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EPIDEMIOLOGY AND SURVEILLANCE

Emergence of Carbapenem-Resistant *Pseudomonas asiatica* Producing NDM-1 and VIM-2 Metallo-β-Lactamases in Myanmar

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ABSTRACT *Pseudomonas asiatica* is a recently proposed species of the genus *Pseudomonas*. This study describes eight isolates of carbapenem-resistant *P. asiatica* harboring bla_{NDM-1} and bla_{VIM-2}, genes encoding metallo-β-lactamase (MBL). These isolates were obtained from urine samples of patients hospitalized in Myanmar. These isolates were resistant to carbapenems but susceptible to colistin. All eight isolates were positive for a carbapenemase inactivation method, CIMTrisII, and seven were positive on an immunochromatographic assay for NDM-type MBL. One isolate was highly resistant to aminoglycosides. Whole-genome sequencing showed that seven isolates harbored bla_{NDM-1} and one harbored bla_{VIM-2}, with these genes located on the chromosome. One isolate harbored bla_{NDM-1} and *rmtC*, a gene encoding 16S rRNA methylase. Five types of genomic environments surrounding bla_{NDM-1} and bla_{VIM-2} were detected in these eight isolates, with four isolates having the same type. These data indicate that *P. asiatica* isolates harboring genes encoding carbapenemases, including bla_{NDM-1} and bla_{VIM-2}, are spreading in medical settings in Myanmar.

KEYWORDS NDM-1 metallo-β-lactamase, *Pseudomonas asiatica*, VIM-2 metallo-β-lactamase

The emergence and spread of metallo-β-lactamase (MBL)-producing isolates of *Pseudomonas* species have become a serious problem in medical settings worldwide (1). MBLs, such as NDM-1 and VIM-2, confer resistance to all β-lactams, except monobactams, and are characterized by their efficient hydrolysis of carbapenems (2, 3).

Pseudomonas asiatica is a recently proposed species belonging to the *Pseudomonas putida* group and is located close to *P. putida* and *Pseudomonas monteilii* (4). *P. asiatica* isolates were obtained from patients hospitalized in Japan and Myanmar and thought to be a human pathogen (4).

The present study describes eight isolates of carbapenem-resistant *P. asiatica* producing NDM-1 and VIM-2 MBLs obtained from patients in Myanmar.

RESULTS

Bacterial surveillance. During the surveillance, 152 isolates of multidrug-resistant (MDR) *Pseudomonas* species were obtained. Of them, 14 were identified as *P. putida* using Vitek 2. Bacterial identification analysis based on average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values revealed that, of these 14 MDR

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isolates, eight were *P. asiatica* species, one was a *P. monteilii* species, one was a *Pseudomonas mendocina* species, one was a *Stenotrophomonas maltophilia* species, and three were unidentified *Pseudomonas* species. The eight MDR *P. asiatica* isolates were obtained from eight individual patients, including four patients admitted to hospital A (in the center of Yangon), one admitted to hospital B (in the northern part of Yangon), and three admitted to hospital C (Mandalay, located 700 km north of Yangon). Seven strains were isolated from urine samples and one from a urine sample containing urinary stones. Of the eight patients, five were men and three were women, ranging in age from 27 to 77 years (mean ± standard deviation [SD] age, 58.8 ± 14.0 years) (see Table S1 in the supplemental material). Other clinical information was not obtained.

Drug susceptibility testing and carbapenemase production. All eight *P. asiatica* isolates tested were resistant to ampicillin-sulbactam, aztreonam, cefepime, ceftazidime, ciprofloxacin, imipenem, levofloxacin, and meropenem, with MICs of >16 μg/ml, but were susceptible to colistin, with MICs of ≤1 μg/ml (Table 1). Of these isolates, one, MY34, was highly resistant to all four aminoglycosides tested with MICs of >2,048 μg/ml, whereas the remaining seven isolates were resistant to one to three of four aminoglycosides tested (Table 1). All eight isolates were positive for the carbapenemase inactivation method, CIMTrisII. Seven were positive for an NDM immunochromatographic assay, but one, MY660, was negative.

