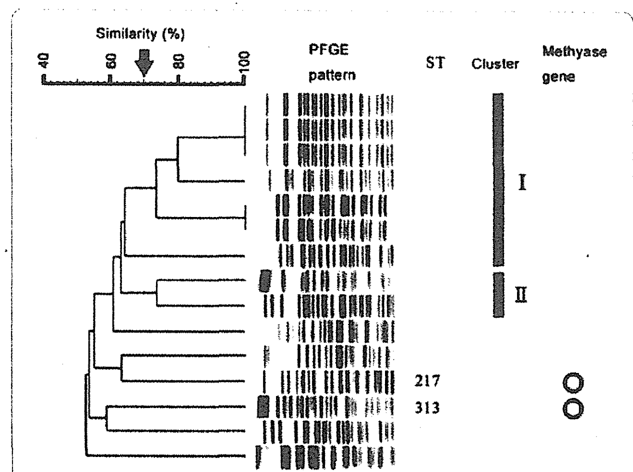

Figure 2 PFGE pattern and MLST analysis analysis of 15 *Pseudomonas aeruginosa* isolates. Eight clusters with more than 70% similarity were detected. Two clusters (I and II) with more than 70% similarity were identified. Isolates harboring *rmtB* (○) are shown in the column on the right.

Malaysia and 1 in China;, 5 strains showing ST231 have been isolated in Brazil and 1 strain showing ST254 has been isolated in China (http://pubmlst.org/abaumannii/). ST136 and ST195 belong to clonal complex 92, the most widely disseminated complex worldwide [19]. Two strains of *P. aeruginosa* producing RmtB, showing ST217 and ST313, may have originally derived from Europe or Australia, because, to date, *P. aeruginosa* ST217 isolates were obtained only in the United Kingdom and ST313 isolates only in Australia, France and Hungary [20] (http://pubmlst.org/paeruginosa/).

To our knowledge, this is the first report showing that *A. baumannii* strains harboring a 16S rRNA methylase (ArmA or RmtB) and with *bla*OXA-23-like and *bla*OXA-51-like genes are emerging in medical settings in Vietnam. ArmA and OXA-23-like producing Gram-negative pathogens have been reported in Bulgaria [21], France [22], India [23], Korea [24], Norway [25] and the United States of America [26], and ArmA and OXA-51-like producing strains have been reported in Japan [27]. Moreover, *armA* and *rmtB* have been linked to *bla*CTX-Ms [28,29], but almost all ArmA producing isolates in Vietnam did not harbor *bla*CTX-Ms.

We found that some *A. baumannii* clinical isolates harbored *rmtB*. The genetic environment of the *rmtB* regions was very similar to the nucleotide sequence, from nt 1 to nt 8,337, of the plasmid pXD2. However, the plasmid of NCGM36 likely differs from pXD2 (Gen bank accession no. JN315966), in that the former NCGM36 did not have *aac(6')-Ib* or *bla*CTX-Ms.

Since 16S rRNA methylase genes in *A. baumannii* and *P. aeruginosa* are located in transferable plasmids [5], the absence of methylase genes was found in the same PFGE clusters. The details of these plasmids will be reported elsewhere.

We plan to survey Gram-negative pathogens producing 16S rRNA methylases in 2013 in Vietnam, since more than 9 Gram-negative bacteria producing 16S rRNA methylases have been reported, including *A. baumannii*, *Citrobacter freundii*, *Enterobacter* spp (including *E. cloacae*), *Escherichia coli*, *Klebsiella pneumonia*, *Morganella morganii*, *Proteus mirabilis*, *Providencia* spp (including *P. stuartii*), and *P. aeruginosa* [5].

Conclusions

This is the first report describing the presence of methylase producing Gram-negative bacteria in medical settings in Southeast Asia, specifically in Vietnam. *A. baumannii* isolates from northern and southern regions of Vietnam may be of different lineages.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT: Performed PCR and sequencing, analyzed data and drafted the manuscript. TMA: Performed MLST analyses. YK, NO, NT and TAT: Performed epidemiological analysis at BMH. NVH, NGB and NQA: Designed protocols and supervised this study at BMH. DMP, TTTN and PHT: Performed clinical bacterial analyses. PTX: Performed epidemiological analysis at CRH. LTAT and NTS: Designed protocols and supervised this study at CRH. TK: Designed protocols and supervised this study. All authors read and approved the final version manuscript.

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IMP-43 and IMP-44 Metallo-β-Lactamases with Increased Carbapenemase Activities in Multidrug-Resistant Pseudomonas aeruginosa

Tatsuya Tada, Tohru Miyoshi-Akiyama, Kayo Shimada, Masahiro Shimojima and Teruo Kirikae
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IMP-43 and IMP-44 Metallo-β-Lactamases with Increased Carbapenemase Activities in Multidrug-Resistant *Pseudomonas aeruginosa*

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Two novel IMP-type metallo-β-lactamase variants, IMP-43 and IMP-44, were identified in multidrug-resistant *Pseudomonas aeruginosa* isolates obtained in medical settings in Japan. Analysis of their predicted amino acid sequences revealed that IMP-43 had an amino acid substitution (Val67Phe) compared with IMP-7 and that IMP-44 had two substitutions (Val67Phe and Phe87Ser) compared with IMP-11. The amino acid residue at position 67 is located at the end of a loop close to the active site, consisting of residues 60 to 66 in IMP-1, and the amino acid residue at position 87 forms a hydrophobic patch close to the active site with other amino acids. An *Escherichia coli* strain expressing bla_{IMP-43} was more resistant to doripenem and meropenem but not to imipenem than one expressing bla_{IMP-7}. An *E. coli* strain expressing bla_{IMP-44} was more resistant to doripenem, imipenem and meropenem than one expressing bla_{IMP-11}. IMP-43 had more efficient catalytic activities against all three carbapenems than IMP-7, indicating that the Val67Phe substitution contributed to increased catalytic activities against carbapenems. IMP-44 had more efficient catalytic activities against all carbapenems tested than IMP-11, as well as increased activities compared with IMP-43, indicating that both the Val67Phe and Phe87Ser substitutions contributed to increased catalytic activities against carbapenems.

Metallo-β-lactamases (MBLs) confer resistance to all β-lactams, except the monobactams, and are characterized by their efficient hydrolysis of carbapenems (1). Acquired MBLs are produced by Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp., and several enterobacteria (1). MBLs are categorized by their amino acid sequences into various types (2–4), including AIM (5), DIM (6), FIM (7), GIM (8), IMPs (9), KHM (10), NDMs (11), SMB (12), SIM (13), SPM (14), TMBs (15) and VIMs (16). The most prevalent types of MBLs are IMP-, VIM-, and NDM-type enzymes (1, 2, 17). We describe here the bacteriological and biochemical characterization of two novel IMP-type MBL variants in multidrug-resistant *P. aeruginosa* isolates obtained in medical settings in Japan.

MATERIALS AND METHODS

Bacterial strains. A total of 161 clinical isolates of multidrug-resistant *P. aeruginosa*, which were resistant to IPM (MIC ≥ 16 μg/ml), AMK (MIC ≥ 32 μg/ml), and CIP (MIC ≥ 4 μg/ml), were obtained between July and September 2011 from 161 hospitals located in 30 of 47 prefectures in Japan by BML Biomedical Laboratories R&D Center (Kawagoe, Saitama, Japan). These strains were isolated from urinary tracts ($n = 93$), respiratory tracts ($n = 62$), and other tissues of patients ($n = 6$). An isolate of *P. aeruginosa* harboring bla_{IMP-7} (NCGM1438) was used to clone bla_{IMP-7}, and *Enterobacter cloacae* harboring bla_{IMP-11} (NCGM5) (18) was used to clone bla_{IMP-11}. *E. coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of bla_{IMP-7}, bla_{IMP-11}, bla_{IMP-43}, and bla_{IMP-44}, respectively.

