



Emergence of Carbapenem-Resistant *Providencia rettgeri* and *Providencia stuartii* Producing IMP-Type Metallo- β -Lactamase in Japan

Shu Iwata,^a Tatsuya Tada,^a Tomomi Hishinuma,^a Mari Tohya,^a Satoshi Oshiro,^a Kyoko Kuwahara-Arai,^a Miho Ogawa,^b Masahiro Shimojima,^b  Teruo Kirikae^a

^aDepartment of Microbiology, Juntendo University School of Medicine, Tokyo, Japan

^bBML, Inc., Kawagoe, Saitama, Japan

ABSTRACT Four *Providencia rettgeri* isolates and one *Providencia stuartii* isolate were obtained from urine samples of five patients in 2018 in Japan. All of the isolates were resistant to imipenem and meropenem, and three were highly resistant to both carbapenems, with MICs of 512 μ g/ml. The three highly carbapenem-resistant isolates harbored *bla*_{IMP-70}, encoding a variant of IMP-1 metallo- β -lactamase with two amino acid substitutions (Val67Phe and Phe87Val), and the other two harbored *bla*_{IMP-1} and *bla*_{IMP-11}, respectively. Whole-genome sequencing revealed that an isolate harbored two copies of *bla*_{IMP-1} on the chromosome and that the other four harbored a copy of *bla*_{IMP-11} or *bla*_{IMP-70} in a plasmid. Expression of *bla*_{IMP-70} conferred carbapenem resistance in *Escherichia coli*. Recombinant IMP-70 and an IMP-1 variant with Val67Phe but without Phe87Val had significant higher hydrolytic activities against meropenem than recombinant IMP-1, indicating that an amino acid substitution of Val67Phe affects increased activities against meropenem in IMP-70. These results suggest that *Providencia* spp. become more highly resistant to carbapenems by acquisition of two copies of *bla*_{IMP-1} or by mutation of *bla*_{IMP} genes with amino acid substitutions, such as *bla*_{IMP-70}.

KEYWORDS *Providencia rettgeri*, *Providencia stuartii*, IMP-70, metallo- β -lactamase

Providencia is a genus of Gram-negative bacteria belonging to the family Enterobacteriaceae. Among *Providencia* spp., *Providencia rettgeri* and *Providencia stuartii* are the common causes of hospital-acquired infections (1, 2). They mainly cause travelers' diarrhea and catheter-related urinary tract infections (3, 4). However, there are several case reports of other infections with *P. rettgeri* and *P. stuartii*, including neonatal sepsis caused by *P. rettgeri* (5) and conjunctivitis (6), meningitis after neurosurgery (7), and endocarditis (8) caused by *P. stuartii*, indicating that these pathogens have the potency to cause serious infections.

P. rettgeri clinical isolates producing IMP-1 metallo- β -lactamase (MBL) were first obtained in 2000 during a laboratory-based surveillance in western Japan (9). A *P. stuartii* clinical isolate producing VIM-19 MBL was first reported in 2008 in Algeria (10). Since then, there have been at least 12 reports of carbapenem-resistant *P. rettgeri* isolates and 5 reports of carbapenem-resistant *P. stuartii* isolates (Table 1). All of these isolates produced MBLs, and the majority of carbapenem-resistant *P. rettgeri* isolates produced IMP-type or NDM-type MBLs (Table 1), i.e., IMP-type MBL-producing isolates were detected in Japan, the Republic of Korea, and the United States, and NDM-type MBL-producing isolates were detected worldwide. The majority of carbapenem-resistant *P. stuartii* isolates, however, produced VIM-type MBLs, and these were detected in Algeria and Greece (Table 1). There is a report describing an NDM-type MBL-producing *P. stuartii* isolate in Afghanistan (Table 1).

Citation Iwata S, Tada T, Hishinuma T, Tohya M, Oshiro S, Kuwahara-Arai K, Ogawa M, Shimojima M, Kirikae T. 2020. Emergence of carbapenem-resistant *Providencia rettgeri* and *Providencia stuartii* producing IMP-type metallo- β -lactamase in Japan. Antimicrob Agents Chemother 64:e00382-20. <https://doi.org/10.1128/AAC.00382-20>.

Copyright © 2020 American Society for Microbiology. All Rights Reserved.

Address correspondence to Teruo Kirikae, t-kirikae@juntendo.ac.jp.

Received 28 February 2020

Returned for modification 16 March 2020

Accepted 8 August 2020

Accepted manuscript posted online 17 August 2020

Published 20 October 2020

TABLE 1 Previous reports of *P. rettgeri* and *P. stuartii* isolates producing MBLs

Species	Metallo- β -lactamase	Location of MBL-encoding gene ^a	Yr(s) of isolation	Country of isolation	Reference
<i>P. rettgeri</i>	IMP-1	–	2000	Japan	9
	IMP-1	Plasmid (2.5 kb)	2002	Japan	30
	IMP-27	Plasmid	2016	USA	31
	IMP-27	–	2018	Republic of Korea	32
	NDM-1	–	2008, 2011	Israel	33
	NDM-1	Plasmid	2012	Nepal	34
	NDM-1	Plasmid (178 kb)	2012	Mexico	35
	NDM-1	Plasmid (190 kb)	2014	China	36
	NDM-1	–	2017	Bulgaria	37
	NDM	–	2013	Brazil	38
NDM-18	Plasmid	2017	South Africa	39	
<i>P. stuartii</i>	VIM-2	–	2004	Republic of Korea	40
	NDM-1	Plasmid (178kb)	2012	Afghanistan	41
	VIM-1	–	2011	Greece	42
	VIM-1	Plasmid (180 kb, IncA/C)	2012	Greece	43
	VIM-1	Plasmid	2013	Greece	44
	VIM-19	Plasmid (180 kb)	2008	Algeria	10

^aA dash (–) indicates that the location of MBL-encoding genes was unspecified.

IMP-type MBL was first detected in a clinical isolate of *Pseudomonas aeruginosa* in Japan in 1991 (11). Since then, IMP-type MBL producers have been spreading nationwide in Japan and in east and southeast Asian countries, although they have been globally detected (12).

