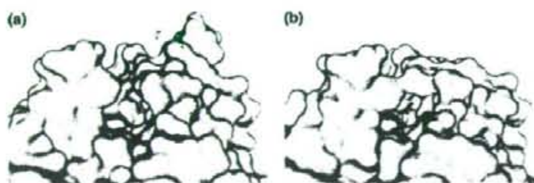

Figure 5. Molecular surface representations of native VIM-2 (a) and the phenylC3SH complex (b). In the phenylC3SH complex, molecule A is depicted as a molecular surface. The amino acid residues of VIM-2 are designated with a BBL number and an amino acid sequence number; the latter is in parentheses. Zn(II) ion (Zn2) is shown as an orange sphere. PhenylC3SH is presented as a stick (carbon, oxygen, and sulfur atoms are shown in magenta, red, and light-green, respectively). The figure was prepared with PyMol software (http://pymol.sourceforgen.net/).

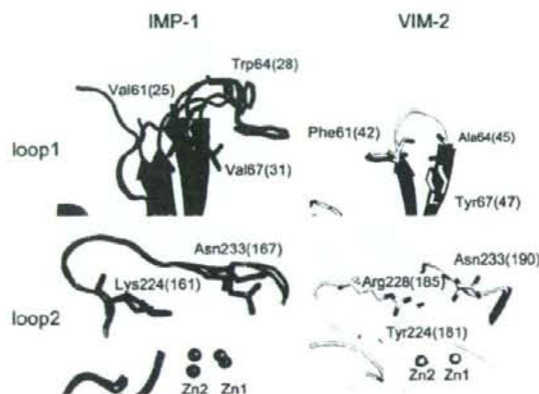

Figure 6. Comparison of loops 1 and 2 between IMP-1 and VIM-2 enzymes. The amino acid residues of the IMP-1 and VIM-2 enzymes are designated with a BBL number and an amino acid sequence number; the latter is in parentheses. In the IMP-1 enzyme (left), the structures of IMP-1 metallo-β-lactamase from *P. aeruginosa* (PDB code 1DDK) (red) and its complex with a mercaptocarboxylate inhibitor (PDB code 1DD6, molecule A is depicted) (blue) were used for comparison,[22] but the inhibitor 2-[5-(1-tetrazoylmethyl)thien-3-yl]-*N*-[2-(mercaptomethyl)-4-(phenylbutyrylglycine)] was omitted for clarity. Val61(25), Trp64(28), and Val67(31) residues on loop1 and Lys224(161) and Asn233(167) residues on loop2 are presented as sticks (carbon, gray; nitrogen, blue; oxygen, red). Zn(II) ions are shown as orange spheres. In the VIM-2−phenylC3SH complex (right), molecule A is depicted and phenylC3SH was omitted for clarity. Phe61(42), Ala64(45), and Tyr67(47) residues on loop1 and Tyr224(181), Arg228(185), and Asn233(190) residues of the VIM-2 enzyme are presented as sticks (carbon, gray; nitrogen, blue; oxygen, red). Zn(II) ions are shown as orange spheres. The figure was prepared with the PyMol software program (http://pymol.sourceforgen.net/).

in inhibitory activity of phenylC3SH between the VIM-2 and IMP-1 enzymes.

Lys224(161) in the IMP-1 enzyme is located on loop2, which is ~5 Å away from the Zn(II) center. In the mercaptocarboxylate inhibitor complex, the carboxyl group of inhibitor interacts with the side chain NZ of Lys224(161) with a distance of 2.8 Å, and this result shows the importance of Lys224(161) in inhibitor binding. Moreover, Lys224(161) is assumed to interact with the carboxylate of the β-lactam antibiotics.[22,31] However, in the case of the VIM-2 enzyme, Lys224(161) is replaced with Tyr224(181). On the basis of the crystal structure of the phenylC3SH complex, Arg228(185) near Tyr224(181) on loop2 might aid in carrying out the role of Lys224(161) in IMP-1.

It is noteworthy that the movement and rotation of Asn233(190) occur upon inhibitor binding to the VIM-2 enzyme. In the crystal structure of the IMP-1−mercaptocarboxylate inhibitor complex,[22] a similar behavior was observed, where the carbonyl oxygen of the inhibitor is oriented for interaction with the side chain ND2 of Asn233(167) (4.1 Å) in an oxyanion hole.[31] From the above results it can be concluded that the role of Asn233(190) in the VIM-2 structure is to stabilize either a substrate or an inhibitor.

Conclusion

We determined the three-dimensional structure of a VIM-2 enzyme complexed with a mercaptocarboxylate inhibitor, phenylC3SH, which is the first VIM family member to be characterized as being complexed with an inhibitor. From the results of the crystal structure, the precise inhibition mode of the VIM-2 enzyme with phenylC3SH was ascertained: in particular, Phe61(42) and Tyr67(47), located in loop1 and Arg228(185) and Asn233(190) in loop2 play an important role in the binding and recognition of the inhibitor to the VIM-2 enzyme and in the stabilization of the VIM-2 structure. Moreover, Phe61 and Tyr67 residues are conserved in the other members of subclass B1 MBLs: VIM-1,[38] BlaB,[39] and IND-1.[40] Therefore, in these MBLs, two residues seem to play the same role, as can been seen in this study.

These findings would aid in the design of inhibitors that target not only VIM-2 but also other MBLs.

Experimental Section

Expression and Purification. The VIM-2 metallo-β-lactamse was expressed by *Escherichia coli* NCB326-1B2 harboring pVM4k/VIM-2 and purified as previously described.[30]

Synthesis of Inhibitor. *rac*-2-*ω*-Phenylpropyl-3-mercaptopropionic acid, phenylC3SH, was synthesized by the method described by Park et al.[41]

Crystallization of the VIM-2 Enzyme Complexed with PhenylC3SH. Prior to the X-ray diffraction experiments, a buffer solution of the VIM-2 enzyme was converted from Tris-HCl (50 mM, pH 7.4, 0.5 M NaCl) to HEPES−NaOH (20 mM, pH 7.5) and the VIM-2 protein was then concentrated to about 5 mg/mL (160 μM) on a Centricon. Drops of the VIM-2 protein with phenylC3SH were prepared by mixing 2 μL of a concentrated protein solution, 2 μL of a reservoir solution (30% PEG MME5000, 0.1 M MES−NaOH, and 0.2 M ammonium sulfate (pH 6.5)), and 1 μL of a methanolic phenylC3SH solution (10 mM). The crystals were grown for two months as plates (0.4 mm × 0.4 mm × 0.2 mm) at 20 C°.