Drug susceptibility tests. MICs of amikacin, cefoxitin, ceftazidime, cefuroxime, cephradine, colistin, piperacillin, ticarcillin, tigecycline (Sigma-Aldrich, St. Louis, MO), ampicillin, gentamicin, penicillin G (Nacalai Tesque, Kyoto, Japan), arbekacin, fosfomycin (Meiji Seika Pharma, Tokyo, Japan), aztreonam (Eizai, Tokyo, Japan), cefepime (Bristol-Myers Squibb, New York, NY), cefotaxime, ceftriaxone (Chugai Pharmaceutical, Tokyo, Japan), cefmetazole, ciprofloxacin, panipenem (Daiichi-Sankyo Pharmaceutical Co, Tokyo, Japan), imipenem (Banyu Pharmaceutical, Tokyo, Japan), meropenem (Sumitomo Pharmaceutical, Osaka, Japan), doripenem, moxalactam (Shionogi, Osaka, Japan), piperacillin-tazobactam (Toyama Pure Chemical Industries, Tokyo, Japan), cefozopran, cefsulodin (Takeda Pharmaceutical, Tokyo, Japan), cefoselis (Fujisawa Pharmaceutical, Tokyo, Japan), ampicillin-sulbactam (Pfizer Pharmaceutical, Tokyo Japan), and cefpirome (Chemix, Kanagawa, Japan) were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (19).

Detection and typing of IMP-type MBLs. IMP-type enzymes were detected using an immunochromatographic assay kit (Mizuho Medy Co., Saga, Japan) (20). The *bla*IMP genes were amplified using PCR primers as described previously (20). All PCR products were sequenced using an ABI Prism 3130 sequencer (Applied Biosystems, Foster City, CA).

Pulsed-field gel electrophoresis (PFGE). PFGE analysis was performed as described previously (21). Fingerprinting patterns were analyzed by the unweighted-pair-group method by using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA) to create an average linkage-based dendrogram.

Whole-genome sequencing. The entire genomes of NCGM1496 and NCGM1663 were sequenced by Illumina GAIIx (Illumina, San Diego, CA). We obtained 764,108 reads and 6,811,220 bp from 1,006 contigs in NCGM1496, and 2,762,006 reads and 6,911,518 bp from 1,532 contigs in NCGM1663. The multilocus sequence types (MLSTs) according to the *P. aeruginosa* MLST Database website (http://pubmlst.org/paeruginosa/) and the genetic environments surrounding *bla*IMP genes, β-lactamase encoding genes, and efflux pump encoding genes were determined using

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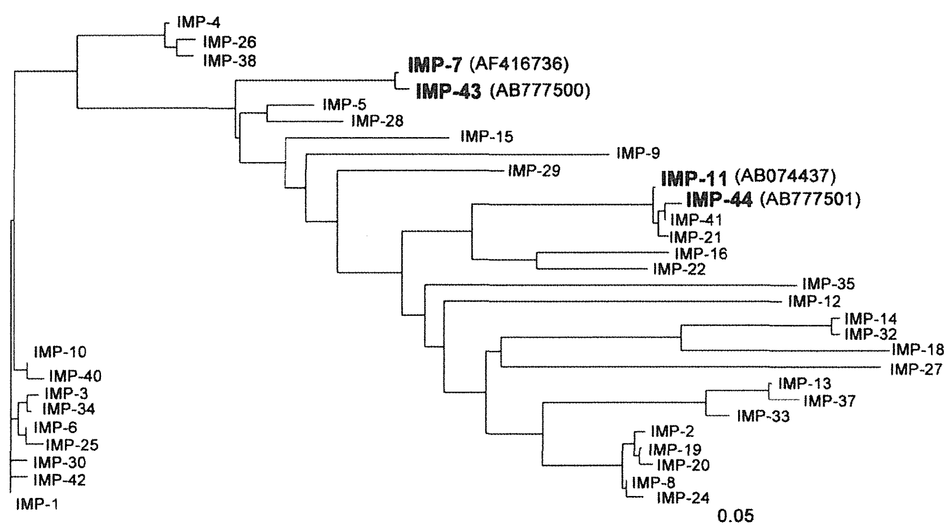

FIG 1 Dendrogram of 43 IMP-type MBLs for comparison with IMP-43 and IMP-44. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for IMP-type enzymes.

the entire genome data. β-Lactamase- and efflux pump-encoding genes were found and sequenced in the genomes.

Cloning of bla_{IMP-7}, bla_{IMP-11}, bla_{IMP-43}, and bla_{IMP-44}. The open reading frames (ORFs) of bla_{IMP-7} and bla_{IMP-43} were PCR amplified using the primers EcoRI-IMP-7/43-F (5′-CCGCATGCGATGAAAAAGTTATC AGTATTC-3′) and PstI-IMP-7/43-R (5′-GGGCGGCCGCTTAGTTACT TGGTTTTGAT-3′), and the ORFs of bla_{IMP-11} and bla_{IMP-44} were amplified using the primers EcoRI-IMP-11/44-F (5′-CCGCATGCGATGAAA AAACTATTTGTTTTA-3′) and PstI-IMP-11/44-R (5′-GGGCGGCCGC TTAATGAACAGTGTTACTTT-3′). The PCR products of each were digested with EcoRI and PstI and ligated into pHSG398 (TaKaRa Bio, Shiga, Japan). The plasmids were used to transform DH5α, transformants were selected on LB agar containing 100 μg/ml of chloramphenicol, and their susceptibility to various β-lactams was assayed.

Purification of recombinant IMP-7, IMP-11, IMP-43, and IMP-44. To determine the kinetic parameters of these IMP-type enzymes, the ORFs of IMP-7, IMP-11, IMP-43, and IMP-44 without signal peptide regions were amplified by PCR. IMP-7, and IMP-43 were amplified using the primers BamHI-IMP-7/43-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGC GGAGAGGCTTTGCCAGATTT-3′) and XhoI-IMP-7/43-R (5′-ATCCT CGAGTTAGTTACTTGGTTTTGATAG-3′), whereas IMP-11 and IMP-44 were amplified using the primers BamHI-IMP-11/44-F (5′-ATG GATCCGAAAACCTGTATTTCCAAGGCGGAGCGTCTTTGCCTGAT TT-3′) and XhoI-IMP-11/44-R (5′-ATCCTCGAGTTAATGAACAGTG TTACTTT-3′). These PCR products were digested with BamHI and XhoI and ligated into pET28a (Novagen, Inc., Madison, WI). The plasmids were used to transform *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA), and transformants were selected on LB agar containing 20 μg/ml of kanamycin. The bacterial cells were lysed by sonication and the recombinant IMP proteins were purified from the soluble fraction on nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instructions (Qiagen, Tokyo, Japan). His-tagged proteins were digested with TurboTEV protease (Accelagen, San Diego, CA), and both the His tag and the protease were removed on Ni-NTA agarose. SDS-PAGE analysis showed that each target protein was over 90% pure. During the purification procedures, the presence of β-lactamase activities was monitored with 100 μM nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Kinetic analysis was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 μM $Zn(NH_3)_2$ at 37°C using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m, k_{cat} and k_{cat}/K_m ratio of each enzyme were determined by analyzing β-lactam hydrolysis under initial-rate conditions using Lineweaver-Burk plots (22–24).

Nucleotide sequence accession numbers. The nucleotide sequences of class I integrons, including bla_{IMP-43} and bla_{IMP-44}, have been deposited in GenBank with the accession numbers AB777500 and AB777501, respectively.

RESULTS

Identification of bla_{IMP-43} in *P. aeruginosa* NCGM1496 and bla_{IMP-44} in NCGM1663. Of the 161 *P. aeruginosa* clinical isolates, 101 were positive for IMP-type enzymes using an immunochromatographic assay kit to detect IMP-type enzymes. Of these, 61 had bla_{IMP-1}, 20 had bla_{IMP-10}, 12 had bla_{IMP-7}, 5 had a new bla_{IMP-7} variant, 2 had bla_{IMP-6}, and 1 had a new bla_{IMP-11} variant. The new bla_{IMP-7} and bla_{IMP-11} variants were designated bla_{IMP-43} and bla_{IMP-44}, respectively. Dendrograms of IMP-type enzymes, including IMP-43 and IMP-44, are shown in Fig. 1. Analysis of the amino acid sequences revealed that IMP-43 had one amino acid substitution (Val67Phe) compared with IMP-7 and IMP-44 had two substitutions (Val67Phe and Phe87Ser) compared with IMP-11. To clarify whether these amino acid substitutions affect enzymatic activities, IMP-43 was compared with IMP-7 and IMP-44 with IMP-11, due to their similar amino acid sequences (Fig. 1). The amino acid sequences of IMP-7 and IMP-11 were 85% identical, with differences in 36 amino acids, and those of IMP-43 and IMP-44 were also 85% identical, with differences in 37 amino acids.