Detection of antimicrobial resistance genes. Eight types of β-lactamase-encoding genes were detected in the eight *P. asiatica* isolates (Table 1). Of these, two genes encoded MBL, whereas the remaining six genes encoded extended-spectrum β-lactamase (ESBL). The ESBL-encoding genes were detected in four of the eight isolates (Table 1), whereas a gene encoding 16S rRNA methylase, *rmtC*, was detected in one isolate, MY34. These isolates contained 16 genes encoding aminoglycoside modification enzymes (Table 1). All isolates had two point mutations encoding amino acid substitutions associated with quinolone resistance in *P. putida* (T83I in GyrA and S87W in ParC) (5, 6) and in *Pseudomonas aeruginosa* (T83I in GyrA and S87L in ParC) (7) (Table 1).

Genetic environments of bla_{NDM-1} and bla_{VIM-2}. The genomic environments surrounding bla_{NDM-1} are shown in Fig. 1. Of the eight isolates, four, MY371, MY545, MY601, and MY680, had nearly identical genomic environments surrounding bla_{NDM-1}, with the 14.7-kbp sequences having >99.6% sequence similarity with each other (GenBank accession numbers LC459616, LC460196, LC460198, and LC460200) (Fig. 1). The genomic environments of MY34 (GenBank accession number LC459615), MY569 (GenBank accession number LC460197), and MY756 (GenBank accession number LC460201) differed from each other (Fig. 1). The genomic environments surrounding bla_{VIM-2} of MY660 (GenBank accession number LC460199) are shown in Fig. 1 and contained a unique structure of class 1 integron with 5′-conserved segments (CS) but not 3′-CS (containing the *qacEΔ1*, *sul1*, and *orf5*), two of which class 1 integrons frequently harbored (8).

Location of bla_{NDM-1} and bla_{VIM-2} in their genomes. To determine the locations of the MBL-encoding genes of all eight isolates, the DNA sequences of short reads obtained from MiSeq and of long reads from MinION were assembled. In all isolates, bla_{NDM-1} and bla_{VIM-2} were detected on a single contig harboring the 16S rRNA genes and the other seven housekeeping genes, indicating that both bla_{NDM-1} and bla_{VIM-2} were located on the chromosomes of these isolates (Fig. 2). Contigs from three isolates, MY545, MY601, and MY756, were circularized and assembled as complete chromosomal sequences. However, those from the remaining five were not circularized (Fig. 2). Genetic maps were constructed of these contigs, based on distances between *dnaA* and other genes, including 16S rRNA genes and housekeeping genes (Fig. 2). Because three contigs, from MY34, MY371, and MY569, did not contain *dnaA*, the genetic map was constructed by comparisons with those of the circular contigs based on distances between 16S rRNA and each housekeeping gene (Fig. 2). The bla_{NDM-1} genes were located at ≈1.4 Mbp in MY569; ≈1.7 Mbp in MY34, MY371, MY545, and MY601; and

TABLE 1 Drug susceptibility profiles and drug-resistance genes of clinical isolates of *P. asiatica*

Strain no.	Antimicrobial susceptibility (MIC in μg/ml)[a]													Genes or mutations associated with drug resistance				
	ABK	AMK	ATM	CAZ	CIP	CST	FEP	GEN	IPM	LVX	MEM	SAM	TOB	β-lactamase(s)	16S rRNA methylase	Aminoglycoside acetyl-, adenylyl-, phospho-, and nucleotidyl-transferase	Mutation in DNA gyrase	
																	GyrA	ParC
MY34	>2,048	>2,048	2,048	>2,048	1,024	1	2,048	>2,048	32	512	1,024	>2,048	>2,048	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{VEB-1}, bla_{OXA-10}, $bla_{OXA-101}$	*rmtC*	*aac(6′)-Il*, *aadA1*, *aadB*, *aph(3′)-VI*, *aph(3′′)-Ib*, *aph(6)-Id*, *ant(2′′)-Ia*	T83I	S87W
MY371	2	64	64	>2,048	512	1	2,048	4	512	1,024	>2,048	>2,048	64	bla_{NDM-1}		*aph(3′′)-Ib*, *aph(6)-Id*, *ant(4′)-IIb*	T83I	S87W
MY545	2	128	128	>2,048	512	0.25	>2,048	4	256	1,024	>2,048	>2,048	64	bla_{NDM-1}		*aph(3′′)-Ib*, *aph(6)-Id*, *ant(4′)-IIb*	T83I	S87W
MY569	2	16	512	>2,048	64	1	2,048	256	256	128	2,048	>2,048	2	bla_{NDM-1}, bla_{PME-1}		*aac(3)-IIa*, *aph(3′)-VI*, *aph(3′)-Ib*, *aph(6)-Id*	T83I	S87L
MY601	0.5	16	1,024	>2,048	512	0.5	>2,048	256	512	256	1,024	>2,048	8	bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{TEM-1}		*aph(3′′)-Ib*, *aph(6)-Id*, *aac(3)-IId*, *ant(2′′)-Ia*, *ant(4′)-IIb*	T83I	S87L
MY660	8	32	64	32	256	1	32	>2,048	64	256	64	1,024	128	bla_{VIM-2}		*aac(3)-Id*, *aac(6′)-Il*, *aadA2*, *aadB*, *aph(3′′)-Ib*, *aph(6)-Id*	T83I	S87L
MY680	2	32	64	>2,048	1,024	1	2,048	256	512	2,048	2,048	>2,048	32	bla_{NDM-1}		*aadA15*, *aadB*, *aph(3′)-Ia*, *aph(3′)-IIa*, *aph(3′′)-Ib*, *aph(6)-Id*, *ant(4′)-IIb*	T83I	S87L
MY756	2	256	256	>2,048	256	1	512	256	256	256	1,024	>2,048	512	bla_{NDM-1}, bla_{PME-1}		*ant(4′)-IIb*	T83I	S87L