This report describes the detection of 5 clinical isolates of carbapenem-resistant *P. rettgeri* and *P. stuartii* producing IMP-type metallo- β -lactamase in 3 hospitals located in different regions in Japan.

RESULTS

Drug susceptibility. As shown by the drug susceptibility profiles of 5 *Providencia* sp. isolates shown in Table 2, *P. rettgeri* BML2496 was susceptible to amikacin and aztreonam but resistant to amoxicillin, amoxicillin-clavulanate, ceftazidime, cephadrine, ciprofloxacin, and meropenem. The MICs of imipenem and meropenem were 16 and 64 μ g/ml, respectively. *P. rettgeri* BML2531 showed a similar profile of drug susceptibility to that of BML2496 (Table 2). However, against *P. rettgeri* BML2526 and BML2576, MICs of imipenem and meropenem (512 μ g/ml) were much higher than those against BML2496 and BML2531 (Table 2). BML2526 and BML2576 were highly resistant to ciprofloxacin (MICs of 512 μ g/ml and 256 μ g/ml, respectively). *P. stuartii* BML2537 was intermediate to amikacin but resistant to amoxicillin, amoxicillin-clavulanate, aztreonam, ceftazidime, cephadrine, ciprofloxacin, and meropenem, and MICs of imipenem and meropenem (>512 μ g/ml and 512 μ g/ml, respectively)

TABLE 2 Drug susceptibility profiles of *Providencia* sp. clinical isolates

Antibiotic(s)	MIC (μ g/ml) of antibiotic against:				
	<i>P. rettgeri</i>				<i>P. stuartii</i>
	BML2496	BML2531	BML2526	BML2576	BML2537
Arbekacin	4	8	2	8	2
Amikacin	8	4	32	32	32
Amoxicillin	512	>512	>512	>512	>512
Amoxicillin-clavulanate ^a	256	128	128	128	512
Aztreonam	2	<0.25	8	8	32
Ceftazidime	128	256	128	128	512
Cephadrine	>512	>512	>512	>512	>512
Ciprofloxacin	32	64	512	256	16
Colistin	>64	>64	>64	>64	>64
Imipenem	16	32	512	512	>512
Meropenem	64	32	512	512	512

^aThe compound ratio of amoxicillin versus clavulanate is 1:5 (wt/wt).

were much higher (Table 2). For all isolates tested, arbekacin MICs were low (2 to 8 $\mu\text{g/ml}$), but colistin MICs were high (64 $\mu\text{g/ml}$) (Table 2).

Drug-resistant genes. All five isolates had several genes and mutations associated with resistance to aminoglycosides, β -lactams, and fluoroquinolones (Table 3). All had one or two of five genes encoding aminoglycoside-modifying enzymes, namely *aac(2')-Ia*, *aac(6')-Iae*, *aac(6')-Ib4*, *aac(6')-II*, and/or *aadA1* (Table 3). The *aac(6')-Iae* gene may be associated with the increased susceptibility to amikacin of BML2526, BML2537, and BML 2576 (Table 3). Isolates had one of three genes encoding metallo- β -lactamases, namely *bla*_{IMP-1}, *bla*_{IMP-11}, or *bla*_{IMP-70} (Table 3). In addition, they had one or two of four other genes encoding β -lactamases, namely *bla*_{CTX-M-2}, *bla*_{OXA-1}, *bla*_{OXA-2}, and/or *bla*_{TEM-1B} (Table 3). All isolates had three or four mutations in the quinolone resistance-determining regions of *gyrA*, *parC*, and *gyrB* with amino acid substitutions that included Ser83Ile, Asp87Ala, and Asp87Glu in *gyrA*; Ser87Ile and Ser87Arg in *parC*; and Glu468Asp in *gyrB* (Table 3).

Complete whole-genome sequences. Analysis using MiSeq and MinION platforms revealed the complete whole-genome sequences of all five isolates (Table 3). Four isolates of *P. rettgeri* had a 4.3- to 4.7-Mbp chromosome with a GC content of 49.6 to 51.0%, and an isolate of *P. stuartii* had a 4.4-Mbp chromosome with a GC content of 49.8% (Table 3). BML2496 did not have any plasmid, but the others had one or two plasmid(s) of 30 to 200 kbp (Table 3). BML2526 had two plasmids of 70 kbp and 200 kbp, and BML2576 had two with the same sizes as those of BML2526. The sequences of the two 70-kbp and 200-kbp plasmids were identical between the two isolates.

Locations of *bla*_{IMP} genes and their genetic environments. The *bla*_{IMP-1} gene was located on the chromosome of BML2496 (Table 3). The *bla*_{IMP-11} gene was located in an 85-kbp plasmid of BML2531. The *bla*_{IMP-70} gene was located in a 200-kbp plasmid of BML2526/BML2576 and in a 150-kbp plasmid of BML2537 (Table 3).

Two copies of *bla*_{IMP-1} were detected on the chromosome of BML2496, and the two copies existed in a row at nucleotide positions 3,384,049 to 3,384,789 and 3,387,611 to 3,387,958, i.e., the following genetic structure of *int1* Δ -*bla*_{IMP-70}-*qacE* Δ 1-*sul1* was repeated (Fig. 1). To confirm the existence of the two copies of *bla*_{IMP-1}, PCR was conducted using a primer set (IMP-1 DF and IMP-1 DR) targeting the two copies, as shown in Fig. 1. Consequently, a 3.5-kbp PCR product was detected as expected based on the whole-genome sequence of BML2496 (Fig. 1).