Data Collection and Processing. Cocrystals of the VIM-2 enzyme with phenylC3SH were mounted in nylon loops directly from the mother liquor and flash-cooled in liquid nitrogen stream (100 K). All diffraction data were collected on beamline BL40B2 using an ADSC Quantum-4R detector at 100 K and beamline BL41XU using a marCCD 165 detector at 100 K at SPring-8 (Harima, Japan). A full MAD data collection around the zinc edge was performed with a single frozen crystal at SPring-8 BL40B2. Diffraction data were collected by the oscillation method at three carefully selected wavelengths: λ_1 = 1.2826 Å (edge, f' minimum), λ_2 = 1.2817 Å (peak, f'' maximum), and λ_3 = 1.2906 Å (remote high-energy wavelength). After completion of the MAD data collection at a resolution of 2.55 Å, a data set of 360 frames at λ = 1.0000 Å was collected at a resolution of 2.3 Å with 0.5° oscillation steps at SPring-8 BL41XU. The data were integrated and scaled with HKL2000.[42]

Phasing, Structure Determination, Model Building, and Refinement. Phase determination for the VIM-2 enzyme complexed with phenylC3SH was performed using the SOLVE program[43] and density modification, and model building was performed with the RESOLVE program.[44] This led to an interpretable density map and

an initial map. The O[45] and Coot[46] programs were further used in modeling and remodeling. The refinement was carried out using REFMAC5,[47] a component of the CCP4 suite,[48] and CNS[49] programs without a noncrystallographic symmetry (NCS). PhenylC3SH was built and minimized in MOE (CCG Inc., Canada), and the topology and parameter files of phenylC3SH were utilized in PRPDRG (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/).[50] The quality of the model was inspected by the program PROCHECK.[51] Data collection and refinement statistics can be found in Table 1. The atomic coordinates and structure factors (PDB code 2YZ3) have been deposited at the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

In the case of the IMP-1 enzyme, however, we were unable to examine the IMP-1 enzyme complexed with phenylC3SH by X-ray crystallography because of the poor quality of the crystals obtained.

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Molecular Characterization of Vancomycin-Resistant *Enterococcus faecium* Isolates from Mainland China[▽]

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Little is known about vancomycin-resistant enterococci in China. Thirteen pulsed-field gel electrophoresis-confirmed heterogeneous VanA-type vancomycin-resistant *Enterococcus faecium* (VRE) isolates were obtained from five Chinese hospitals from 2001 to 2005. The isolates were typed by multilocus sequence typing into nine different sequence types (STs), including five new STs (ST18, ST25, ST78, ST203, ST320, ST321, ST322, ST323, and ST335). Vancomycin resistance in each isolate was encoded on conjugative plasmids; two of the plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were highly conjugative and were able to transfer at high frequencies of around 10^{-4} and 10^{-7} per donor cell in broth mating, respectively. None of the plasmids identified in these isolates carried *traA*, which is usually conserved in the pMG1-like highly conjugative plasmid for *E. faecium*, implying that pZB18 and pZB22 were novel types of a highly conjugative plasmid in enterococci. Thirteen Tn*1546*-like elements encoding VanA-type VRE on the conjugative plasmids were classified into six types (types I to VI), and most of them contained both IS*1216V* and IS*1542* insertions. The isolates carrying the type II element were predominant. The six type elements were different from that of a VanA-type *Enterococcus faecalis* strain isolated from Chinese chicken meat. The results suggested that the disseminations of VRE in these areas were by Tn*1546*-like elements being acquired by the conjugative plasmids and transferred among *E. faecium* strains.

The isolation of vancomycin-resistant enterococci (VRE) was first reported in 1988 in the United Kingdom and France (17, 29) and then in hospitals in the United States (20). VRE are now encountered in many countries, especially in Europe and the United States (2). There are several reports describing the isolation of VRE in East Asian regions and countries, including Japan, Korea, and Taiwan, and isolation frequencies of VRE from patients and food animals have been increasing both in Korea and in Taiwan (15, 34). Since the first Japanese report of a VanA-type VRE (*Enterococcus faecium*) clinical isolate in 1996, the frequencies of VRE isolation from patients have also increased (7, 18). However, little information is available on the prevalence of VRE and their molecular makeup from mainland China, although glycopeptide antimicrobials have been used there for decades.

VanA-type resistance, characterized by high-level inducible vancomycin resistance (MICs of 64 to >1,024 μg/ml) and teicoplanin resistance (MICs of 16 to >512 μg/ml), is most frequently encountered (19). The genes encoding VanA-type vancomycin resistance are located on mobile Tn*1546*-like elements; therefore, the horizontal transfer of resistance genes among enterococci has a more significant impact on the dissemination of VRE than does the clonal spread of resistant enterococci (14). Epidemiological studies of VanA-type enterococci indicate that there are geographic differences (22). Considerable diversity has been identified in the Tn*1546*-related elements. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been exploited in several epidemiological studies (33, 31).

Vancomycin has been used in patient care in mainland China for 40 years, and its usage is increasing. Our group has previously described vancomycin-dependent VanA-type VRE strains isolated in Japan from retail chicken meat imported from China (25). Clinical isolates of VanA-type VRE (*E. faecium*) are rarely obtainable from China. Over the past five years, we have obtained a total of 13 clinical strains of VanA-type VRE (*E. faecium*) from China. The current report is the first to describe molecular characterization of VanA-type VRE from mainland China.

MATERIALS AND METHODS

Bacterial strains and culture media. Thirteen clinical isolates of vancomycin-resistant *E. faecium* recovered from blood cultures and urine and sputum samples from patients in China were used in this study (Table.1). VanA-type vancomycin-resistant *Enterococcus faecalis* strain KC122.1, isolated in Japan from chicken meat imported from China in 2001, was used for the comparative analysis of Tn*1546*-like elements (25). *E. faecium* strains BM4105RF and BM4105SS were used as recipient strains for transfer experiments (13). Enterococci were grown in Todd-Hewitt broth (THB).

Antimicrobial susceptibility testing. Glycopeptide resistance levels were determined by the agar dilution method. An overnight pure culture of each strain grown in Mueller-Hinton broth (Nissui, Tokyo, Japan) was diluted 100-fold with fresh broth. An inoculum of approximately 5×10^5 cells was plated on a series of Mueller-Hinton agar plates (Eiken, Tokyo, Japan) containing a range of concentrations of the test drug. The plates were incubated at 37°C, and the susceptibility results were finalized at 24 h of incubation. Susceptibility testing and interpretation of results were in compliance with standards recommended by

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TABLE 1. Chinese VanA-type vancomycin-resistant *E. faecium* clinical isolates[a]