[a]CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; SAM, ampicillin-sulbactam; AMK, amikacin; ABK, arbekacin; GEN, gentamicin; CIP, ciprofloxacin; LVX, levofloxacin; CST, colistin; TOB, tobramycin.

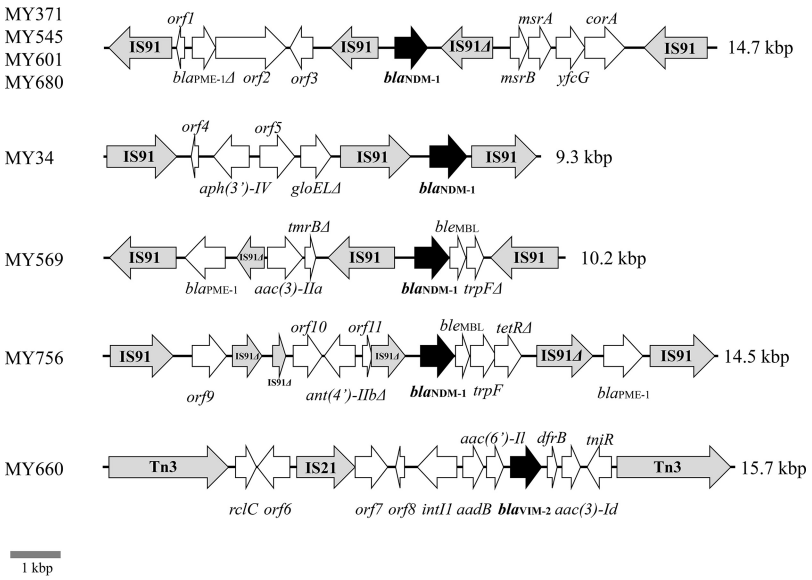

FIG 1 Genomic environments of bla_{NDM-1} and bla_{VIM-2} in *P. asiatica* clinical isolates. Genes are represented as arrows, which indicate their transcription orientations and relative lengths. MBL genes, *tnp* genes, and truncated genes are shown as black arrows, gray arrows, and Δ, respectively. *orf1*, *orf3*, *orf8*, *orf10*, and *orf11* are genes encoding hypothetical proteins; *orf2* is a gene encoding an ABC transporter ATP-binding protein; *orf4* is a gene encoding a mobile element protein; *orf5* is a gene encoding a NADH dehydrogenase; *orf6* is a gene encoding an AraC family transcriptional regulator; *orf7* is a gene encoding an AAA family ATPase; and *orf9* is a gene encoding a mechanosensitive ion channel protein.

≈4.0 Mbp in MY680 and MY756. The bla_{VIM-2} gene in MY660 was located at −64,552 bp of *dnaA* (Fig. 2).

Phylogenetic analysis. Maximum-likelihood phylogenetic and neighbor-joining phylogenetic trees were constructed using core genome single nucleotide polymorphisms (SNPs) of the eight isolates and the seven type strains belonging to the *P. putida* group (Fig. 3). The *P. asiatica* isolates were divided into two groups, with isolates belonging to each group obtained from two hospitals (A and C) and one hospital (B) (Fig. 3).