Drug susceptibility of *Escherichia coli* DH5 α expressing *bla*_{IMP-1}, *bla*_{IMP-70}, and two variants of *bla*_{IMP-1}. IMP-70 is a variant of IMP-1 with two amino acid substitutions, Val67Phe and Phe87Val (GenBank accession no. [LC348383.1](https://www.ncbi.nlm.nih.gov/nucl/NC_049173)). A variant of *bla*_{IMP-1} encoding an amino acid substitution of Val67Phe was *bla*_{IMP-10} (accession no. [NG_049173](https://www.ncbi.nlm.nih.gov/nucl/NC_049173)). Another variant of *bla*_{IMP-1} encoding an amino acid substitution of Phe87Val [*bla*_{IMP-1(Phe87Val)}] was prepared as described in Materials and Methods. *E. coli* DH5 α expressing *bla*_{IMP-1}, *bla*_{IMP-10}, *bla*_{IMP-1(Phe87Val)}, and *bla*_{IMP-70} showed significantly increased MICs for all antibiotics tested, including four carbapenems, compared with those of a vector control (Table 4). Compared with *E. coli* expressing *bla*_{IMP-1}, *E. coli* expressing *bla*_{IMP-70} showed slightly, but not significantly, increased MICs of doripenem and meropenem, while it showed the same MICs of imipenem and panipenem (Table 4). However, *E. coli* expressing *bla*_{IMP-10} showed a significantly higher MIC of doripenem and a slightly increased MIC of meropenem (Table 4). *E. coli* expressing *bla*_{IMP-1(Phe87Val)}, in contrast, showed no increased MIC for all carbapenems tested (Table 4).

Enzymatic activities of IMP-1, IMP-70, and two variants of IMP-1. IMP-70 is a variant of IMP-1 with Val67Phe and Phe87Val. Two variants of IMP-1 with an amino acid substitution of Val67Phe (IMP-10) and that of Phe87Val [IMP-1(Phe87Val)] were prepared as described in Materials and Methods. Enzymatic activities of recombinant IMP-70, IMP-10, and IMP-1(Phe87Val) were compared with those of recombinant IMP-1. IMP-70 and IMP-10 showed greater hydrolytic activities than IMP-1 against meropenem, i.e., the k_{cat}/K_m values of IMP-70 and IMP-10 were 2.3- and 3.4-fold higher, respectively, than those of IMP-1. However, IMP-70 showed similar enzyme activities against dorip-

TABLE 3 Genetic characterization of carbapenem-resistant *Providencia* sp. isolates

Isolate	Genome	Plasmid type	Size (bp)	GC content (%)	Antibiotic resistance gene(s) encoding:		Amino acid substitution(s) in quinolone resistance gene:		
					Aminoglycoside(s)	β -lactam(s)	GyrA	ParC	GyrB
<i>P. rettgeri</i> BML2496	Chromosome		4,651,003	49.6	<i>aac(6)-Ib4</i>	<i>bla_{IMP-11}</i> , <i>bla_{OXA-1}</i>	Ser83Ile, Asp87Ala	Ser87Ile	
<i>P. rettgeri</i> BML2526	Chromosome	IncA/C2	4,342,905	51.0	<i>aadA1</i>				
	Plasmid	Nontypeable	204,791	50.2	<i>aac(6)-Iae</i>	<i>bla_{IMP-70'}</i> , <i>bla_{CTX-M-2'}</i> , <i>bla_{TEM-1B}</i>	Ser83Ile, Asp87Glu	Ser87Ile	Glu468Asp
<i>P. rettgeri</i> BML2531	Chromosome	IncT	4,696,377	50.1					
	Plasmid		84,930	50.3	<i>aac(6)-II</i>	<i>bla_{IMP-11'}</i> , <i>bla_{OXA-2}</i>	Ser83Ile, Asp87Glu	Ser87Ile	
<i>P. rettgeri</i> BML2576	Chromosome	IncA/C2	4,351,300	51.0	<i>aadA1</i>				
	Plasmid	Nontypeable	204,791	50.2%	<i>aac(6)-Iae</i>	<i>bla_{IMP-70'}</i> , <i>bla_{CTX-M-2'}</i> , <i>bla_{TEM-1B}</i>	Ser83Ile, Asp87Glu	Ser87Ile	Glu468Asp
<i>P. stuartii</i> BML2537	Chromosome	IncA/C	4,418,649	49.8%	<i>aac(2)-Ia</i>				
	Plasmid	Nontypeable	152,754	50.0%	<i>aac(6)-Iae</i>	<i>bla_{IMP-70}</i> , <i>bla_{CTX-M-2}</i>	Ser83Ile, Asp87Glu	Ser87Arg	

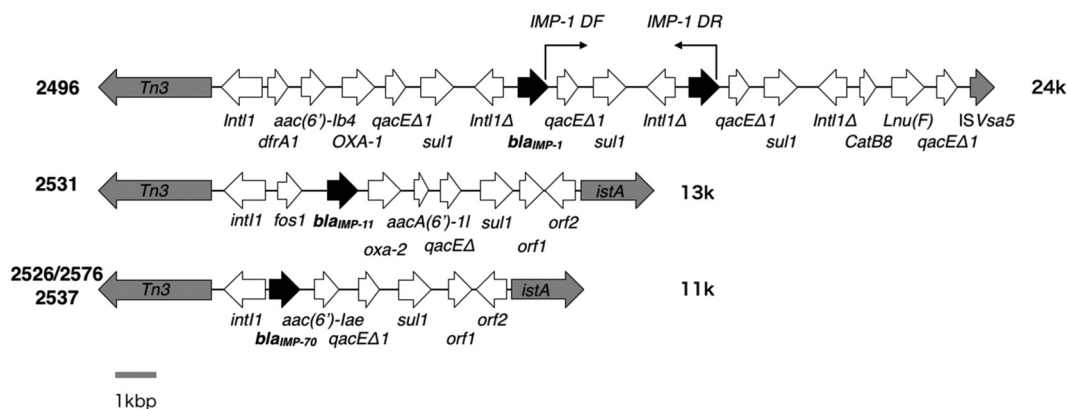


FIG 1 Genomic environments of *bla*_{IMP-1} and *bla*_{IMP-70} in *P. rettgeri* and *P. stuartii* clinical isolates. Genes are represented as arrows, which indicate their transcription orientations and relative lengths. MBL genes, *tnp* genes, and truncated genes are shown as black arrows, gray arrows, and Δ, respectively. *orf1* is a gene encoding a hypothetical protein, and *orf2* is a gene encoding an ATP-binding protein.

enem, imipenem, and panipenam to those of IMP-1 (Table 5). IMP-1(Phe87Val) showed similar or reduced enzymatic activities against all carbapenems tested (Table 5).