Strain	Hospital (city)	Date of isolation (yr/mo/day)	Source	Diagnosis/underlying disease[b]	Antibiotic(s) used	MLST result[c]								
						Sequence type (nearest)	Clonal complex	Allelic profile						
								atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adkC*
I125	E (Dalian)	2001/12/9	Sputum	Cachexia/gastric cancer	CEPs, CLI	ST78	CC78	15	1	1	1	1	1	1
C264	A (Beijing)	2003/8/21	Bile	Cholangitis/hepatocirrhosis	VAN, CEPs	ST320* (ST80)	CC117	9	1	1	1	12	29	1
ZB11	A (Beijing)	2004/1/15	Urine	Pyelitis/gastric cancer	VAN	ST335* (ST172)	CCS	45*	13	34*	15	19	29	18*
ZB14	B (Beijing)	2004/2/13	Urine	Pyelitis/leukemia	CEPs	ST321* (ST31)	CC280	7	3	1	1	1	1	3
ZB15	B (Beijing)	2004/2/27	Urine	Pyelitis/kidney dysfunction	TEC	ST78	CC78	15	1	1	1	1	1	1
ZB16	B (Beijing)	2004/4/4	Urine	Pyelitis/hepatocirrhosis	CEPs	ST18	CC18	7	1	1	1	5	1	1
ZB18	B (Beijing)	2004/4/9	Blood	Sepsis/leukemia	VAN, CEPs	ST25	CC25	9	3	1	6	1	1	1
ZB19	C (Beijing)	2005/10/18	Sputum	Pneumonia/pulmonary dysfunction	VAN, CEPs	ST322* (ST262)	CC18	7	1	6	1	5	7	1
ZB20	C (Beijing)	2005/10/20	Urine	Pyelitis/rectal cancer	VAN, CEPs	ST203	CC78	15	1	1	1	1	20	1
ZB21	D (Tianjin)	2005/10/12	Blood	Sepsis/hepatocellular cancer	VAN, MEM	ST78	CC78	15	1	1	1	1	1	1
ZB22	B (Beijing)	2005/10/28	Sputum	Pneumonia/COPD	VAN, MEM	ST323* (ST17)	CC17	5	1	1	1	1	1	1
ZB23	C (Beijing)	2005/11/7	Sputum	Pneumonia/bronchiectasis	CEPs	ST203	CC78	15	1	1	1	1	20	1
ZB24	C (Beijing)	2005/11/8	Urine	Pyelitis/COPD	CEPs	ST203	CC78	15	1	1	1	1	20	1

[a] Drug abbreviations: CEPs, broad-spectrum cephalosporins; CLI, clindamycin; VAN, vancomycin; TEC, teicoplanin; MEM, meropenem; AMP, ampicillin; GEN, gentamicin; STR, streptomycin; TET, tetracycline; ERY, erythromycin; CHL, chloramphenicol; RIF, rifampin; LVX, levofloxacin; LZD, linezolid.
[b] COPD, chronic obstructive pulmonary disease.
[c] *, new ST/allele in this study.
[d] The *esp* gene was detected by PCR amplification, as described in the text. P, positive; N, negative.
[e] Tn*1546*-like elements were typed by DNA sequence structure, as shown in Fig. 4.
[f] The wild-type strain and *E. faecium* BM4105RF were used as donor and recipient, respectively (13).
[g] Plasmids were identified in our previous report (35).

Clinical Laboratory Standards Institute (formerly NCCLS). *E. faecium* ATCC 9790 was used as a control strain.

Plasmid DNA methods. Recombinant DNA methodology, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (21). PCR was performed with a Perkin-Elmer Cetus apparatus. Specific primers for DNA sequencing of the Tn*1546*-like element and insertion sequences were designed as previously reported and purchased from Invitrogen (11, 31, 22, 34). Sequence analysis was performed with a dye terminator cycle sequencing kit (Applied Biosystems) and a model 310 gene analyzer (ABI PRISM).

Conjugation experiments. Broth matings were performed as previously described with a donor/recipient ratio of 1:10 (5, 12). Overnight cultures of 0.05 ml of the donor and 0.45 ml of the recipient were added to 4.5 ml of fresh THB, and the mixtures were incubated at 37°C with gentle agitation for the appropriate times and then vortexed. Portions of the mixed cultures were then plated on solid media with appropriate selective antibiotics. Colonies were counted after 48 h of incubation at 37°C. Filter matings were performed as previously described with a donor/recipient ratio of 1:10 (6). Overnight cultures were prepared, 0.05 ml of the donor and 0.5 ml of the recipient were added to 4.5 ml of fresh THB, and the cells were then trapped on a membrane filter (Millipore, Bedford, MA). The cells on the filters were incubated at 37°C overnight and were then suspended in 1 ml of THB. Appropriate dilutions of the mixture were transferred to plates of solid medium containing selective antibiotics. Throughout the mating experiments, the antibiotic concentration used for the selection of vancomycin-resistant transconjugants was 6 μg/ml. The antibiotic concentrations used for the selection of rifampin- and fusidic acid-resistant recipient strains or streptomycin- and spectinomycin-resistant recipient strains were 25 and 25 μg/ml or 250 and 250 μg/ml, respectively.

PFGE. Pulsed-field gel electrophoresis (PFGE) was then carried out in a 1% agarose gel with 0.5% Tris-borate-EDTA buffer, and the following settings were applied: 1 to 23 s, 6 V/cm, and 22 h (with the CHEF Mapper system [Bio-Rad]) (18).

DNA-DNA hybridization. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual (21).

MLST analysis. Multilocus sequence typing (MLST) analysis of *E. faecium* isolates was performed as previously reported (10, 32). The alleles and sequence types (STs) were analyzed and determined through the MLST database (http://efaecium.mlst.net/). The new alleles and new STs identified in this study have been deposited in the database.

Detection of the *esp* gene. To detect the *esp* gene of the *E. faecium* isolates, PCR amplification was performed as previously described with specific primers (16).

Detection of the *traA* gene of a pMG1-like plasmid. To detect the *traA* gene in the conjugative plasmids, PCR amplification was performed with specific primers (traA-F, TGAGAAAGAAATCGCTGATG; traA-R, TGAAGGCGTTCTTCTTTCAG), as previously described (24, 28).

RESULTS AND DISCUSSION

Isolation and characterization of VanA-type vancomycin-resistant *E. faecium*. The characteristics of the 13 isolates of vancomycin-resistant *E. faecium* are listed in Table 1. Each VRE strain was isolated from an individual patient in the hospital. In all cases, glycopeptide antibiotics and/or broad-spectrum cephalosporins were administrated to the patient before the isolation of VRE. All isolates were multidrug resistant, with MICs of 256 to 512 μg/ml and 16 to 512 μg/ml for vancomycin and teicoplanin, respectively. All 13 *E. faecium* isolates were resistant to erythromycin, and 11 isolates (85%) also showed high-level resistance to ampicillin and gentamicin. All isolates were sensitive to linezolid.

The PFGE profiles of SmaI-digested chromosomal DNA demonstrated that strain ZB23 was closely related to strain ZB24, differing by only one band; the other 11 isolates were largely heterogeneous in nature (Fig. 1), indicating that these isolates were unrelated and suggesting that vancomycin resistance was able to emerge in different *E. faecium* strains.