Whole-genome analysis revealed that both NCGM1496 and NCGM1663 did not have any other genes encoding β-lactamase except for those encoding PDC-11 (an AmpC variant) and PoxB (an intrinsic β-lactamase). They had efflux pump-encoding genes, including *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY*, and had a point mutation in *mexR* resulting in an amino acid substitution of Val126Glu. The mutation is known to be associated with the overexpression of the MexAB-OprM efflux pump (25).

The MLSTs of both NCGM1496 and NCGM1663 were ST357 (*Pseudomonas aeruginosa* MLST Database [http://pubmlst.org/paeruginosa/]). Four isolates harboring bla_{IMP-43} except for

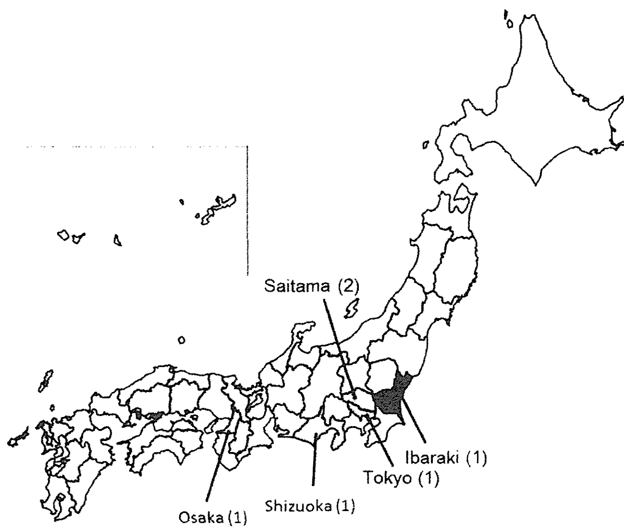

FIG 2 Geographical distribution of *P. aeruginosa* isolates producing IMP-43 and IMP-44 in Japan. The 5 isolates producing IMP-43 were obtained from prefectures marked in gray, and the isolate producing IMP-44 was obtained from the prefecture marked in black. The numbers of isolates are in parentheses.

NCGM1496 were ST235. The PFGE pattern of NCGM1496 had 66.7% similarity to that of NCGM1663 (data not shown).

bla_{IMP-43} and bla_{IMP-44} in class I integrons. bla_{IMP-43} and bla_{IMP-44} were located in class I integrons. These integrons had the different structures; i.e., one had a unique gene cassette array of *aac(6')-Ib*, bla_{IMP-43}, *aac(6')-Ib*, and bla_{OXA-2} (accession no. AB777500), whereas the other had an array similar (with more than 96% identity) to that in a *P. aeruginosa* isolate (accession no. AJ628135) obtained in Italy (26).

Geographic distribution. A total of 5 isolates harboring bla_{IMP-43} and 1 harboring bla_{IMP-44} were detected in this study. The isolates harboring bla_{IMP-43} were obtained in Osaka, Shizuoka, Tokyo, and Saitama prefectures, whereas the isolate harboring bla_{IMP-44} was from Ibaraki prefecture (Fig. 2). Although isolates harboring bla_{IMP-7} were obtained in these prefectures, these isolates were not obtained at the hospitals where the isolates harboring bla_{IMP-43} were obtained.

Drug susceptibility of *E. coli* DH5α expressing IMP-43 and IMP-44. *P. aeruginosa* NCGM1496 harboring bla_{IMP-43} and NCGM1663 harboring bla_{IMP-44} were resistant to all antibiotics tested, except for colistin (MICs of 2 and 0.5 μg/ml, respectively). The MICs of β-lactams in NCGM1496 and NCGM1663 are shown in Table 1; the MICs of other antibiotics in these two strains were 32 and 32 μg/ml, respectively, for arbekacin; 32 and 32 μg/ml, respectively, for amikacin; 64 and 512 μg/ml, respectively, for gentamicin; 16 and 64 μg/ml, respectively, for ciprofloxacin; and 4 and 4 μg/ml, respectively, for tigecycline.

When the drug susceptibility of *E. coli* DH5α expressing IMP-43, an IMP-7 variant, was compared with that of the same strain expressing IMP-7, the MICs of doripenem and meropenem were each 4-fold higher for *E. coli* expressing IMP-43 than *E. coli* expressing IMP-7, whereas the MICs of cefmetazole, cefoxitin, ticarcillin, and ticarcillin-clavulanic acid were 8-fold lower for *E. coli* expressing IMP-43 than IMP-7 (Table 1). When drug susceptibility of *E. coli* expressing IMP-44, an IMP-11 variant, was compared with that expressing IMP-11, the MICs of doripenem, imipenem, meropenem, and panipenem were at least 4-fold higher for *E. coli* expressing IMP-44 than IMP-11, whereas the MICs of ampicillin, ampicillin/sulbactam, cefmetazole, cefoselis, cefoxitin, cefpirome, cephradine, ticarcillin, and ticarcillin-clavulanic acid were at least 4-fold lower for *E. coli* expressing IMP-44 than IMP-11 (Table 1).

Catalytic activities of IMP-43 and IMP-44. All four recombinant IMP-type enzymes tested, including IMP-7, IMP-11, IMP-

TABLE 1 MICs of β-lactams for *P. aeruginosa* NCGM1496 and NCGM1663 and *E. coli* strains transformed with IMP-7, IMP-43, IMP-11, and IMP-44

	MIC (μg/ml)						
Antibiotic(s)[a]	*P. aeruginosa* NCGM1496	*P. aeruginosa* NCGM1663	*E. coli* DH5α(pHSG398/IMP-7)	*E. coli* DH5α(pHSG398/IMP-43)	*E. coli* DH5α(pHSG398/IMP-11)	*E. coli* DH5α(pHSG398/IMP-44)	*E. coli* DH5α(pHSG398)
Ampicillin	>512	>512	16	8	32	8	2
Ampicillin-sulbactam	>512	>512	8	4	16	4	1
Aztreonam	32	64	<0.25	<0.25	<0.25	<0.25	<0.25
Cefepime	>512	>512	4	4	16	16	<0.25
Cefmetazole	>512	>512	256	32	256	64	1
Cefoselis	>512	>512	8	8	32	4	<0.25
Cefotaxime	>512	>512	32	32	128	128	<0.25
Cefoxitin	>512	>512	512	64	512	128	2
Cefozopran	512	>512	8	8	32	32	<0.25
Cefpirome	64	>512	32	32	4	1	<0.25
Cefsulodin	>512	>512	>512	>512	>512	>512	256
Ceftazidime	>512	>512	512	512	256	256	<0.25
Ceftriaxone	>512	>512	64	64	128	256	<0.25
Cefuroxime	>512	>512	512	512	512	>512	4
Cephradine	>512	>512	512	512	512	128	16
Doripenem	>512	>512	0.5	2	8	32	<0.25
Imipenem	>512	>512	0.5	0.5	2	8	<0.25
Meropenem	>512	>512	0.5	2	16	64	<0.25
Moxalactam	>512	>512	256	256	256	>512	<0.25
Panipenem	>512	>512	1	1	2	16	<0.25
Penicillin G	>512	>512	32	32	64	32	32
Piperacillin	64	64	2	2	4	2	2
Piperacillin-tazobactam	32	32	2	2	1	1	1
Ticarcillin	256	>512	512	64	512	128	2
Ticarcillin-clavulanic acid	256	>512	256	64	512	128	2

[a] The ratio of ampicillin to sulbactam was 2:1. The ratio of piperacillin to tazobactam was 4:1. The ratio of ticarcillin to clavulanic acid was 15:1.