Production of IMP-type MBL in clinical isolates of *P. rettgeri* and *P. stuartii*. As shown by Western blotting in Fig. 2, all five isolates tested (BML2496, BML2526, BML2531, BML2537, and BML2576) produced IMP-type MBLs (IMP-1 was produced by BML2496, and IMP-70 was produced by the other three isolates). *P. rettgeri* BML2496, an IMP-1 producer, produced the largest amount of IMP-type MBL among all isolates tested (Fig. 2). *P. rettgeri* BML2526 and BML2576 and *P. stuartii* BML2537 produced relatively large amounts of IMP-type MBLs, whereas BML2531 produced a smaller amount of IMP-type MBL (IMP-11) than did the other four isolates tested. IMP-type MBL (IMP-11) produced by BML2531 was detected as a band with a lower molecular weight than those of other bands produced by the remaining four isolates, which correspond to their molecular weights calculated with amino acid sequences (27.06 kDa and 27.12 kDa, respectively).

DISCUSSION

Two copies of *bla*_{IMP-1} in *P. rettgeri* BML2496 may be necessary for effective IMP-1 production and high hydrolytic activities against carbapenems. Western blot analysis revealed that BML2496 produced a relatively high level of IMP-1 (Fig. 2). There are at least four reports describing carbapenem-resistant Gram-negative pathogens harboring two copies of genes encoding carbapenemases (13–16). An isolate of *P. aeruginosa* obtained from a patient in Serbia harbored two *bla*_{NDM-1} copies in the genome on

TABLE 4 Drug susceptibility profiles of *E. coli* isolates expressing IMP-1, IMP-10, a variant of IMP-1 with an amino acid substitution (Phe87Val), and IMP-70

Antibiotic(s)	Antibiotic MIC (μg/ml) against:				
	<i>E. coli</i> DH5α (pHSG398)	<i>E. coli</i> DH5α (pHSG398/IMP-1)	<i>E. coli</i> DH5α (pHSG398/IMP-10) ^a	<i>E. coli</i> DH5α [pHSG398/IMP-1(Phe87Val)]	<i>E. coli</i> DH5α (pHSG398/IMP-70) ^b
Amoxicillin	4	512	32	32	16
Amoxicillin-clavulanate ^c	4	128	32	16	8
Ceftazidime	≤0.25	128	256	16	64
Cephadrine	16	256	128	128	64
Doripenem	≤0.06	2	8	1	4
Imipenem	0.125	1	1	1	1
Meropenem	≤0.06	4	8	2	8
Panipenem	0.125	2	2	1	2

^aIMP-10 is a variant of IMP-1 with an amino acid substitution of Val67Phe.

^bIMP-70 is a variant of IMP-1 with two amino acid substitutions, Val67Phe and Phe87Val.

^cThe compound ratio of amoxicillin versus clavulanate is 1:5 (wt/wt).

TABLE 5 Kinetic parameters of β -lactamases IMP-1 and IMP-70 with substrates

Substrate	Kinetic parameter for β -lactamase:											
	K_m (μM) ^a				K_{cat} (s^{-1}) ^a				K_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$) ^a			
	IMP-1	IMP-10	IMP-1(F87V)	IMP-70	IMP-1	IMP-10	IMP-1(F87V)	IMP-70	IMP-1	IMP-10	IMP-1(F87V)	IMP-70
Penicillin G	941 ± 53	305 ± 17	818 ± 48	203 ± 6	390 ± 11	14 ± 1	13 ± 1	7 ± 0	0.42	0.047	0.016	0.036
Ceftazidime	71 ± 3	259 ± 5	363 ± 5	106 ± 6	7 ± 0	43 ± 1	4 ± 0	2 ± 0	0.1	0.17	0.0097	0.021
Doripenem	32 ± 3	10 ± 1	2626 ± 61	27 ± 0	4 ± 0	8 ± 0	239 ± 5	5 ± 0	0.13	0.82	0.091	0.18
Imipenem	205 ± 14	472 ± 18	534 ± 26	88 ± 6	46 ± 2	112 ± 3	92 ± 3	21 ± 0	0.23	0.24	0.17	0.24
Meropenem	40 ± 2	79 ± 3	1799 ± 67	36 ± 2	6 ± 0	40 ± 2	310 ± 11	13 ± 0	0.15	0.51	0.17	0.35
Panipenem	60 ± 6	46 ± 2	744 ± 19	83 ± 6	24 ± 1	16 ± 0	178 ± 4	19 ± 1	0.4	0.35	0.24	0.23

^a K_m and k_{cat} were calculated as means ± SD from three independent experiments.

the chromosome (13). Carbapenem-resistant *P. aeruginosa* harboring two copies of *bla*_{IMP-34} on the chromosome was isolated from a patient in Japan (14). A clinical isolate of *E. coli* obtained from a patient in China harbored *bla*_{NDM-1} on the chromosome along with two tandem copies of an *ISCR1* element (15). An *E. coli* isolate obtained from a patient in China had an IncF plasmid carrying two copies of *bla*_{NDM-5} (16). Our present study, together with these reports, strongly suggests that acquisition of two copies of a gene encoding carbapenemase is a strategy of Gram-negative pathogens to obtain high resistance to carbapenems.

The amino acid substitution of Val67Phe, but not that of Phe87Val, in IMP-70 seems to play a crucial role in a significant increase compared with IMP-1 of enzymatic activities against meropenem. Amino acid residue 67 in IMP-1 is located at the end of a loop close to the active site that consists of residues 60 to 66 (17). This loop is a major determinant for the tight binding of substrates in the active site (17). A Val67Phe amino acid substitution in IMP-43, a variant of IMP-7, increased catalytic activities against imipenem and meropenem (18). Amino acid substitutions at position 67 in IMP-1 MBLs affect the ability to hydrolyze and confer resistance to β -lactams (19). Residue 67 was