MLST analysis. All isolates were analyzed by the MLST scheme for *E. faecium* described previously (10, 32; http://www.mlst.net). Allelic profiles of these *E. faecium* isolates were obtained by sequencing of internal fragments of seven housekeeping genes—*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adkC*—and STs were determined (Table 1). In strain ZB18, three of seven alleles, *atpA*, *adh*, and *pdkC*, belonged to the new alleles, and the closest homologue alleles found were between *atpA* and *atpA15* (81% identity), *adh* and *gdh19* (99% identity), and *pdkC* and *adkC8* (81% identity). Based on the allelic profiles of the 13 isolates, three belonged to ST78 and three to ST203,

TABLE 1—*Continued*

esp status[d]	Tn*1546*-like element		Transfer frequency of vancomycin resistance[f] (per donor cell)		MIC (μg/ml)										
	Type[e]	Location	Broth mating (4 h)	Filter mating (16 h)	VAN	TEC	AMP	GEN	STR	TET	ERY	CHL	RIF	LVX	LZD
N	VI	Plasmid (pI125V^R)	$<10^{-7}$	1.6×10^{-4}	512	16	128	>1,024	2048	0.5	512	8	4	16	2
N	V	Plasmid (pC264V^R)	$<10^{-7}$	2.3×10^{-7}	256	32	128	>1,024	64	0.5	512	32	4	32	2
N	II	Plasmid	$<10^{-7}$	3.0×10^{-5}	512	256	0.25	>1,024	32	0.5	512	16	512	1	2
N	II	Plasmid	$<10^{-7}$	3.7×10^{-6}	512	256	128	>1,024	>4,096	0.25	512	8	4	4	2
N	II	Plasmid	$<10^{-7}$	1.4×10^{-3}	512	256	128	>1,024	32	0.25	512	16	4	64	2
N	II	Plasmid	$<10^{-7}$	5.7×10^{-6}	512	256	128	>1,024	16	8	512	8	4	64	2
N	I	Plasmid (pZB18)	3.1×10^{-4}	7.0×10^{-1}	512	256	2	16	2,048	256	256	64	<0.1	2	2
P	II	Plasmid	$<10^{-7}$	1.7×10^{-5}	512	512	128	>1,024	32	0.25	512	16	8	128	2
P	III	Plasmid	$<10^{-7}$	1.8×10^{-5}	256	64	128	>1,024	64	64	512	16	8	32	2
P	IV	Plasmid	$<10^{-7}$	2.8×10^{-6}	512	16	128	128	32	0.25	512	16	4	64	2
P	II	Plasmid (pZB22)	4.2×10^{-7}	8.5×10^{-3}	512	512	128	>1,024	32	64	512	4	4	64	2
P	II	Plasmid	$<10^{-7}$	2.7×10^{-6}	512	256	128	>1,024	32	0.5	512	16	<0.1	64	2
P	II	Plasmid	$<10^{-7}$	3.1×10^{-6}	512	256	128	>1,024	32	0.25	512	16	<0.1	64	2

and one isolate belonged to ST18 and another to ST25. The remaining five isolates were new STs, designated as ST320, ST335, ST321, ST322, and ST323 (Table 1; http://efaecium.mlst.net/ [accessed 10 February 2007]).

The nearest relation to each of the five new STs was ST80, ST172, ST31, ST262, and ST17, respectively. In total, nine STs (ST323, ST18, ST322, ST25, ST78, ST203, ST320, ST321, and ST335) of the Chinese isolates were categorized into seven clonal complexes (CC), as follows; CC17 (ST323), CC18 (ST18 and ST322), CC25 (ST25), CC78 (ST78 and ST203), CC117 (ST320), CC280 (ST321), and CCS (ST335). Previous reports showed that CC17 strains, which are frequently isolated as hospital outbreak strains, have a genetic lineage to ampicillin resistance and pathogenicity islands containing the *esp* gene (16, 30, 32). The clonal complexes of the Chinese isolates were genetically linked to each other and were close to CC17, except for CCS. All isolates were ampicillin resistant, except for two isolates belonging to CCS and CC25, which were relatively far

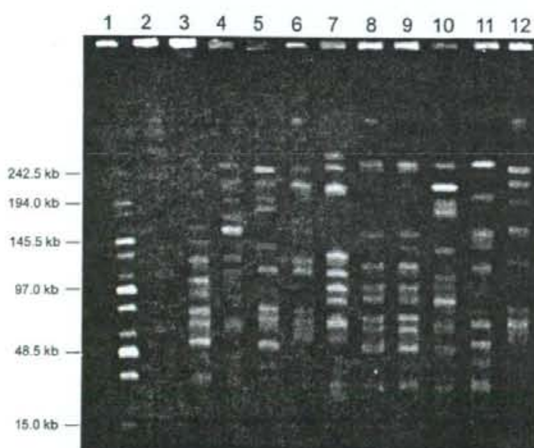

FIG. 1. PFGE of SmaI-digested chromosomal DNAs. Lane 1, molecular mass marker (Midrange Molecular Marker; New England Biolabs); lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

from the other CCs. Most previously reported hospital outbreak isolates are ampicillin resistant and positive for the *esp* gene (16). However, only 6 of the 11 ampicillin-resistant isolates in this study were found to carry the *esp* gene.

Analysis of VanA-type vancomycin resistance genes encoded on Tn*1546*-like elements. Since, most Tn*1546*-like elements encoding VanA-type vancomycin resistance are plasmid borne (4), all of the isolates in this study were examined for plasmid content and location of the *vanA* gene by Southern hybridization. The EcoRI restriction profiles of total plasmid DNAs isolated from the VRE strains showed that the plasmids of strain ZB23 were identical to those of strain ZB24 and that the plasmids of strain ZB14 were closely related to those of strain ZB15 (Fig. 2A). Other strains showed heterogeneous plasmid patterns. The *vanA* probe hybridized to an EcoRI fragment in plasmid DNA from each of the strains (Fig. 2B). These results indicated that all of the VanA determinants (Tn*1546*-like elements) were carried on plasmids in these VRE strains. The transferabilities of the vancomycin-resistant traits of the strains were examined by broth mating and filter mating with *E. faecium* BM4105RF used as the recipient strain (Table 1). Vancomycin resistance was transferred at frequencies from 10^{-1} to 10^{-7} per donor cell by filter mating among the strains studied. The vancomycin resistance of ZB18 and ZB22 was transferred at frequencies of 10^{-4} and 10^{-7} per donor cell by broth mating, respectively. All of the vancomycin resistance plasmids were self-transferable or mobile. Two highly conjugative vancomycin resistance plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were isolated from strains ZB18 and ZB22, respectively (Fig. 3). There are two kinds of highly conjugative plasmids found in enterococci, including pheromone-responsive plasmids of *E. faecalis* and pMG1-like plasmids of *E. faecium* (3, 13). We previously discovered the highly conjugative gentamicin resistance plasmid pMG1 (65 kbp) from an *E. faecium* clinical isolate in Japan (13). pMG1-like plasmids were widely disseminated in vancomycin-resistant *E. faecium* clinical isolates obtained from a hospital in the United States (27). Recently, we also isolated pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *E. faecium* clinical isolates in Japan (26, 28). All of the pMG1-like plasmids carry a conserved *traA* gene which is involved in the *tra* gene