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TABLE 2 Kinetic parameters of β-lactamases IMP-7, IMP-11, IMP-43, and IMP-44 with various substrates

	Km (μM)[a]				$kcat$ (s^{-1})[a]				$kcat/Km$ ($\mu M^{-1} \cdot s^{-1}$)[a]			
Substrate	IMP-7	IMP-43	IMP-11	IMP-44	IMP-7	IMP-43	IMP-11	IMP-44	IMP-7	IMP-43	IMP-11	IMP-44
Penicillin G	207 ± 19	3176 ± 236	574 ± 18	482 ± 53	25.3 ± 0.7	64 ± 3	36 ± 2	14 ± 1	0.12	0.02	0.063	0.029
Ampicillin	180 ± 10	494 ± 40	230 ± 21	627 ± 80	9.1 ± 0.6	3.5 ± 0.2	7.4 ± 0.6	11 ± 1	0.051	0.0072	0.032	0.017
Cephradine	27 ± 2	69 ± 5	39 ± 4	119 ± 8	8.0 ± 0.3	10.3 ± 0.8	14.6 ± 0.7	14.1 ± 0.2	0.29	0.15	0.38	0.12
Cefoxitin	33 ± 1	55 ± 5	4.4 ± 1.2	53 ± 5	4.18 ± 0.05	3.48 ± 0.05	3.2 ± 0.1	12 ± 1	0.13	0.062	0.76	0.22
Cefotaxime	24 ± 2	6.8 ± 0.9	35 ± 4	27 ± 3	1.88 ± 0.07	7.2 ± 0.1	4.3 ± 0.2	44 ± 1	0.08	1.1	0.12	1.6
Ceftazidime	59 ± 4	14 ± 2	29 ± 3	63 ± 4	0.89 ± 0.05	1.9 ± 0.1	1.33 ± 0.09	5.6 ± 0.2	0.015	0.14	0.046	0.089
Cefepime	50 ± 6	30 ± 4	40 ± 5	64 ± 5	1.34 ± 0.04	4.6 ± 0.1	2.00 ± 0.04	19.4 ± 0.7	0.027	0.16	0.05	0.31
Aztreonam	NH[b]	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH
Doripenem	63 ± 5	59 ± 7	101 ± 10	257 ± 27	6.8 ± 0.2	10.0 ± 0.4	11.3 ± 0.6	589 ± 40	0.11	0.17	0.11	2.3
Imipenem	254 ± 20	268 ± 23	142 ± 7	119 ± 11	20 ± 1	49 ± 2	19.2 ± 0.5	165 ± 5	0.078	0.18	0.13	1.4
Meropenem	59 ± 7	24 ± 2	50 ± 5	137 ± 16	2.6 ± 0.2	8.2 ± 0.3	5.9 ± 0.2	335 ± 13	0.044	0.34	0.12	2.5

[a] The K_m and k_{cat} values are the means ± standard deviations from three independent experiments.

[b] NH, no hydrolysis detected with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

43, and IMP-44, hydrolyzed all β-lactams tested except for aztreonam (Table 2). The k_{cat}/K_m ratios of IMP-43 and IMP-44 against cefotaxime, ceftazidime, cefepime, doripenem, imipenem, and meropenem were higher than those of IMP-7 and IMP-11, respectively, whereas the k_{cat}/K_m ratios of IMP-43 and IMP-44 against penicillin, ampicillin, cephradine, and cefoxitin were lower than those of IMP-7 and IMP-11, respectively. The K_m value of IMP-43 for penicillin was markedly higher than that of other IMP-type enzymes tested (Table 2). The k_{cat}/K_m ratios for IMP-44 against all carbapenems tested were higher than those of the other IMP-type enzymes, and its k_{cat} values were 9 to 57 times higher than those of IMP-11 (Table 2).

DISCUSSION

This is the first report of *P. aeruginosa* ST357 isolates found in Japan, although these isolates have been found in other countries. To date, 5 *P. aeruginosa* ST357 isolates from France, Nigeria, Poland, Senegal, and Singapore have been registered on the PubMLST website (http://pubmlst.org/paeruginosa/). Regional spread of *P. aeruginosa* ST357 producing IMP-7 has been reported in central Europe (27). The two isolates of NCGM1496 and NCGM1663 from different regions belonged to ST357, indicating that ST357 isolates will exist widely in Japan, although PFGE analysis revealed that the isolates were not very close to each other (with 66.7% similarity).

The Val67Phe amino acid substitution seems to have a significant impact on the catalytic efficiency for meropenem, whereas the efficiencies for imipenem and doripenem are slightly influenced in IMP-43. The presence of both the amino acid substitutions Val67Phe and Phe87Ser results in a significant increase of catalytic efficiencies for all the carbapenems in IMP-44. The amino acid residue 67 in IMP-1 is located at the end of a loop close to the active site consisting of residues 60 to 66 (28). This loop may be a major determinant for the tight binding of substrates in the active site (28). The active-site loop of MBL BcII has been reported to contribute to substrate binding by providing hydrophobic interactions between the phenyl and methyl groups of penicillin G and residues located at both ends of the loop (Phe61 and Val67) (29). The Phe87Ser amino acid substitution observed in IMP-44 could also contribute to enzymatic activity against carbapenems. Mutational analysis of VIM-2 revealed that the aromatic residue Trp87 is critical for the stability and folding of this protein (30). Residues Phe-61, Tyr-67, and Trp-87 of VIM-2, which form a hydrophobic patch close to the active site, were close to the second Zn binding site in VIM-2 (28). Two aromatic side chains in the hydrophobic pocket (the phenyl groups of Phe61 and Trp87) interact with the phenyl group of penicillin G (29, 31). The Phe87Ser amino acid substitution may therefore affect stability and folding, resulting in changes in substrate specificities due to the absence of a benzene ring.

The amino acid substitutions Val67Phe and Phe87Ser affected catalytic activities of β-lactams other than the carbapenems. The Val67Phe amino acid substitution reduced affinity with penicillin G and the Phe87Ser marginally affected the affinity (Table 2). Both amino acid substitutions increased catalytic activities against cefotaxime, ceftazidime, and cefepime, although the MICs of these antibiotics are intrinsically high and did not change regardless of substitutions. These substitutions may specifically affect their affinity for carbapenems. These IMP-type enzymes did not show catalytic activities against aztreonam and did not confer aztreonam resistance on *E. coli* strains expressing these IMP-type enzymes. Nevertheless, NCGM1496 and NCGM1663 were resistant to aztreonam, perhaps due to the presence of genes encoding efflux pumps and PDC-11, an AmpC variant (32). Both strains had 4 operons encoding efflux pump systems (*mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY*) as described for *P. aeruginosa* (33). Among these systems, *mexAB-oprM* and a point mutation in *mexR* may be associated with aztreonam resistance in these strains (34).

IMP-7 and IMP-11 also showed epidemiological differences. IMP-7 producers were observed globally, whereas IMP-11 producers have been reported only in Japan. IMP-7 was originally isolated from a patient in Canada (35) and IMP-11 from a patient in Japan (accession no. AB074437). IMP-7 has been detected in patients in Canada (35), the Czech Republic (36), Japan (37), Malaysia (38), and Slovakia (39).

In conclusion, multidrug-resistant *P. aeruginosa* strains producing IMP-43 and IMP-44 are emerging in medical settings in Japan. In particular, IMP-43-producing *P. aeruginosa* isolates were disseminated throughout Japan. The amino acid substitutions Val67Phe and Phe87Ser found in IMP-44 appear to significantly increase the hydrolytic efficiency of IMP-type enzymes. Therefore, it is necessary to carefully investigate these isolates.

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Table 1
Description of the 23 cefotaxime-resistant *Salmonella enterica* isolates.