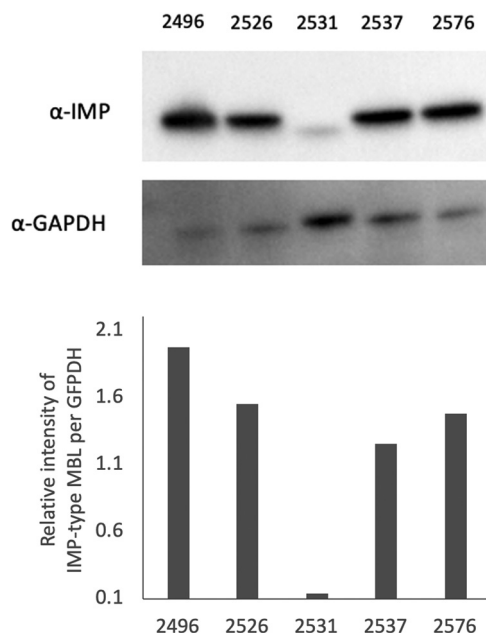


FIG 2 IMP-type metallo- β -lactamase expression in carbapenem-resistant clinical isolates of *P. rettgeri* and *P. stuartii*. Bacterial cells of *P. rettgeri* BML2496, BML2526, BML2531, and BML2576 and of *P. stuartii* BML2537 were solubilized in 2×10^7 CFU, which was applied to an SDS-PAGE gel and transferred onto Immobilon-P. IMP-type MBL and GAPDH were visualized using monoclonal antibodies (upper panel), respectively, as described in Materials and Methods. Relative intensity of IMP-type MBL per GAPDH was calculated using NIH ImageJ software (<https://imagej.nih.gov/ij/>) (lower).

important for substrate binding in VIM-type MBLs (20). Residue 87 plays a critical role in the stability and folding of VIM-2 (21).

IMP-44, a variant of IMP-11 with two substitutions (Val67Phe and Phe87Ser), had more efficient catalytic activities against carbapenems than those of IMP-11 (18), suggesting that coexistence of two amino acid substitutions at positions 67 and 87 may be necessary to increase enzymatic activities and change substrate profiles in IMP-44. However, a substitution of Phe87Val in IMP-70 did not affect enzymatic activities (Table 5). A substitution of Phe87 to a polar amino acid such as Ser, but not to a hydrophobic amino acid such as Val, may affect enzymatic activities.

Regarding the naming of IMP-70, two sequences of IMP-70 were registered at the NCBI database (GenBank accession no. [LC348383](#) and [AUM56801](#)). One sequence (accession no. [LC348383](#)) was registered in December 2017, and the other (accession no. [AUM56801](#)) in January 2018. We describe here IMP-70, a variant of IMP-1 in *P. rettgeri* and *P. stuartii* isolates, the sequences of which were deposited in the NCBI database (accession no. [LC507075](#) and [AP022377](#), respectively). These sequences correspond to that of IMP-70 (accession no. [LC348383](#)). To avoid confusion, IMP-70 sequences should be mentioned with their accession numbers, such as IMP-70 (accession no. [LC348383](#)).

MATERIALS AND METHODS

Bacterial strains and identification. Four *P. rettgeri* isolates and a *P. stuartii* isolate were obtained from urine samples of five patients (76.6 ± 7.6 years old) collected from October to September 2018 in 3 hospitals in Japan (BML Biomedical Laboratories R&D Center, Kawagoe, Saitama, Japan). *P. rettgeri* BML2496 and BML2531 were isolated at two hospitals in Saitama and Kochi, Japan, respectively. Isolates of *P. rettgeri* BML2526 and BML 2576 and of *P. stuartii* BML2537 were obtained at a hospital in Osaka, Japan. Bacterial species were identified by WalkAway System (Beckman Coulter, Brea, CA), and confirmed by 16S RNA sequencing. A clinical isolate of *Pseudomonas aeruginosa* harboring *bla*_{IMP-10} was used to clone the *bla*_{IMP-10} gene (18).

Drug susceptibility testing. MICs of ceftazidime, aztreonam, imipenem, meropenem, arbekacin, amikacin, and cephalosporin were determined using the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (22). MICs of colistin were determined using the microdilution method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (23).

Whole-genome sequencing. DNA of these isolates was extracted using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan), and their complete genomes were sequenced using the MiSeq platform (Illumina, San Diego, CA). The raw reads were assembled using CLC Genomics Workbench v10.0.1 (CLC bio, Aarhus, Denmark). The sequences of drug resistance genes, including those encoding β -lactamase and aminoglycoside resistance, were determined using ResFinder v3.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>). The complete genome sequences of all 5 isolates were determined using MinION (Oxford Nanopore Technologies, Oxford, UK). The raw data were base called by Albacore v2.3.1, and adapters were removed by Porechop v0.2.3 (<https://github.com/rrwick/Porechop>). The long reads generated by MinION and the short reads generated by MiSeq were assembled using Unicycler. Drug resistance genes encoding β -lactamases, aminoglycoside modification enzymes, and 16S rRNA methylases (24) were detected using ResFinder 2.1 (<https://cge.cbs.dtu.dk/services/ResFinder/>). The sequences of the quinolone resistance genes (25) *gyrA*, *parC*, and *gyrB* were determined using CLC Genomics Workbench v8.0. Multilocus sequence types (MLSTs) were deduced as described in the protocols of the PubMLST database (<https://pubmlst.org/paeruginosa/>).

PCR. PCR was conducted using a set of primers, IMP-1 DF (5'-GTAAGCCTCAGCATTTACAAGAACC-3') and IMP-1DR (5'-CAAGTCACAGTGAAGTTGGAGAC-3'), to determine a distance between these sequences of *bla*_{IMP-1} on the genome of an isolate (BML2496).

Cloning of *bla*_{IMP-1}, *bla*_{IMP-10}, and *bla*_{IMP-70}. The open reading frames (ORFs) of *bla*_{IMP-1}, *bla*_{IMP-10}, and *bla*_{IMP-70} were PCR amplified using the primers EcoRI-IMP-1/70-F (5'-GGGGAATTCATGAGCAAGTTATCTGTATTC-3') and PstI-IMP-1/70-R (5'-AAACTGCAGTTAGTTAGTTGGTTTGTATGG-3'), and 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 DH5 α , transformants were selected on LB agar containing 100 μ g/ml of chloramphenicol, and their susceptibility to various β -lactams was assayed. A mutant clone of *bla*_{IMP-1} (TTT \rightarrow GTT at nucleotide positions 205 to 207) encoding IMP-1 with an amino acid substitution of Phe87Val [IMP-1(Phe87Val)] was introduced into plasmid pHSG398-IMP-1 and pET28a-IMP-1 using the KOD Plus mutagenesis kit (Toyobo, Osaka, Japan) using a set of primers, Phe87Val F (5'-TAATTGACACTCCAGTTCAGGCTAAAGAT-3') and Phe87Val R (5'-ATCTTTAGCCGTAAGTGGAGTGTCAATTA-3').