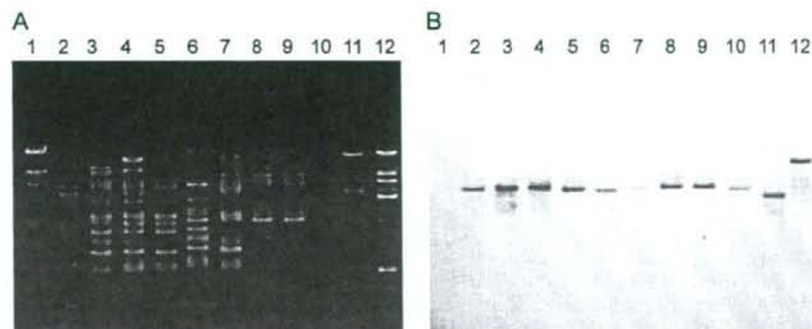

FIG. 2. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs from *E. faecium* isolates (A) and Southern hybridization analysis with the *vanA* probe (B). Lanes 1, HindIII-digested lambda DNA; lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

system for conjugation and is pMG1 specific (24). pZB18 and pZB22 were examined by PCR amplification to determine whether *traA* was conserved in each of these plasmids. Neither of plasmids carried the *traA* gene. The result implied that both pZB18 and pZB22 were different from the pMG1-like plasmids and could be a new type of highly conjugative *E. faecium* plasmid, as previously reported (23).

The DNA sequences of the Tn*1546*-like elements encoding the *vanA* operon for vancomycin resistance on the plasmids were determined (Fig. 4) (1). Specific primers for the insertion

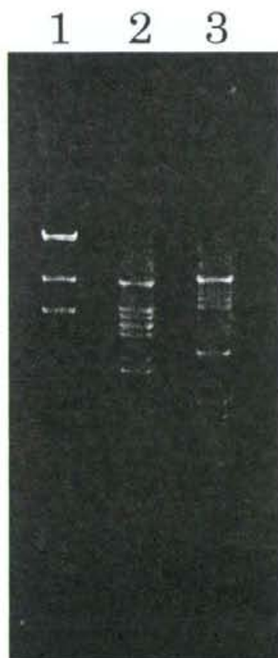

FIG. 3. Agarose gel electrophoresis of the EcoRI-digested highly conjugative plasmid DNA of pZB18 and pZB22. Lane 1, HindIII-digested lambda DNA; lane 2, pZB18; lane 3, pZB22.

sequences IS*1216V* (809bp) and IS*1542* (1,324bp), which are often found in Tn*1546*-like elements, were used in the sequence analysis (8, 31, 33).

A summary of the sequence analysis of the plasmid Tn*1546*-like elements and their comparison to the prototype element (designated type I in this study) are shown in Fig. 4. The *vanS* genes of all of the Chinese isolates were identical to that of the BM4147 strain and had no substitutions. Three specific substitutions within VanS result in low-level teicoplanin resistance, which is frequently found in East Asian VRE isolates (9, 15, 18, 34). The Tn*1546*-like elements of the 13 isolates were classified into six types based on sequence analysis and were designated type I to type VI (Fig. 4). We have reported two VanA-type VRE (*E. faecium*) clinical isolates, C264 and I125, which were originally isolated from patients in China (35). The Tn*1546*-like elements of both strains contained the insertion sequences IS*1216V* and IS*1542* and are classified as type V and type VI, respectively (Fig. 4).

Our group reported the first case of VanA-type *Enterococcus faecalis*: strain KC122.1, isolated in Japan from chicken meat imported from mainland China in 2001 (25). The Tn*1546*-like element was encoded on a conjugative plasmid and had three specific substitutions of VanS, resulting in low-level teicoplanin resistance, as mentioned above. The Tn*1546*-like element of KC122.1 was also examined in this study (Fig. 4). The element was identical to the prototype (type I) except for five point mutations, including the three substitutions within VanS, and had no insertions, suggesting that the origins of the elements of VRE clinical isolates were different from that of the VRE isolate from food animals.

Analytical data for the Tn*1546*-like elements of Chinese VRE isolates can be summarized as follows: (i) all of the elements are plasmid borne; (ii) 12 of the 13 isolates had multiple insertions of IS*1216V* and IS*1542* in the Tn*1546*-like elements; (iii) IS*1542* was inserted into the 8-bp target sequence CTATAATC, from bp 3817 to 3924 of Tn*1546*; (iv) the origins of the two IS*1216V* elements differed from each other, and the IS*1216V* in the *vanXY* region had one base pair substitution (T662C); (v) the distributions and insertions of IS*1216V* and IS*1542* associated with the Tn*1546*-like elements of the Chinese isolates were similar to those previously re-