Year of isolation	Isolate ID	Serovar	Phage type	Genes encoding cephalosporin resistance	Sex	Age (years)	Travel abroad	Resistance profile
2008	0812M7303	Typhimurium	193	$bla_{CTX-M-55}$	M	50	Thailand	CHL, CIP, FFN, GEN, SUL, STR, TET
	0811R10895	Typhimurium	RDNC	$bla_{CTX-M-1}$	M	1	Unknown	SUL,TET
	0809W37247	Stanley		$bla_{CMY-2-like}$	F	37	No	AMC, CHL, FFN, SUL, STR, TET
	0809F35063	Stanley		$bla_{CMY-2-like}$	F	6	Unknown	AMC, CHL, FFN, GEN, SUL, STR, TET
	0808S63221	Typhimurium	NT	$bla_{CMY-2-like}$	M	20	Thailand	AMC, CHL, FFN, SUL, STR, TET
	0807F21428	Stanley		$bla_{CMY-2-like}$	F	22	Thailand	AMC, CHL, FFN, GEN, SUL, STR, TET
	0806H16365	Stanley		$bla_{CMY-2-like}$	M	2	Unknown	AMC, CHL, FFN, GEN, SUL, STR, TET
	0806R9615	Typhimurium	U292	$bla_{CTX-M-3}$	M	12	No	None
	0805R9530	Typhimurium	NT	$bla_{CTX-M-14}$	M	47	Greece	AMC, CHL, GEN, SUL, STR, TMP
2009	0911W58164	Heidelberg		$bla_{CTX-M-14}$	M	40	Egypt	GEN, SUL, STR
	0910W56953	subsp. enterica (I)		$bla_{CMY-2-like}$	M	55	Thailand	AMC, CHL, CIP, FFN, GEN, NAL, SUL, STR, TET
	0910F48822	Isangi		$bla_{CMY-2-like}$, bla_{OXA-10}	M	<1	South Africa	AMC, CHL, CIP, FFN, GEN, NAL, SUL, STR, TET, TMP
	0909F36769	O:6,8; H:e,h:-		$bla_{CMY-2-like}$	M	49	No	AMC, CHL, FFN, SUL, STR, TET, TMP
	0905W18230	O:4,5,12; H:i:-	U302	$bla_{CTX-M-15}$	M	48	Unknown	CHL, CIP, GEN, SUL, STR, TET, TMP
	0904R11448	Enteritidis	1	$bla_{CTX-M-15}$	F	44	Egypt	GEN
	0904W9384	Typhimurium	193	$bla_{CTX-M-15}$	F	54	No	CHL, CIP, FFN, GEN, SUL, STR, TET
	0903T66197	O:4,5,12; H:i:-	193	$bla_{CTX-M-55}$	F	46	Unknown	GEN, SUL, STR, TET, TMP
2010	1003F13978	O:4,12; H:i: -	193	$bla_{CTX-M-55}$	M	16	Thailand	CHL, CIP, FFN, SUL, STR, TET
	1001M23541	Infantis		$bla_{CTX-M-55}$	F	56	Thailand	CHL, CIP, FFN, GEN, NAL, SUL, STR, TET, TMP
	1010H59657	Senftenberg		$bla_{CTX-M-15}$	M	36	Egypt	SUL, TMP
	1008R13307	Typhimurium	193	$bla_{CTX-M-55}$	F	21	Thailand	CHL, CIP, FFN, GEN, SUL, STR, TET
	1002H3270	Stanley		$bla_{CMY-2-like}$	F	58	Thailand	AMC, CHL, FFN, SUL, STR, TET
	1002W11208	O:4,5,12; H:i:-	193	$bla_{CTX-M-55}$	F	58	Unknown	CHL, CIP, FFN, GEN, SUL, STR, TET

CHL, chloramphenicol; CIP, ciprofloxacin, FFN, florfenicol; GEN, gentamicin; SUL, sulfamethoxazole; STR, streptomycin; TET, tetracycline; AMC, amoxicillin/clavulanic acid; TMP, trimethoprim; NAL, nalidixic acid.

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Dissemination of multidrug-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal

Sir,

The carbapenemases NDM-1 and OXA-72 hydrolyse almost all β-lactams. NDM-1-producing Enterobacteriaceae and OXA-72-producing *Acinetobacter* spp. have been reported in various countries [1,2]. To date, OXA-72-producing isolates of bacterial species other than *Acinetobacter* spp. have not been reported.

Acquired 16S rRNA methylase genes responsible for high-level resistance to various aminoglycosides have been widely distributed among Enterobacteriaceae, including *Klebsiella pneumoniae* and glucose-non-fermentative bacteria [3]. 16S rRNA methylase-producing Gram-negative pathogens have been isolated in various countries [3], including Nepal [4]. The 16S rRNA methylases ArmA and RmtC are widely spread among various bacterial species, including Enterobacteriaceae and *Acinetobacter* spp.

In this study, 25 *K. pneumoniae* isolates were obtained from 25 inpatients during the period May–October 2012 at Tribhuvan University Teaching Hospital (Kathmandu, Nepal), of which 13 isolates were obtained from sputa and 12 were from pus samples. Isolates were identified phenotypically and species identification

Table 1
Summary of the characteristics of the 25 *Klebsiella pneumoniae* strains, including antimicrobial resistance profiles and resistance genes.

Strain	MIC (μg/mL)												Carbapenemases	ESBL	16S rRNA methylases	Mutations in DNA gyrase	
	PIP	TZP	CAZ	CTX	FEP	IPM	MEM	ATM	ABK	AMK	GEN	CIP				*gyrA*	*parC*
IOMTU 23	>1024	512	>1024	1024	256	16	32	512	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-158, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 25	>1024	1024	>1024	512	128	32	64	512	1024	1024	>1024	128	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83I	S80I
IOMTU 40	>1024	256	>1024	1024	256	16	32	128	1024	>1024	1024	128	NDM-1	CTXM-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 46	>1024	512	>1024	>1024	128	16	32	512	>1024	>1024	>1024	128	NDM-1	CTX-M-15, SHV-11, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 53	>1024	512	>1024	>1024	512	32	32	512	>1024	>1024	>1024	64	NDM-1, OXA-72	CTX-M-15, SHV-11, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 67	512	8	64	512	128	<0.5	<0.5	32	1	4	64	32	–	CTX-M-15, SHV-28, TEM-1	–	S83F, D87A	S80I
IOMTU 74	>1024	512	32	256	128	4	8	128	>1024	>1024	>1024	2	NDM-1, OXA-72	CTX-M-15, SHV-1	ArmA	S83F, D87A	S80I
IOMTU 76	>1024	1024	>1024	512	128	32	64	512	>1024	>1024	>1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83I	S80I
IOMTU 83	1024	512	128	1024	128	32	32	128	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 89	>1024	512	>1024	>1024	256	32	32	1024	>1024	>1024	>1024	128	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83Y, D87F	S80I
IOMTU 100	>1024	1024	>1024	>1024	128	32	64	512	>1024	>1024	>1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83F, D87A	S80I
IOMTU 102	>1024	512	>1024	1024	256	32	32	256	512	>1024	>1024	128	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 103	>1024	8	64	>1024	64	<0.5	<0.5	128	1	8	32	128	–	CTX-M-15, SHV-28, TEM-1	–	S83F, D87A	S80I
IOMTU 111	>1024	1024	>1024	>1024	512	16	32	512	>1024	>1024	1024	64	NDM-1, OXA-72	CTX-M-15, TEM-1	RmtC	S83I	S80I
IOMTU 116.1	>1024	512	>1024	1024	512	8	8	256	>1024	>1024	512	64	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 116.2	>1024	512	>1024	>1024	>1024	8	16	256	>1024	>1024	1024	64	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 117	>1024	512	>1024	1024	128	4	4	128	1024	>1024	1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	ArmA	S83F, D87A	S80I
IOMTU 120	>1024	16	512	>1024	>1024	<0.5	<0.5	1024	>1024	>1024	>1024	128	–	CTX-M-15, SHV-28,TEM-1	RmtF	S83Y, D87N	S80I
IOMTU 122	>1024	512	>1024	>1024	512	16	16	256	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 125	>1024	4	32	512	256	<0.5	<0.5	64	>1024	>1024	>1024	8	OXA-72	CTX-M-15, SHV-28, TEM-1	ArmA	S83F, D87A	S80I
IOMTU 138	>1024	128	256	>1024	>1024	<0.5	2	512	>1024	>1024	>1024	>1024	OXA-72	CTX-M-15, SHV-11, TEM-1	RmtF	S83F, D87N	No mutation
IOMTU 139	>1024	128	128	512	256	2	2	512	32	32	64	256	–	CTX-M-15, SHV-28, TEM-1	–	S83Y, D87G	S80I
IOMTU 145	>1024	8	32	1024	256	<0.5	<0.5	64	4	4	<0.5	2	–	CTX-M-15, TEM-1	–	No mutation	No mutation
IOMTU 154	>1024	1024	>1024	1024	256	16	32	64	>1024	>1024	1024	16	NDM-1, OXA-72	CTX-M-15, SHV-28, TEM-1	RmtC	S83F, D87A	S80I
IOMTU 164	>1024	8	64	512	256	<0.5	<0.5	64	4	4	<0.5	<0.5	–	CTX-M-15, SHV-83, TEM-1	–	No mutation	No mutation