DISCUSSION

P. asiatica is a likely pathogen in humans and causes nosocomial infections. We have detected a total of 10 clinical isolates of *P. asiatica* to date, including type strains in Japan and Myanmar (4), and an additional seven strains from clinical isolates in Myanmar. All of the isolates were obtained from hospitalized patients (4) (Table 1). The isolates obtained from hospitals A and C were closely related to each other; nonetheless, the location of hospital A (Yangon) is different from that of hospital C (Mandalay), and these isolates were also closely related to the type strain, which was originally isolated in Japan (4). *P. asiatica* isolates may be clonally spreading in Asian countries.

The bla_{NDM-1} and bla_{VIM-2} genes were located on the chromosome of all *P. asiatica* isolates (Fig. 2), suggesting that foreign genes tend to be inserted into the chromosome. In comparison, the bla_{VIM-1} and bla_{VIM-2} genes were located on the chromosome and/or a plasmid in carbapenem-resistant *P. putida* isolates (9), and bla_{VIM-2} was located on a plasmid in clinical isolates of carbapenem-resistant *P. monteilii* (10).

This is the first report of *rmtC* detected in an isolate belonging to the *P. putida* group as well as in an isolate of *P. asiatica*. The *rmtC* gene was located on the chromosome, as it was present in a contig harboring a housekeeping gene, *nth* (endonuclease III) (data not shown). The *rmtC* gene was first reported in clinical isolates of *Proteus mirabilis* in Japan and then reported in MDR isolates of *Enterobacteriaceae* (11). More recently, *rmtC* was found in clinical isolates of pan-aminoglycoside-resistant *P. aeruginosa* in India (12, 13). The *rmtD3* gene, obtained from a clinical isolate of *P. aeruginosa* in

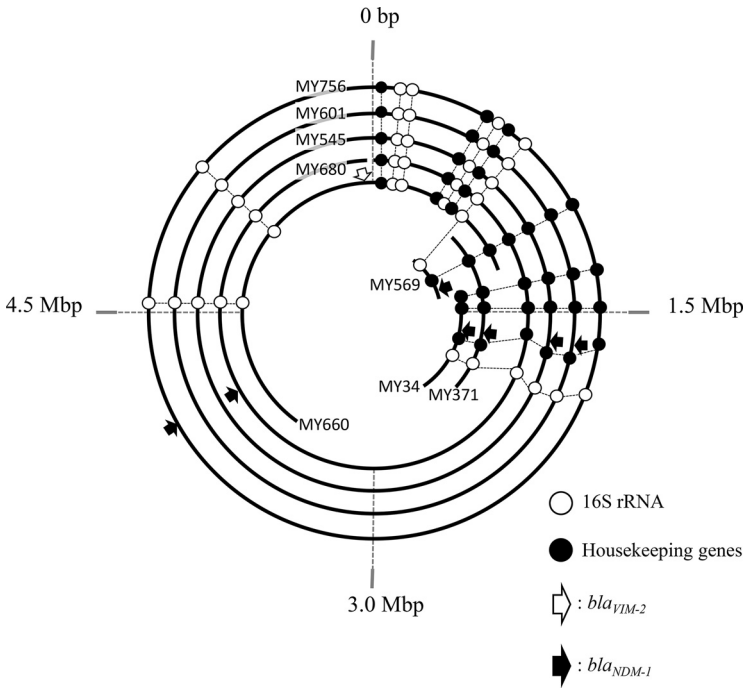

FIG 2 Circular visualization of *Pseudomonas asiatica* contigs for location of MBL genes. The circles and curve lines represent chromosomal DNA sequences of the following *P. asiatica* isolates from the innermost curved or circularized line: MY569 (742,245 bp; accession number SWEI00000000), MY34 (1,297,202 bp; SWEL00000000), MY371 (1,475,821 bp; SWEK00000000), MY660 (4,005,680 bp; SWEG00000000), MY680 (6,101,876 bp; SWEF00000000), MY545 (5,914,934 bp; SWEJ00000000), MY601 (6,139,917 bp; SWEH00000000), and MY756 (6,110,371 bp; SWEE00000000), respectively. The start point at 0 bp was *dnaA*. The 16S rRNA genes are shown in white circles, housekeeping genes (*gyrB*, *rpoD*, *rpoB*, *rpoN*, *rpoS*, *gyrA*, and *fabD*) are shown in black circles, bla_{VIM-2} is a white arrow, and bla_{NDM-1} is represented by black arrows.