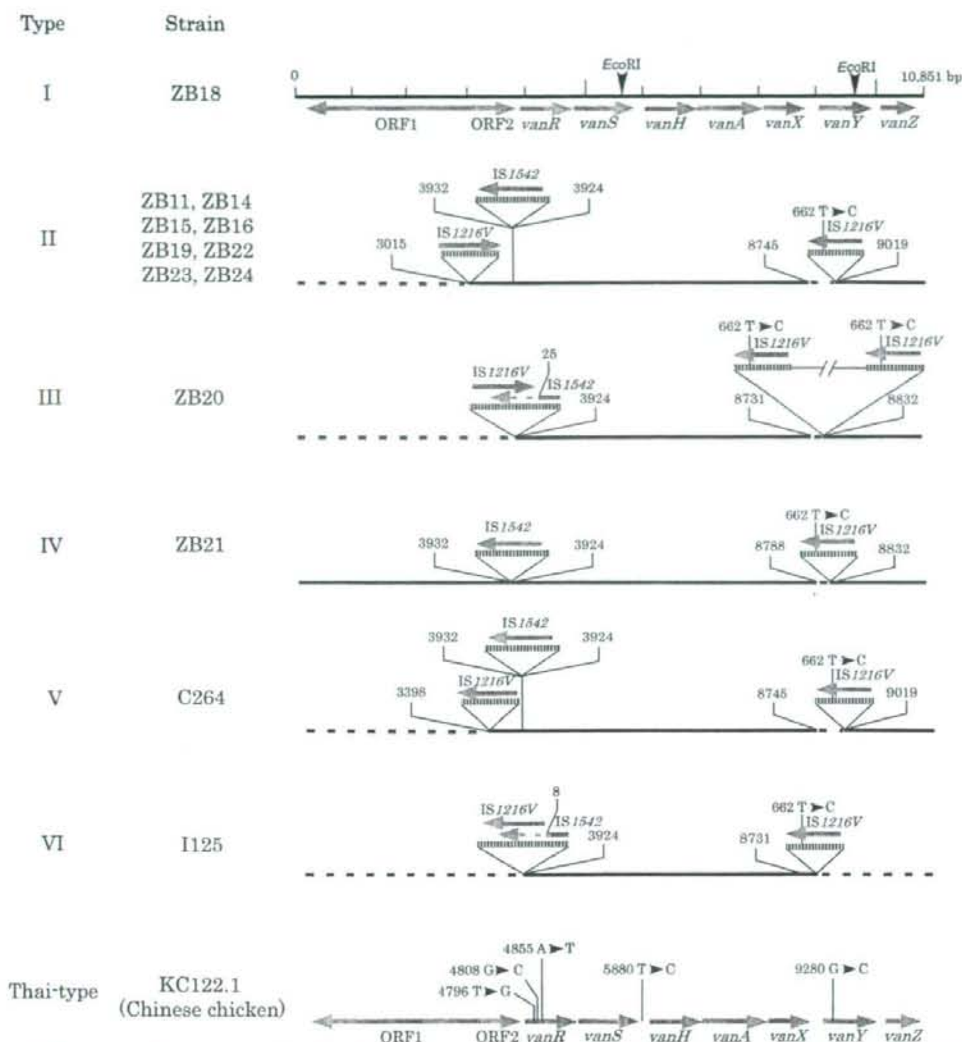

FIG. 4. Genetic organization and typing of Tn*1546*-like elements found in Chinese clinical isolates. The upper horizontal arrows show the genes and open reading frames (ORFs) encoded on the prototype Tn*1546* element of plasmid pIP816 (1). Boxes with vertical lines represent IS elements. The numbers at the IS insertions show the positions of the first nucleotides upstream and downstream of the inserts. The horizontal arrows on the IS elements indicate the transcriptional orientation of the transposase encoded on the ISs. The dotted horizontal lines indicate the deleted region. KC122.1 is a VanA-type *E. faecalis* strain isolated from chicken meat imported from mainland China (25). The Tn*1546*-like element of KC122.1 was temporarily classified as Thai-type, which is often found in VRE isolates from chicken meats imported from Thailand (9, 18).

ported for European and Korean VanA-type VRE isolates (11, 22, 31, 34); (vi) Tn*1546*-like elements were classified into six types, based on DNA sequencing (type I to VI), and type II elements were predominantly isolated from hospitals in Beijing and could be disseminated among different *E. faecium* strains; and (vii) there was no linkage between VRE isolates from humans (patients) and the VRE isolate from a food animal (chicken meat).

This study is the first to provide detailed molecular analyses of VRE clinical isolates from mainland China. To further elucidate the characteristics of Chinese VRE strains, a nationwide surveillance of VRE and systemic analyses of other types of VRE strains are necessary. In the meantime, the current recommended hospital infection control measures for developed countries may be readily implemented to prevent further spread of VRE in mainland China.

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JAC

Investigation of isolation rates of *Pseudomonas aeruginosa* with and without multidrug resistance in medical facilities and clinical laboratories in Japan

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Objectives: To perform a large-scale investigation of *Pseudomonas aeruginosa* strains with and without drug resistance in Japan.

Methods: We distributed questionnaires to assess isolation rates of *P. aeruginosa* with and without drug resistance at medical facilities and clinical laboratories throughout Japan during the period January 2003 through June 2006. Completed questionnaires were obtained from 339 medical facilities and 4 clinical laboratories.

Results: The total number of *P. aeruginosa* strains isolated at the medical facilities was 549 746 and that at clinical laboratories was 640 232. Strains resistant to carbapenems, fluoroquinolones (ciprofloxacin or levofloxacin) and amikacin were defined as multidrug-resistant (MDR) strains, and strains resistant to two of these drugs were defined as two-drug-resistant (TDR) strains. The percentage of MDR at medical facilities and clinical laboratories was 2.4% and 1.1%, respectively, and that of TDR isolates was 6.4% and 4.2%, respectively. MDR and TDR isolates were found nationwide. No MDR isolates were found at approximately one-third of the medical facilities each year. The percentages of MDR and TDR isolates increased significantly from 2003 to 2005. *P. aeruginosa* strains were obtained mainly from the respiratory and urinary tracts, and the percentages of MDR and TDR isolates were particularly increased in the urinary tract during these years.

Conclusions: MDR *P. aeruginosa* was prevalent nationwide in Japan. The incidence was low, except in a limited number of facilities, but it increased significantly.

Keywords: nationwide surveillance, retrospective questionnaire, laboratory-based surveillance

Introduction

Pseudomonas aeruginosa has intrinsic resistance to many antimicrobial agents, and only a few antimicrobial agents show potent antibacterial activity against this bacterium. The emergence of multidrug-resistant (MDR) *P. aeruginosa* strains is a serious problem.[1–3] Nosocomial outbreaks of *P. aeruginosa* infection, particularly by MDR strains, have become problematic in hospitals in various countries,[4–8] including in Japan.[3,9,10] Many cases of MDR *P. aeruginosa* infection have been reported in Japan. However, there have been few nationwide investigations of the prevalence *P. aeruginosa* infection at medical facilities. We investigated isolation rates of *P. aeruginosa* strains with and without drug resistance in cooperation with various medical facilities and clinical laboratories throughout Japan. This is the first surveillance study of clinically isolated *P. aeruginosa* with and without drug resistance in Japan.

Materials and methods

Methods and subjects

Information was gathered by means of a questionnaire. Questionnaires were sent on 27 July 2006 to 538 medical facilities and 4 clinical laboratories across Japan, including all 350 facilities with

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500 or more beds and 188 regional core hospitals with 120 or more but <500 beds and 4 large clinical laboratories. The regional core hospitals were selected by considering their geographic locations across the country. The four clinical laboratories had numerous branch laboratories (a total of 300 sites) covering all areas of Japan, and they are commissioned by clinics and hospitals to analyse clinical samples, including pathogenic bacterial samples.

Completed questionnaires were returned by 339 medical facilities (63% response rate) and the 4 clinical laboratories (100% response rate) as of 6 October 2006. The average number of beds in these medical facilities was 577 ± 230 (median 550; range 120–1505). The period of investigation was January 2003 through June 2006.

Questionnaires

The questionnaire enquired about (i) the number of beds, (ii) the total number of *P. aeruginosa* strains isolated each year with or without TDR or MDR, (iii) the number of patients with TDR or MDR strains and (iv) the tissue sources of the isolated strains.

Clinical isolation of P. aeruginosa strains

P. aeruginosa strains were isolated from inpatients and outpatients with suspected *P. aeruginosa* infection and subjected to drug-susceptibility testing. Repeat testing of single patients was assumed when repeat examinations were ordered. Strains isolated for analysis in this study were not from the environment, carriers, non-symptomatic patients or healthy staff.

Drug-resistant strains

Strains that were resistant to carbapenems, fluoroquinolones (ciprofloxacin or levofloxacin) and amikacin were defined as MDR strains. Strains that were resistant to two of these drugs were defined as two-drug-resistant (TDR) strains. Drug resistance was assessed by determining the MIC in culture medium containing the drugs or by determining the diameter of the growth inhibition zone (DGIZ) on culture agar with the use of drug-containing discs. Breakpoints were determined in accordance with the criteria for MDR strains specified by the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections of the Japanese Ministry of Health, Labour and Welfare. MIC breakpoints for carbapenems, amikacin, ciprofloxacin and levofloxacin were ≥ 16, ≥ 32, ≥ 4 and ≥ 8 mg/L, respectively; DGIZ breakpoints for these drugs were ≤ 13, ≤ 14, ≤ 15 and ≤ 13 mm, respectively.