MIC, minimum inhibitory concentration; PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; ATM, aztreonam; ABK, arbekacin; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; ESBL, extended-spectrum β-lactamase.

was confirmed by 16S rRNA sequencing. Minimum inhibitory concentrations of antibiotics were determined by the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M07-A9.

Entire genomes of the isolates were sequenced by MiSeq™ (Illumina, San Diego, CA). CLC Genomics Workbench v.5.5 (CLC bio, Tokyo, Japan) was used to search 923 drug resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in *gyrA* and *parC* associated with quinolone resistance. The genetic environments surrounding bla_{NDM-1}, bla_{OXA-72} and 16S rRNA methylase-encoding genes were determined. Multilocus sequence typing (MLST) and clonal complexes (CCs) were determined according to the *K. pneumoniae* MLST database website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) and eBURST v.3 (http://eburst.mlst.net), respectively.

Pulsed-field gel electrophoresis (PFGE) analysis was performed and fingerprinting patterns were analysed by the unweighted pair-group method.

All isolates were resistant to piperacillin, of which 19 isolates were resistant to piperacillin/tazobactam. All isolates were resistant to ceftazidime, cefotaxime and cefepime. Seventeen isolates were resistant to carbapenems (imipenem and meropenem). All isolates are resistant to aztreonam. Twenty isolates were resistant to all aminoglycosides tested (arbekacin, amikacin and gentamicin). Twenty-two isolates were resistant to ciprofloxacin (Table 1).

The majority of isolates had various combinations of genes encoding carbapenemases (bla_{NDM-1} and bla_{OXA-72}) and 16S rRNA methylases (*armA*, *rmtC* and *rmtF*) (Table 1). These isolates also had extended-spectrum β-lactamase-encoding genes, including $bla_{CTX-M-15}$, bla_{TEM-1} and/or bla_{SHV}-type, as well as aminoglycoside-modifying enzymes, including *aac(6′)-Ib* and/or *aadA2*. Twenty-three isolates had two or three point mutations in the quinolone resistance-determining regions of *gyrA* and *parC*.

The genetic environment surrounding bla_{NDM-1} (GenBank accession no. AB824738) including *rmtC* was a unique structure, which was *orf1–tniB–orf2–orf3–rmtC–*bla_{NDM-1}*–*ble_{MBL}*–trpF–dsbC–cutA1–groL*. The genetic environment surrounding *armA* (GenBank accession no. AB825954) from nucleotides 19 to 14 138 had >99.9% sequence identity to a nucleotide sequence from nucleotides 65 492 to 79 611 of the plasmid pCTX-M3 (GenBank accession no. AF550415). The genetic environment surrounding *rmtF* (GenBank accession no. AB824739) from nucleotides 268 to 9812 had >99.9% sequence identity to a nucleotide sequence from nucleotides 49 291 to 58 835 of the plasmid pKPX-1 (GenBank accession no. AP012055). The genetic environment surrounding bla_{OXA-72} (GenBank accession no. AB825955) from nucleotides 1 to 8970 was identical to that of pAB-NCGM253 (GenBank accession no. AB823544).

The clinical isolates of *K. pneumoniae* tested belonged to one of the following sequence types (STs): ST11; ST14; ST15; ST29; ST43; ST340; ST378; ST395; ST437; ST1231; and ST1232. Of these isolates, 14 belonged to CC14 and 5 belonged to CC11. These results mostly corresponded with the results of PFGE pattern analysis, which revealed two clusters showing >60% similarity (clusters I and II). Cluster I comprised 12 isolates belonging to CC14 and cluster II comprised 4 isolates belonging to CC11.

NDM-1-producers have epidemiological links to the Indian subcontinent as of 2011 [5]. There was, nevertheless, no report of NDM-1-producers in Nepal. We recently found NDM-1-producing *Pseudomonas aeruginosa* and a novel variant NDM-8-producing *Escherichia coli* isolates in Nepal [4].

This is the first report describing OXA-72-producers in South Asia, suggesting that OXA-72-producers have disseminated in this region. This is also the first report of OXA-72-producing *K. pneumoniae* clinical isolates. Up to now, OXA-72-producers were reported to be only *Acinetobacter* spp.

The present study suggests that aminoglycoside-resistant Gram-negative pathogens producing ArmA, RmtC and RmtF disseminated in medical settings in Nepal. These pathogens producing 16S rRNA methylases may also disseminate in neighbouring countries. Hidalgo et al. [6] recently reported that 14% of Enterobacteriaceae isolates from an Indian hospital had 16S rRNA methylases, of which 24% produced RmtF.

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Ethical approval: This study protocol was ethically reviewed and approved by the Institutional Review Board of the Institute of Medicine, Tribhuvan University (Kathmandu, Nepal) [ref. 6-11-E].

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CASE REPORT

Three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* bloodstream infection in Japan

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Abstract We report three cases of IMP-type metallo-β-lactamase-producing *Enterobacter cloacae* bloodstream infection, which showed minimum inhibitory concentration values for imipenem with 2 μg/ml in all isolates. Although carbapenems were initiated empirically in all cases, two of three cases died. The Clinical and Laboratory Standards Institute lowered the breakpoints of carbapenems for *Enterobacteriaceae* in 2010. However, the previous breakpoints are still used in many clinical laboratories, which can result in failure to detect carbapenem-resistant *Enterobacteriaceae*. Therefore, lower breakpoints of carbapenems should be used in clinical settings, and alternative tests for detecting metallo-β-lactamase such as polymerase chain reaction and immunochromatographic assays may contribute to better detection of carbapenem-resistant isolates.

Keywords Metallo-β-lactamase · Carbapenemase · *Enterobacter cloacae* · Bloodstream infection

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Introduction

Since metallo-β-lactamase (MBL)-producing *Enterobacter cloacae* was first reported in 2000, there have been increasing reports of carbapenem-resistant *E. cloacae* worldwide [1, 2]. MBL-producing *Enterobacteriaceae*, including *E. cloacae*, often exhibited relatively low minimum inhibitory concentrations (MIC) of carbapenems (0.06–2 μg/ml) [3]. Recently, the Clinical and Laboratory Standards Institute (CLSI) lowered the breakpoints of carbapenems against *Enterobacteriaceae* [4]. However, higher MIC breakpoints are still used in many clinical laboratories because Food and Drug Administration (FDA)-approved breakpoints have not been changed, which can result in failure to detect carbapenem-resistant *Enterobacteriaceae* [5]. Furthermore, it is unclear whether carbapenems are effective against MBL-producing *E. cloacae* [2]. Clinical studies to determine appropriate chemotherapeutic regimens against MBL-producing *E. cloacae* infection are required. Here, we report three cases including two with unsuccessful outcomes of IMP-type MBL-producing *E. cloacae* bloodstream infections in a hospital in Japan.