Purification of recombinant IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val). To determine the kinetic parameters of these IMP-type enzymes, the ORFs of IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val) without signal peptide regions were amplified by PCR. IMP-1, IMP-70, IMP-10, and IMP-1(Phe87Val) were amplified using the primers BamHI-IMP-1/70-F (5'-ATGGATCCGAAAACCTGTATTCCAAGGCAGAGTCTTGCCAGATTTAA-3') and XhoI-IMP-1/70-R (5'-ATCTCGAGTTAGTTGGTTTGTATGGT-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 μ g/ml of kanamycin. The transformants were incubated overnight in 200 ml of LB broth containing 20 μ g/ml of kanamycin, and the progeny were transferred to 2 liters of the same broth, and further incubated for 3 h. Then, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the bacteria at a final concentration of 0.5 mM/ml, and further incubation was allowed for 2 h. The bacterial cells were collected and 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). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 μ M nitrocefin (Oxoid Ltd., Basingstoke, UK). These enzymes were prepared in 50 mM Tris-HCl buffer (pH 7.5) in the range of 0.41 to 0.51 mg/ml. Kinetic analysis was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 μ M Zn(NO₃)₂ (Nakalai Tesque, Inc., Kyoto, Japan) at 37°C using a UV-visible spectrophotometer (V-730; 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 (26–28).

Western blot analysis. Bacterial cells of *P. rettgeri* BML2496, BML2526, BML2531, and BML2576 and of *P. stuartii* BML2537 growing in the log phase were collected and solubilized in 2×10^7 CFU per 10 μ l of SDS-PAGE sample buffer. These solubilized bacterial cells were applied to an SDS-PAGE gel and transferred onto Immobilon-P (Merk Millipore Ltd., Cork, Ireland). IMP-type MBL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on Immobilon-P were visualized using a rat monoclonal antibody against IMP-type MBL (4E7) (29) and a mouse monoclonal antibody against GAPDH (Proteintech, Cambridge, UK), respectively. As secondary antibodies, horseradish peroxidase (HRP)-linked goat anti-rat IgG (SouthernBiotech, Birmingham, Alabama) and goat anti-mouse IgG (Abcam, London, UK), respectively, were used and were detected by chemiluminescence (Western Blot chemiluminescence HRP substrate; TaKaRa Bio, Shiga, Japan). Relative intensities of IMP-type MBL per GAPDH (log relative intensity values) were calculated using NIH ImageJ software (<https://imagej.nih.gov/ij/>).

Ethics approval. The study protocol was approved by the ethics committee of Juntendo University (approval number 809) and by the Biosafety Committee, Juntendo University (approval number BSL2/29-1). Allowed information about patients included age, gender, and sample tissues.

Data availability. The genome sequence data generated by MiSeq and MinION were deposited in the DNA Data Bank of Japan (DDBJ). The data of assembled and annotated nucleotide sequences were deposited in the GenBank database with the accession numbers AP022371 to AP022377 and LC507075, respectively.

ACKNOWLEDGMENTS

This study was supported by grants from the Japan Society for the Promotion of Science (grants 18K07120 and 19K16652) and from the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development (grant 20fk0108061h0303). S.I. was supported by the Training Program for Medical Students in Basic Research, Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (grant 2117016).

Miho Ogawa and Masahiro Shimojima are employed by BMI, Inc. There are no patents, products in development, or marketed products to declare.

REFERENCES

- Stock I, Wiedemann B. 1998. Natural antibiotic susceptibility of *Providencia stuartii*, *P. rettgeri*, *P. alcalifaciens* and *P. rustigianii* strains. *J Med Microbiol* 47:629–642. <https://doi.org/10.1099/00222615-47-7-629>.
- Abdallah M, Balshi A. 2018. First literature review of carbapenem-resistant *Providencia*. *New Microbes New Infect* 25:16–23. <https://doi.org/10.1016/j.nmni.2018.05.009>.
- Yoh M, Matsuyama J, Ohnishi M, Takagi K, Miyagi H, Mori K, Park KS, Ono T, Honda T. 2005. Importance of *Providencia* species as a major cause of travellers' diarrhoea. *J Med Microbiol* 54:1077–1082. <https://doi.org/10.1099/jmm.0.45846-0>.
- O'Hara CM, Brenner FW, Miller JM. 2000. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clin Microbiol Rev* 13:534–546. <https://doi.org/10.1128/CMR.13.4.534>.
- Sharma D, Sharma P, Soni P. 2017. First case report of *Providencia rettgeri* neonatal sepsis. *BMC Res Notes* 10:536. <https://doi.org/10.1186/s13104-017-2866-4>.
- Crane ES, Shum M, Chu DS. 2016. Case report: *Providencia stuartii* conjunctivitis. *J Ophthalmic Inflamm Infect* 6:29. <https://doi.org/10.1186/s12348-016-0097-9>.
- Sipahi OR, Bardak-Ozdemir S, Ozgiray E, Aydemir S, Yurtseven T, Yaman T, Tasbakan M, Ulusoy S. 2010. Meningitis due to *Providencia stuartii*. *J Clin Microbiol* 48:4667–4668. <https://doi.org/10.1128/JCM.01349-10>.
- Krake PR, Tandon N. 2004. Infective endocarditis due to *Providencia stuartii*. *South Med J* 97:1022–1023. <https://doi.org/10.1097/01.smj.0000141308.19657.ba>.
- Nishio H, Komatsu M, Shibata N, Shimakawa K, Sueyoshi N, Ura T, Satoh K, Toyokawa M, Nakamura T, Wada Y, Orita T, Kofuku T, Yamasaki K, Sakamoto M, Kinoshita S, Aihara M, Arakawa Y. 2004. Metallo- β -lactamase-producing gram-negative bacilli: laboratory-based surveillance in cooperation with 13 clinical laboratories in the Kinki region of Japan. *J Clin Microbiol* 42:5256–5263. <https://doi.org/10.1128/JCM.42.11.5256-5263.2004>.
- Robin F, Aggoune-Khinache N, Delmas J, Naim M, Bonnet R. 2010. Novel VIM metallo- β -lactamase variant from clinical isolates of *Enterobacteriaceae* from Algeria. *Antimicrob Agents Chemother* 54:466–470. <https://doi.org/10.1128/AAC.00017-09>.
- Watanabe M, Iyobe S, Inoue M, Mitsuhashi S. 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 35:147–151. <https://doi.org/10.1128/aac.35.1.147>.