Drug susceptibility tests for bacteria, including *P. aeruginosa*, are performed in Japan in accordance with the standards published by the Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Statistical analysis

Chronological trends in the proportions of TDR and MDR isolates were assessed by the Mantel–Haenszel χ^2 test. The numbers of isolates from various tissue sources were analysed by the χ^2 test. P values of <0.0001 were considered statistically significant.

Results

P. aeruginosa isolates and infections at medical facilities

During the study period, a total of 549 746 *P. aeruginosa* strains were isolated at the 339 medical facilities. The numbers of TDR and MDR isolates were 35 030 (6.4%) and 13 296 (2.4%), respectively. As shown in the upper half of Table 1, the total numbers of isolates, including TDR and MDR strains, as well as the adjusted numbers (number of isolates/1000 beds), increased gradually from 2003 to 2005. The percentages of TDR and MDR strains also increased significantly during the period ($P < 0.0001$). In addition, the number of patients with TDR or MDR strains (and the number of patients per 1000 beds/year) increased gradually from 2003 to 2005. The reason for the lack of increase in the first half of 2006 was unclear. Investigation of the entire year may yield different results.

We also analysed the number of patients with MDR per 1000 beds per month. The numbers ranged from 0 to 110.7 in 2003, 0 to 100.5 in 2004, 0 to 150.5 in 2005 and 0 to 106.7 in the first half of 2006. The median values for these years were 2.8, 3.6, 4.6 and 4.0, respectively. The 90 percentile values were 22.9, 25.0, 25.6 and 25.3, respectively. Some of the 339 medical facilities reported that no MDR *P. aeruginosa* strains were isolated in a given year. The number of these facilities was 103 (30.4%) in 2003, 93 (27.4%) in 2004, 93 (27.4%) in 2005 and 127 (37.5%) in the first half of 2006. Forty-eight facilities (14.2%) reported isolation of no MDR *P. aeruginosa* strains during the study period. The number of patients with MDR per 1000 beds per month was less than two in 90.4%, 89.0%, 87.9% and 89.3% of the facilities in 2003–06, respectively, whereas two or more patients with MDR strains were identified in ~10% of the facilities during the study period, suggesting that MDR *P. aeruginosa* was prevalent in a limited number of hospitals. We then investigated whether high incidence of MDR occurred geographically. No significant difference between geographic areas was found (data not shown).

P. aeruginosa isolated at clinical laboratories

Completed questionnaires from the four clinical laboratories were also analysed. The number of *P. aeruginosa* strains isolated during the study period was 640 232, and the numbers of TDR and MDR isolates were 26 913 (4.2%) and 6768 (1.1%), respectively. The data for each year are shown in the lower half of Table 1. The numbers increased markedly from 2003 to 2005, but the percentages of TDR and MDR did not. It could not be determined whether the percentages increased in 2006, given that data were obtained for only half of the year.

Comparison of the percentages of TDR and MDR isolates from the laboratories with those from the medical facilities showed lower percentages from the laboratories.

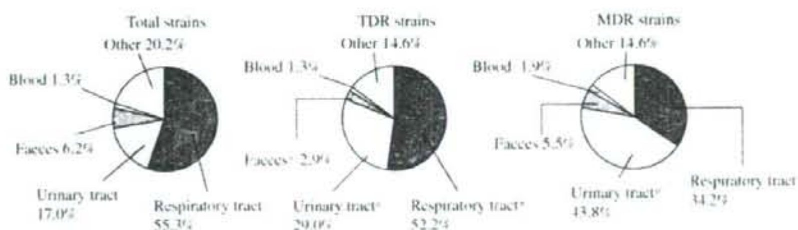
Tissue sources of P. aeruginosa

The tissue sources and percentages of the total *P. aeruginosa* strains isolated at the 339 medical facilities for the study periods and those of TDR and MDR strains are shown in Figure 1a. The percentages for each year were similar to those for the entire study period (data not shown). These results indicated that *P. aeruginosa*, including TDR and MDR strains, affected mainly the respiratory and urinary tracts. However, it is notable that the percentages of TDR and MDR isolates in the urinary tract were significantly greater than that of the total isolates ($P < 0.0001$) and the percentages of MDR

Table 1. Isolation of *P. aeruginosa* with or without multidrug-resistance in medical facilities and clinical laboratories

Strains	2003	2004	2005	2006 (half a year)
Medical facilities				
P. aeruginosa				
total numbers (nos.)[a]	145 148	160 784	168 762	75 052
nos. of isolates per 1000 beds/year	742	822	862	767
TDR strains				
total nos.	8615	10 236	11 125	5054
nos. of isolates per 1000 beds/year	44.0	52.3	56.9	51.7
rate (%)[b]	5.9	6.4	6.6	6.7
patient nos.[c]	3912	5624	4737	2342
nos. of patients per 1000 beds/year	20.0	23.3	24.2	23.9
MDR strains				
total nos.	2941	3894	4437	2024
nos. of isolates per 1000 beds/year	15.0	19.9	22.7	20.7
rate (%)	2.0	2.4	2.6	2.7
patient nos.	1234	1681	1969	888
nos. of patients per 1000 beds/year	6.3	8.6	10.1	9.1
Clinical laboratories				
P. aeruginosa				
total nos.	154 055	167 472	210 311	108 394
TDR strains				
total nos.	5899	7290	9108	4616
rate (%)	3.8	4.4	4.3	4.3
MDR strains				
total nos.	1436	1937	2284	1111
rate (%)	0.9	1.2	1.1	1.0

[a]Numbers of *P. aeruginosa* isolated from the 339 medical facilities and the 4 clinical laboratories that answered the questionnaire.
[b]The ratio of the numbers of TDR or MDR *P. aeruginosa* to the total numbers of isolated *P. aeruginosa* (%).
[c]The numbers of patients with TDR or MDR *P. aeruginosa*.



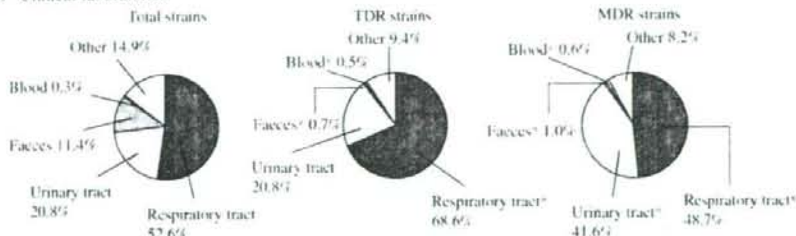


Figure 1. Tissue sources of total *P. aeruginosa* strains, TDR strains and MDR strains isolated during the study period at medical facilities (a) and clinical laboratories (b). An asterisk indicates a significant difference between numbers of TDR and total isolates or between MDR and total isolates. TDR, two-drug-resistant; MDR, multidrug-resistant.

isolates in the urinary tract surpassed those in the respiratory tract.