Case reports

Case 1 was a 91-year-old man who was admitted to the hospital for cerebral infraction. He developed aspiration pneumonia on day 20 and was treated with ampicillin/sulbactam (ABPC/SBT) for 10 days. He developed septic shock consequent to peripheral venous catheter infection on day 33, and administration of meropenem (MEM) was initiated. However, hemodynamic instability persisted, and blood culture revealed *E. cloacae* with elevated MIC for imipenem (IPM) (MIC = 2 μg/ml) and *Proteus vulgaris*. MEM was changed to levofloxacin (LVFX) based on the results of susceptibility testing on day 37. Furthermore, MBL production was tested using Cica-β-test [6] and an immunochromatographic assay [7] because of the elevated MIC for imipenem, which revealed positive. IMP-1 was confirmed by polymerase chain reaction and sequencing in our research institute. Although an additional blood culture was negative for *E. cloacae*, the patient died on day 40.

Case 2 was a 77-year-old man with type 2 diabetes receiving insulin therapy. He was admitted to the hospital for esophageal cancer, and subtotal esophageal resection and subcutaneous reconstruction were performed. He developed infection in the cervical wound because of leakage and received vancomycin and MEM for 36 days, with subsequent oral LVFX for 1.5 months. Although the surgical wound infection improved, he had recurrent aspiration pneumonia. On postoperative day (POD) 105, he developed bacteremia caused by *E. cloacae*, which was possibly caused by central venous catheter infection, and MEM was commenced on POD 106. The central venous catheter was removed on POD 109. *E. cloacae* was still isolated from blood culture despite 3 days of antibiotic therapy, and MEM was considered to be ineffective. The isolate was revealed to be a MBL producer and thus gentamycin was added on POD 116. However, the patient died on POD 117.

Case 3 was an 88-year-old man with an abdominal artery aneurysm for which an endovascular graft was inserted 6 months before admission. He was admitted to the hospital for colon cancer, and right hemicolectomy was performed. On POD 3, MEM was initiated for postoperative fever because of surgical site infection. Although his fever improved on POD 5, blood culture revealed MBL-producing *E. cloacae* and *Bacteroides* sp. Thus, MEM was switched to LVFX + ABPC/SBT on POD 7. Bacterial clearance was documented by a follow-up blood culture. The patient completed a 2-week course of intravenous antibiotic therapy followed by 2 weeks of oral LVFX + metronidazole and was discharged.

Drug susceptibility profiles are shown in Table 1. MIC values for both IPM and MEM were 2–4 μg/ml in all isolates, which were reported as susceptible in our clinical laboratory. All the isolates were positive for MBL by phenotypic, immunochromatographic, and polymerase chain reaction (PCR) assays. Two isolates produced IMP-1 and the remaining isolate produced IMP-11. There was no epidemiological link among the three patients.

Discussion

The reduced breakpoints of carbapenems for *Enterobacteriaceae* as revised recently by CLSI should be applied in clinical laboratories [4]. The MICs of IPM for all *Enterobacter cloacae* isolates from these cases were within the susceptible range according to the criteria recommended by CLSI in 2009 [8]. Nevertheless, these isolates were MBL producers. The breakpoint should have been ≤1 μg/ml for IPM in the present cases. CLSI recently recommended lowering the breakpoints for *Enterobacteriaceae* to improve the detection of carbapenemase producers [4]. However, higher MIC breakpoints are still used in many clinical laboratories, including those in Japan, because FDA-approved breakpoints have not been changed [5]. These higher breakpoints can lead to underestimation of the resistance, which may result in inadequate treatment. Yan et al. [9] recently reported that MBL production was not correlated with clinical outcomes and thus it was unnecessary to test MBL routinely. However, they did not analyze the association between MBL production and mortality by multivariate analysis. Information is still

Table 1 Susceptibility profiles of MBL-producing *Enterobacter cloacae* isolates

Isolates	MBL typing	MIC (μg/ml)									
		IPM	MEM	CTX	CAZ	CPR	AZT	P/T	CIP	AMK	CLS
1	IMP-1	2	2	512	512	6	64	64	1	1	2
2	IMP-11	2	2	32	64	4	0.5	64	32	8	2
3	IMP-1	2	4	256	512	16	32	64	0.5	1	2

IMP subtyping was performed by polymerase chain reaction and sequencing

MBL metallo-β-lactamase, *IPM* imipenem, *MEM* meropenem, *CTX* ceftriaxone, *CAZ* ceftazidime, *CPR* cefpirome, *AZT* aztreonam, *P/T* piperacillin/tazobactam, *CIP* ciprofloxacin, *AMK* amikacin, *CLS* colistin

scarce on this point, and further studies are needed to clarify whether MBL production is truly associated with poor outcome and should be tested routinely in clinical settings.

Tests for detecting MBLs may contribute to improved treatment of infections with carbapenem-resistant *Enterobacteriaceae*. These rapid tests include SMA Eiken (SMA, disk diffusion; Eiken Chemical) [10], Cica-β-test [6], PCR [11], and immunochromatographic assays [7]. Infections with MBL producers that have lower breakpoints than those presented by CLSI have been reported [12]. Therefore, additional methods may be required to accurately diagnose infections caused by MBL producers.

To our knowledge, this is the first report of IMP-type MBL-producing *E. cloacae* bloodstream infection in Japan, although a number of VIM-type MBL-producing *E. cloacae* infections have been reported in European countries [2, 3]. The Center for Disease Control and Prevention recommends active surveillance following isolation of carbapenemase-producing *Klebsiella* spp. or *Escherichia coli* because these isolates represent the majority of carbapenemase-producing *Enterobacteriaceae* in the United States [13]. However, active surveillance of *Enterobacter cloacae* is not included in this recommendation. More information is required to determine the validity of active surveillance of MBL-producing *E. cloacae* in healthcare facilities in Japan.

It is unclear whether carbapenems are effective against infections caused by IMP type MBL-producing *E. cloacae* showing MIC within the susceptible range. Two of our three cases were refractory to MEM, suggesting clinical inefficacy of carbapenems against MBL-producing *E. cloacae* regardless of their MIC. Falcone et al. [3] described seven cases of VIM-1-type MBL-producing *E. cloacae* infections: these cases were difficult to diagnose because of apparent susceptibility to carbapenems and were associated with high relapse rate and a prolonged duration of antibiotic therapy. Clinical studies on appropriate chemotherapies against MBL-producing *E. cloacae* infections will be required.

We reported three cases of MBL-producing *E. cloacae* showing relatively low MICs around the breakpoints for carbapenems. Effective testing strategies should be urgently implemented in medical facilities to adequately detect carbapenem-resistant *E. cloacae.*

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Conflict of interest None declared.

Ethical approval Approved by Human Research Ethics Committee of National Center for Global Health and Medicine (NCGM/G/001232/00).

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RESEARCH ARTICLE Open Access

NDM-1 Metallo-β-Lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal

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Abstract

Background: Drug-resistant *Providencia rettgeri* producing metallo-β-lactamase and 16S rRNA methylase has been reported in several countries. We analyzed *P. rettgeri* clinical isolates with resistance to carbapenems and aminoglycosides in a hospital in Nepal.

Methods: Five clinical isolates of multidrug-resistant *P. rettgeri* were obtained in a hospital in Nepal. Antimicrobial susceptibilities were determined using the microdilution method and entire genomes were sequenced to determine drug-resistant genes. Epidemiological analysis was performed by pulsed-field gel electrophoresis.

Results: Four of the 5 isolates were resistant to carbapenems (imipenem and meropenem), with MICs ≥16 mg/L, with the remaining isolate showing intermediate resistance to imipenem, with an MIC of 2 mg/L and susceptibility to meropenem with an MIC ≤1 mg/L. All 5 isolates had bla_{VEB-1}. Of the 4 carbapenem-resistant strains, 3 had bla_{NDM-1} and 1 had bla_{OXA-72}. All isolates were highly resistant to aminoglycosides (MICs ≥1,024 mg/L) and harbored *armA*. As the result of pulsed-field gel electrophoresis pattern analysis in the 5 *P. rettgeri* isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity.

Conclusions: This is the first report describing multidrug-resistant *P. rettgeri* strains harboring bla_{NDM-1} or bla_{OXA-72} and *armA* isolated from patients in Nepal.