12. Mojica MF, Bonomo RA, Fast W. 2016. B1-metallo- β -lactamases: where do we stand? *Curr Drug Targets* 17:1029–1050. <https://doi.org/10.2174/1389450116666151001105622>.
13. Jovčić B, Lepšanović Z, Begović J, Rakonjac B, Perovanović J, Topisirović L, Kojić M. 2013. The clinical isolate *Pseudomonas aeruginosa* MMA83 carries two copies of the *bla*_{NDM-1} gene in a novel genetic context. *Antimicrob Agents Chemother* 57:3405–3407. <https://doi.org/10.1128/AAC.02312-12>.
14. Tada T, Miyoshi-Akiyama T, Shimada K, Shiroma A, Nakano K, Teruya K, Satou K, Hirano T, Shimojima M, Kirikae T. 2016. A carbapenem-resistant *Pseudomonas aeruginosa* isolate harboring two copies of *bla*_{IMP-34} encoding a metallo- β -lactamase. *PLoS One* 11:e0149385. <https://doi.org/10.1371/journal.pone.0149385>.
15. Shen P, Yi M, Fu Y, Ruan Z, Du X, Yu Y, Xie X. 2017. Detection of an *Escherichia coli* sequence type 167 strain with two tandem copies of *bla*_{NDM-1} in the chromosome. *J Clin Microbiol* 55:199–205. <https://doi.org/10.1128/JCM.01581-16>.
16. Feng Y, Liu L, McNally A, Zong Z. 2018. Coexistence of two *bla*_{NDM-5} genes on an IncF plasmid as revealed by nanopore sequencing. *Antimicrob Agents Chemother* 62:e00110-62. <https://doi.org/10.1128/AAC.00110-18>.
17. Moali C, Anne C, Lamotte-Brasseur J, Gros Lambert S, Devreese B, Van Beeumen J, Galleni M, Frère JM. 2003. Analysis of the importance of the metallo- β -lactamase active site loop in substrate binding and catalysis. *Chem Biol* 10:319–329. [https://doi.org/10.1016/s1074-5521\(03\)00070-x](https://doi.org/10.1016/s1074-5521(03)00070-x).
18. Tada T, Miyoshi-Akiyama T, Shimada K, Shimojima M, Kirikae T. 2013. IMP-43 and IMP-44 metallo- β -lactamases with increased carbapenemase activities in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 57:4427–4432. <https://doi.org/10.1128/AAC.00716-13>.
19. LaCuran AE, Pegg KM, Liu EM, Bethel CR, Ai N, Welsh WJ, Bonomo RA, Oelschlaeger P. 2015. Elucidating the role of residue 67 in IMP-type metallo- β -lactamase evolution. *Antimicrob Agents Chemother* 59:7299–7307. <https://doi.org/10.1128/AAC.01651-15>.
20. Yamaguchi Y, Jin W, Matsunaga K, Ikemizu S, Yamagata Y, Wachino J, Shibata N, Arakawa Y, Kurosaki H. 2007. Crystallographic investigation of the inhibition mode of a VIM-2 metallo- β -lactamase from *Pseudomonas aeruginosa* by a mercaptocarboxylate inhibitor. *J Med Chem* 50:6647–6653. <https://doi.org/10.1021/jm701031n>.
21. Borgianni L, Vandenameele J, Matagne A, Bini L, Bonomo RA, Frère JM, Rossolini GM, Docquier JD. 2010. Mutational analysis of VIM-2 reveals an essential determinant for metallo- β -lactamase stability and folding. *Antimicrob Agents Chemother* 54:3197–3204. <https://doi.org/10.1128/AAC.01336-09>.
22. Clinical and Laboratory Standards Institute. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 11th ed. Approved standard M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
23. EUCAST. 2016. Recommendations for MIC determination of colistin (polymyxin E), as recommended by the Joint CLSI-EUCAST Polymyxin Breakpoints Working Group. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf.
24. Galimand M, Courvalin P, Lambert T. 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. *Antimicrob Agents Chemother* 47:2565–2571. <https://doi.org/10.1128/aac.47.8.2565-2571.2003>.
25. Akasaka T, Tanaka M, Yamaguchi A, Sato K. 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. *Antimicrob Agents Chemother* 45:2263–2268. <https://doi.org/10.1128/AAC.45.8.2263-2268.2001>.
26. Boschi L, Mercuri PS, Riccio ML, Amicosante G, Galleni M, Frère JM, Rossolini GM. 2000. The *Legionella* (*Fluoribacter*) *gormanii* metallo- β -lactamase: a new member of the highly divergent lineage of molecular-subclass B3 β -lactamases. *Antimicrob Agents Chemother* 44:1538–1543. <https://doi.org/10.1128/aac.44.6.1538-1543.2000>.
27. Crowder MW, Walsh TR, Banovic L, Pettit M, Spencer J. 1998. Overexpression, purification, and characterization of the cloned metallo- β -lactamase L1 from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 42:921–926. <https://doi.org/10.1128/AAC.42.4.921>.
28. Queenan AM, Shang W, Flamm R, Bush K. 2010. Hydrolysis and inhibition profiles of β -lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. *Antimicrob Agents Chemother* 54:565–569. <https://doi.org/10.1128/AAC.01004-09>.