Myanmar, was found to encode a new variant of 16S rRNA methylase (14). Taken together, these findings suggest that *P. asiatica* may be a reservoir of *rmtC*.

Five types of genomic environments were found to surround bla_{NDM-1} and bla_{VIM-2} in the *P. asiatica* clinical isolates from Myanmar, indicating the spread in hospitals of carbapenem-resistant *P. asiatica* isolates with several types of genomic environments. Four strains, MY371, MY545, MY601, and MY680, had the same genomic environment but were isolated at three hospitals located in different areas, indicating that *P. asiatica* with this genomic environment had spread among hospitals in Myanmar. The partial sequence of this genomic environment was >97% identical with that of *P. aeruginosa* N15-01092 from the United States (accession number CP012901; 4,093,958 to 4,098,533) (15), although sequences upstream and downstream of the partial sequence were unique.

The strategy of using MinION with MiSeq has become a standard protocol for the genetic analysis of bacteria to determine whether drug-resistant genes are located on the chromosome or plasmids. Long-read sequencing by MinION revealed the longer genomic environmental structures surrounding antimicrobial-resistant genes (16, 17).

To our knowledge, this is the first report to describe the molecular epidemiology of MDR *P. asiatica* clinical isolates. These bacteria possess several genes associated with drug resistance located on the chromosome. Further surveillance of *P. asiatica* is necessary in other Asian countries as well as in Myanmar.

MATERIALS AND METHODS

Surveillance. A prospective surveillance study on MDR Gram-negative pathogens was performed from December 2016 to March 2018 in medical settings in Myanmar. A total of 543 carbapenem- and/or aminoglycoside-resistant Gram-negative isolates were obtained from patients in 22 hospitals and one public health laboratory. MDR strains are defined as strains showing no susceptibility to at least one agent in more than three antimicrobial categories, as described (18).