Keywords: NDM-1, OXA-72, 16S rRNA methylase, *Providencia rettgeri*, Molecular epidemiology

Background

Providencia rettgeri has been associated with hospital acquired infections, including catheter-related urinary tract infections, bacteremia, skin infections, diarrhea, and gastroenteritis [1,2]. To date, there have been 5 reports of *P. rettgeri* isolates harboring metallo-β-lactamase (MBL) encoding genes, including IMP-type MBL producers in Japan [3,4]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [5]; and NDM-type MBL in Israel [6] and Brazil [7].

NDM-type MBL was initially identified in *Klebsiella pneumoniae* and *Escherichia coli* in 2009 in Sweden [8]. Since then, NDM-1-producing *Enterobacteriaceae* have been isolated in various parts of the world [9,10].

Exogenously acquired 16S rRNA methylase genes responsible for very high levels of resistance to various aminoglycosides are widely distributed among *Enterobacteriaceae* and glucose-nonfermentative microbes [11]. Gram-negative pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [11].

Although co-production of several resistance determinants is not rare in *Enterobacteriaceae* [12-16], it is less common in *P. rettgeri* [5]. We describe here *P. rettgeri* clinical isolates from Nepal that produce carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA).

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Table 1 Summary of the characteristics of the 5 *P. rettegeri* strains, including antimicrobial resistance profiles and resistant genes

Strains	Tissue sources	Infection	MIC (mg/L)															Antibiotics resistant genes
			PIP	TZP	CAZ	FEP	IPM	DPM	MEM	ATM	ABK	AMK	GEN	CIP	CST	FOF	TIG	
IOMTU1	Pus	SSI	1,024	512	>1,024	64	32	16	64	1,024	>1,024	>1,024	>1,024	128	>128	512	4	bla_{NDM-1}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*, *aadA2*
IOMTU4	Sputum	NLRTI	1,024	128	>1,024	256	16	16	32	1,024	>1,024	>1,024	>1,024	>256	>128	512	4	bla_{OXA-72}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU91	Sputum	NLRTI	>1,024	1,024	>1,024	1,024	64	32	64	1,024	>1,024	>1,024	>1,024	256	128	128	4	bla_{NDM-1}, bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU94	Pus	SSI	1,024	4	>1,024	256	2	1	1	>1,024	1,024	1,024	>1,024	256	>128	1,024	4	bla_{OXA-10}, bla_{VEB-1}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*
IOMTU99	Sputum	NLRTI	>1,024	512	>1,024	128	64	32	64	1,024	>1,024	>1,024	>1,024	>256	>128	1,024	4	bla_{NDM-1}, bla_{VEB-1}, bla_{OXA-10}, bla_{TEM-1}, bla_{ADC-67}, *armA*, *aadA1*

SSI, surgical site infection; NLRTI, nosocomial lower respiratory tract infection PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; DPM, doripenem; MEM, meropenem; ATM, aztreonam; ABK, arbekacin; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; CST, colistin; FOF, fosfomycin; TIG, tigecycline.

Methods

Bacterial strains

Five *P. rettgeri* clinical isolates were obtained from May to July 2012 from 5 patients at Tribhuvan University Teaching Hospital in Kathmandu, Nepal. Three isolates were from sputum and 2 from pus at surgical sites. Samples were obtained as part of standard patient care. Phenotypical identification [17] was confirmed by API 32GN (BioMérieux, Mercy l'Etoile, France) and 16S rRNA sequencing (1,497 bp) [18,19].

Antimicrobial susceptibilities

MICs were determined using the microdilution method, according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [20]. Breakpoints to antibiotics were determined. The modified Hodge test, the meropenem-sodium mercaptoacetic acid double-disk synergy test (Eiken Chemical, Tokyo, Japan) and E-test (imipenem/EDTA) (AB Biodisk, Solna, Sweden) were performed.

Entire genome sequencing

The entire genomes of these isolates were extracted and sequenced by MiSeq (Illumina, San Diego, CA). CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used for de novo assembly of reads and to search for 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acethyl/adenylyltransferases; point mutations in the *gyrA*, *parC* and *pmrCAB* operons; and point mutations in the *fos* genes, including *fosA*, *fosA2*, *fosA3*, *fosC* and *fosC2*.

Pulsed-field gel electrophoresis (PFGE) and southern hybridization

PFGE analysis was performed as described [3]. An 813 bp probe for bla_{NDM-1} was synthesized by PCR amplification using the primers 5′-atggaattgcccaatattatgcac-3′ (forward) and 5′-tcagcgcagcttgtcggccatgcggg-3′ (reverse), and a 780 bp probe for bla_{OXA-72} was synthesized using the primers 5′-agtttctctcagtgcatgttcatctat-3′ (forward) and 5′-agaaccagacattccttctttcatttc-3′ (reverse). Southern hybridization to detect bla_{NDM-1} and bla_{OXA-72} was performed using these probes, which were detected using DIG High Prime DNA labeling and detection starter kit II (Roche Diagnostics, Mannheim, Germany).

Nucleotide sequence accession numbers

The nucleotide sequences surrounding bla_{NDM-1} and bla_{OXA-72} have been deposited in GenBank with the accession number AB828598 and AB857844, respectively.

Ethical approval

The study protocol was reviewed and approved by the Institutional Review Board of the Institute of Medicine, Tribhuvan University (ref. 6-11-E) and the Biosafety Committee, National Center for Global Health and Medicine (approval number: 23-M-49).

Results

Antimicrobial susceptibilities

Four of the 5 isolates were resistant to carbapenems (doripenem, imipenem and meropenem) and piperacillin/tazobactam, whereas the fifth was susceptible to piperacillin/tazobactam, doripenem and meropenem and showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cefepime), aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin, and all 5 showed intermediate resistance to tigecycline. The four isolates resistant to carbapenems were negative with the modified Hodge test, but three of the four isolates were positive with the meropenem-sodium mercaptoacetic acid double-disk synergy test and E-test/EDTA.

Drug-resistant genes

All 5 isolates tested had several genes associated with β-lactam and aminoglycoside-resistance (Table 1). These isolates had bla_{VEB-1}, bla_{OXA-10}, bla_{TEM-1}, bla_{ADC-67} (*ampC*), *armA* and *aadA1*; 3 had bla_{NDM-1}; and 1 had bla_{OXA-72}. None of these isolates had any other β-lactamase encoding genes, including the class A genes bla_{SHVs} and bla_{CTX-Ms}; the class B genes bla_{AIM}, bla_{DIM}, bla_{FIM}, bla_{GIM}, bla_{IMPs}, bla_{INDs}, bla_{KHM}, bla_{SIM}, bla_{SMB}, bla_{SPM}, bla_{TMBs}, and bla_{VIMs}; or the class D gene bla_{OXAs} except for bla_{OXA-10} and bla_{OXA-72}. None had other genes encoding 16S rRNA methylases or aminoglycoside acetyl/adenylyltransferases. All 5 isolates had point mutations in the quinolone-resistance-determining regions of *gyrA* and *parC*, with amino acid substitutions of S83I and D87E in GyrA and S80I in ParC, but none had any mutations in the *pmrCAB* operon and *fos* genes. All sequences of the drug-resistant genes tested were identical to those registered in GenBank.

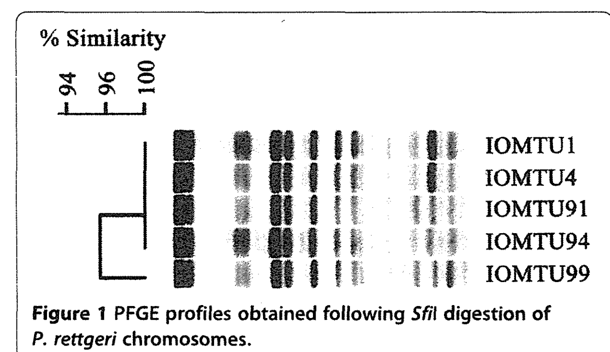

Figure 1 PFGE profiles obtained following *SfiI* digestion of *P. rettgeri* chromosomes.