29. Kitao T, Miyoshi-Akiyama T, Tanaka M, Narahara K, Shimojima M, Kirikae T. 2011. Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo- β -lactamases that mediate carbapenem resistance in *Pseudomonas*. *J Microbiol Methods* 87:330–337. <https://doi.org/10.1016/j.mimet.2011.09.011>.
30. Shiroto K, Ishii Y, Kimura S, Alba J, Watanabe K, Matsushima Y, Yamaguchi K. 2005. Metallo- β -lactamase IMP-1 in *Providencia rettgeri* from two different hospitals in Japan. *J Med Microbiol* 54:1065–1070. <https://doi.org/10.1099/jmm.0.46194-0>.
31. Potter RF, Wallace MA, McMullen AR, Prusa J, Stallings CL, Burnham CAD, Dantas G. 2018. *bla*_{IMP-27} on transferable plasmids in *Proteus mirabilis* and *Providencia rettgeri*. *Clin Microbiol Infect* 24:1019.e5–1019.e8. <https://doi.org/10.1016/j.cmi.2018.02.018>.
32. Pathirana HNSK, Shin GW, Wimalasena SHMP, Hossain S, De Silva BCJ, Dahanayake PS, Heo GJ. 2018. Incidence of antibiogram, antibiotic resistance genes and class 1 and 2 integrons in tribe *Proteaeae* with IMP27 gene for the first time in *Providencia* sp. isolated from pet turtles. *Lett Appl Microbiol* 67:620–627. <https://doi.org/10.1111/lam.13077>.
33. Gefen-Halevi S, Hindiyeh MY, Ben-David D, Smollan G, Gal-Mor O, Azar R, Castanheira M, Belausov N, Rahav G, Tal I, Mendelson E, Keller N. 2013. Isolation of genetically unrelated *bla*_{NDM-1}-positive *Providencia rettgeri* strains in Israel. *J Clin Microbiol* 51:1642–1643. <https://doi.org/10.1128/JCM.00381-13>.
34. Tada T, Miyoshi-Akiyama T, Dahal RK, Sah MK, Ohara H, Shimada K, Kirikae T, Pokhrel BM. 2014. NDM-1 metallo- β -lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal. *BMC Infect Dis* 14:56. <https://doi.org/10.1186/1471-2334-14-56>.
35. Barrios H, Garza-Ramos U, Reyna-Flores F, Sanchez-Perez A, Rojas-Moreno T, Garza-Gonzalez E, Llaca-Diaz JM, Camacho-Ortiz A, Guzmán-López S, Silva-Sanchez J. 2013. Isolation of carbapenem-resistant NDM-1-positive *Providencia rettgeri* in Mexico. *J Antimicrob Chemother* 68:1934–1936. <https://doi.org/10.1093/jac/dkt124>.
36. Zhou G, Guo S, Luo Y, Ye L, Song Y, Sun G, Guo L, Chen Y, Han L, Yang J. 2014. NDM-1-producing strains, family *Enterobacteriaceae*, in hospital, Beijing, China. *Emerg Infect Dis* 20:340–342. <https://doi.org/10.3201/eid2002.121263>.
37. Pfeifer Y, Trifonova A, Pietsch M, Brunner M, Todorova I, Gergova I, Wilharm G, Werner G, Savov E. 2017. Clonal transmission of gram-negative bacteria with carbapenemases NDM-1, VIM-1, and OXA-23/72 in a Bulgarian hospital. *Microb Drug Resist* 23:301–307. <https://doi.org/10.1089/mdr.2016.0059>.
38. Carvalho-Assef AP, Pereira PS, Albano RM, Berião GC, Chagas TP, Timm LN, Da Silva RC, Faldi DR, Asensi MD. 2013. Isolation of NDM-producing *Providencia rettgeri* in Brazil. *J Antimicrob Chemother* 68:2956–2957. <https://doi.org/10.1093/jac/dkt298>.
39. Ntshobeni NB, Allam M, Ismail A, Amoako DG, Essack SY, Chenia HY. 2019. Draft genome sequence of *Providencia rettgeri* APW139_S1, an NDM-18-producing clinical strain originating from hospital effluent in South Africa. *Microbiol Resour Announc* 8:e00259-19. <https://doi.org/10.1128/MRA.00259-19>.
40. Lee HW, Kang HY, Shin KS, Kim J. 2007. Multidrug-resistant *Providencia* isolates carrying *bla*_{PER-1}, *bla*_{VIM-2}, and *armA*. *J Microbiol* 45:272–274.
41. McGann P, Hang J, Clifford RJ, Yang Y, Kwak YI, Kuschner RA, Lesho EP, Waterman PE. 2012. Complete sequence of a novel 178-kilobase plasmid carrying *bla*_{NDM-1} in a *Providencia stuartii* strain isolated in Afghanistan. *Antimicrob Agents Chemother* 56:1673–1679. <https://doi.org/10.1128/AAC.05604-11>.
42. Douka E, Perivolioti E, Kraniotaki E, Fountoulis K, Economidou F, Tsakris A, Skoutelis A, Routsis C. 2015. Emergence of a pandrug-resistant VIM-1-producing *Providencia stuartii* clonal strain causing an outbreak in a Greek intensive care unit. *Int J Antimicrob Agents* 45:533–536. <https://doi.org/10.1016/j.ijantimicag.2014.12.030>.
43. Drieux L, Decré D, Frangeul L, Arlet G, Jarlier V, Sougakoff W. 2013. Complete nucleotide sequence of the large conjugative pTC2 multireplicon plasmid encoding the VIM-1 metallo- β -lactamase. *J Antimicrob Chemother* 68:97–100. <https://doi.org/10.1093/jac/dks367>.
44. Galani L, Galani I, Souli M, Karaiskos I, Katsouda E, Patrozou E, Baziaka F, Paskalis C, Giamarellou H. 2013. Nosocomial dissemination of *Providencia stuartii* isolates producing extended-spectrum β -lactamases VEB-1 and SHV-5, metallo- β -lactamase VIM-1, and RNA methylase RmtB. *J Glob Antimicrob Resist* 1:115–116. <https://doi.org/10.1016/j.jgar.2013.03.006>.