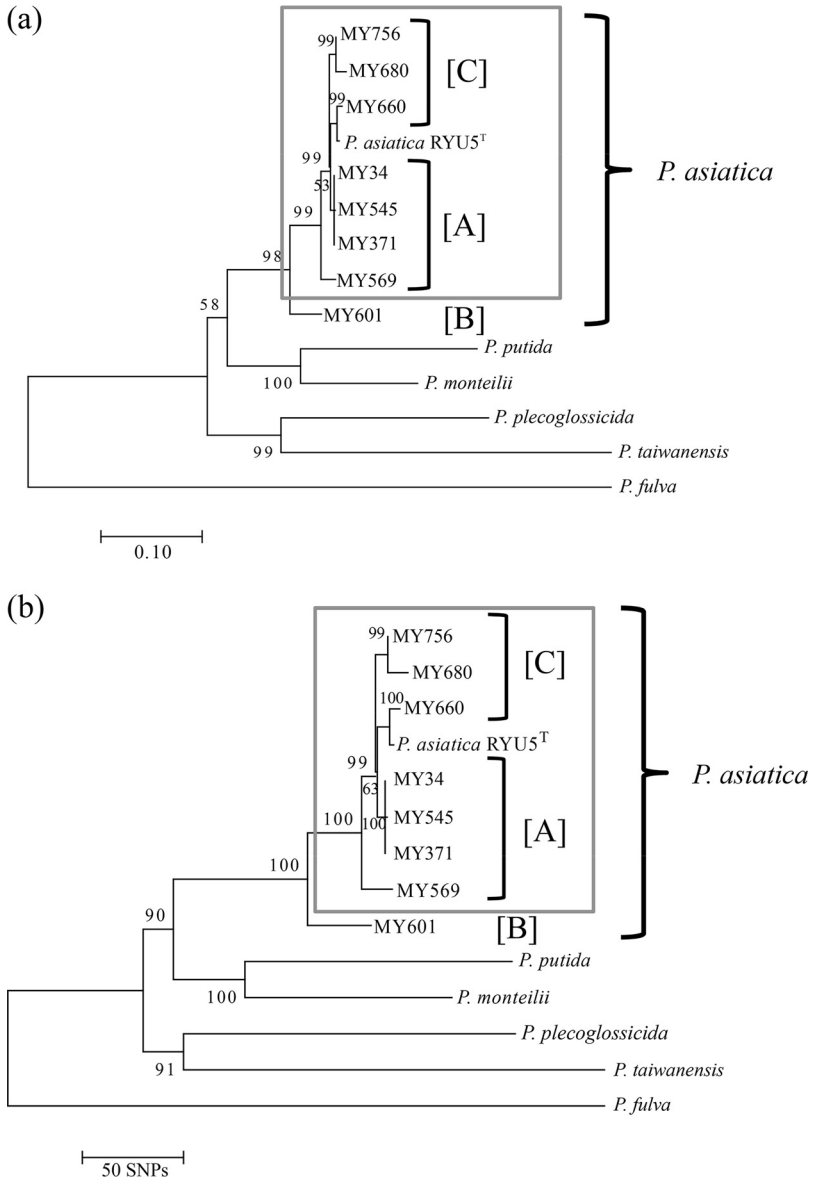

FIG 3 Phylogenetic trees for the eight clinical isolates and the type strain of *P. asiatica*. The trees were constructed using maximum-likelihood (a) and neighbor-joining (b) phylogenetic analysis based on core genome SNPs.

Drug susceptibility testing. The MICs of antibiotics were determined using the microdilution method according to the guidelines of CLSI (M100-S25) (19).

Detection of carbapenemases. Carbapenemase production was detected with the CIMTrisII carbapenem inactivation method (Kohjin Bio, Saitama, Japan) (20), and NDM-type MBL production was detected using a KBM LineCheck NDM immunochromatographic assay (Kojin Bio) (21).

Whole-genome sequencing. The whole genomes of *P. asiatica* isolates were sequenced using MiSeq (Illumina, San Diego, CA) and MinION (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturers' instructions. Quality trimming and filtering of the obtained sequence reads generated by MiSeq were performed using CLC Genomics Workbench v11 (CLC bio, Aarthus, Denmark). MinION data were basecalled by Albacore v2.3.1 (Oxford Nanopore Technologies) and adapters trimmed by Porechop v0.2.3 (https://github.com/rrwick/Porechop). The long reads generated by MinION were assembled using Canu v1.7.1 (22) and polished with the short reads generated by MiSeq using Pilon v1.22 (23).

Bacterial species identification based on whole-genome sequences. The species of the 14 isolates, identified as *P. putida* with Vitek 2 (bioMérieux, Marcy l'Étoile, France), were reidentified using ANI (24, 25) and dDDH (26) based on comparisons of their whole-genome sequences with the sequences of 17 type strains belonging to the *P. putida* group as described (4). The cutoff values of ANI and dDDH between these isolates and the type strains were 95% and 70%, respectively.

Detection of drug resistance genes. The assembled genome sequences were searched for genes associated with drug resistance, including genes encoding β-lactamases, 16S rRNA methyltransferases, and aminoglycoside-acetyl/adenyltransferase using the ABRicate program (https://github.com/tseemann/abricate) and databases of the National Center for Biotechnology Information (NCBI), ResFinder, and the Comprehensive Antibiotic Resistance Database (CARD). The amino acid sequences of GyrA and ParC in each isolate were compared with those of *P. putida* KT2440 (5), a reference strain. The type strain of *P. asiatica* was resistant to ciprofloxacin and levofloxacin; therefore, *P. putida*, which is a closed species to *P. asiatica* and sensitive to these antibiotics, was used as a reference for quinolone-sensitive strains.

Phylogenic analysis. Phylogenetic analysis was performed using kSNP3 v3.1 software with a k-mer length of 31 (27). Maximum-likelihood phylogenetic and neighbor-joining phylogenetic trees were estimated based on the core genome single nucleotide polymorphisms (SNPs) among contigs of the eight *P. asiatica* isolates and its type strain RYU5. Trees were visualized using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Ethics approval. The study protocol was approved by the Ministry of Health and Sports in the Republic of the Union of Myanmar (letter number Ethical Committee 2016), by the ethics committee of Juntendo University (number 809), and by the Biosafety Committee, Juntendo University (approval numbers 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) Sequence Read Archive under accession numbers DRA007940 and DRA007946. The data of assembled and annotated nucleotide sequences were deposited in the GenBank database with the accession numbers SWEE00000000 to SWEL00000000, LC459615, LC459616, and LC460196 to LC460201.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00475-19.

SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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We declare no conflict of interest.

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