The tissue sources and percentages of *P. aeruginosa* isolated at the four clinical laboratories during the study period were also analysed (Figure 1b). The results showed that the percentage of MDR isolates in the urinary tract was significantly greater than that of the total and TDR strains ($P < 0.0001$).

Discussion

It was not practical to send questionnaires to all medical facilities and laboratories in Japan. Therefore, we selected all large-scale facilities and many regional core hospitals, as well as highly respected laboratories, by considering their geographic locations across the country. Data collected by these laboratories may provide information for various clinics and small-scale hospitals, and may shed light on different aspects of *P. aeruginosa* prevalence. By analysing questionnaires completed by 339 medical facilities and 4 laboratories, we believe that we obtained accurate information regarding the present state of *P. aeruginosa* prevalence in Japan. However, the use of retrospective data implies inherent biases.

Our survey showed that MDR *P. aeruginosa* was prevalent nationwide but the incidence was low, except in a limited number of facilities. The survey also showed that MDR strains were isolated from the urinary tract as well as the respiratory tract, suggesting the importance of management of patient's urine in the prevention and control of nosocomial MDR *P. aeruginosa* infection.[3,9]

The samples from clinical laboratories may provide information on the prevalence of *P. aeruginosa* infection at clinics and small-scale hospitals. The percentages of TDR and MDR *P. aeruginosa* isolates from the laboratories were lower than those from the medical facilities. Although the exact reasons are unclear, this may be related to differences in the use of antibiotics, the severity of cases, scales of nosocomial outbreaks and populations of inpatients and outpatients.

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Transparency declarations

None to declare.

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Outbreaks of Multidrug-Resistant *Pseudomonas aeruginosa* in Community Hospitals in Japan

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We previously reported an outbreak in a neurosurgery ward of catheter-associated urinary tract infection with multidrug-resistant (MDR) *Pseudomonas aeruginosa* strain IMCJ2.S1, carrying the 6′-*N*-aminoglycoside acetyltransferase gene [*aac(6′)-Iae*]. For further epidemiologic studies, 214 clinical isolates of MDR *P. aeruginosa* showing resistance to imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 64 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml) were collected from 13 hospitals in the same prefecture in Japan. We also collected 70 clinical isolates of *P. aeruginosa* that were sensitive to one or more of these antibiotics and compared their characteristics with those of the MDR *P. aeruginosa* isolates. Of the 214 MDR *P. aeruginosa* isolates, 212 (99%) were serotype O11. We developed a loop-mediated isothermal amplification (LAMP) assay and a slide agglutination test for detection of the *aac(6′)-Iae* gene and the AAC(6′)-Iae protein, respectively. Of the 212 MDR *P. aeruginosa* isolates, 212 (100%) and 207 (98%) were positive in the LAMP assay and in the agglutination test, respectively. Mutations of *gyrA* and *parC* genes resulting in amino acid substitutions were detected in 213 of the 214 MDR *P. aeruginosa* isolates (99%). Of the 214 MDR *P. aeruginosa* isolates, 212 showed pulsed-field gel electrophoresis patterns with ≥70% similarity to that of IMCJ2.S1 and 83 showed a pattern identical to that of IMCJ2.S1, indicating that clonal expansion of MDR *P. aeruginosa* occurred in community hospitals in this area. The methods developed in this study to detect *aac(6′)-Iae* were rapid and effective in diagnosing infections caused by various MDR *P. aeruginosa* clones.

Pseudomonas aeruginosa causes nosocomial infections as a result of its ubiquitous nature, ability to survive in moist environments, and resistance to many antibiotics and antiseptics. A serious problem is the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains resistant to β-lactams, aminoglycosides, and quinolones (34, 39, 46). Although intrinsically sensitive to β-lactams (e.g., ceftazidime [CAZ] and imipenem [IPM]), aminoglycosides (e.g., amikacin [AMK] and tobramycin), and fluoroquinolones (e.g., ciprofloxacin [CIP] and ofloxacin [OFX]), *P. aeruginosa* resistant to these antibiotics has emerged and is widespread (34, 39, 46).

We previously reported a nosocomial outbreak of catheter-associated urinary tract infection involving new MDR *P. aeruginosa* strain IMCJ2.S1, which occurred in a neurosurgery ward of a hospital located in the Tohoku area of Japan (46). This strain showed broad-spectrum resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, sulfonamide, and chlorhexidine. We found that IMCJ2.S1 harbored a novel class 1 integron, In113, containing an array of three gene cassettes of the metallo-β-lactamase (MBL) bla_{IMP-1} gene, aminoglycoside 6′-acetyltransferase *aac(6′)-Iae* gene, and aminoglycoside 3′-adenylyltransferase *aadA1* gene (46). This strain possessed mutations of the *gyrA* (83Thr→Ile) and *parC* (87Ser→Leu) genes involving amino acid substitutions, resulting in high-level resistance to fluoroquinolones.

In the geographic area where the MDR *P. aeruginosa* outbreak occurred (46), hospitals and a commercial clinical laboratory were surveyed for similar organisms. Because 99% of the MDR *P. aeruginosa* isolates analyzed were found to harbor the *aac(6′)-Iae* gene, we developed a loop-mediated isothermal amplification (LAMP) assay (31) and a slide agglutination assay to detect the *aac(6′)-Iae* gene and AAC(6′)-Iae protein, respectively. These methods were evaluated for their usefulness in detecting new MDR *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial strains. Criteria for multidrug resistance of *P. aeruginosa* were in accordance with the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections of the Japanese Ministry of Health, Labor, and Welfare; the criteria are resistance to imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 64 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml). The criterion for amikacin resistance (MIC ≥ 64 μg/ml) was different from that of a guideline of the Clinical and Laboratory Standards Institute (MIC ≥ 32 μg/ml) (4). Two

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hundred eighty-four clinical isolates of *P. aeruginosa* were obtained from 284 inpatients in 13 hospitals in Japan during the period October 2003 to September 2004; 214 isolates were MDR, and 70 were non-MDR. Information regarding the origins of the specimens was available for 99 of the 214 MDR isolates: 72 (73%) were from urine specimens, 18 (18%) were from respiratory tract specimens, 5 (5%) were from feces, 2 (2%) were from catheter tips, and 2 (2%) were from wounds. Of the 72 isolates from urine, 55 were from patients with urinary catheters. All *P. aeruginosa* isolates were originally identified by the submitting laboratories. Isolates that did not have typical characteristics (pigment and colony morphology) for *P. aeruginosa* were analyzed biochemically with an API 20NE kit (API-bioMerieux, La Balme les Grottes, France) to confirm identity as *P. aeruginosa*. *P. aeruginosa* M207 possessing bla_{IMP-1}, *P. aeruginosa* NCB326 possessing bla_{IMP-2}, and *Acinetobacter baumannii* NCB0211-439 possessing bla_{VIM-2} were provided by Y. Arakawa (National Institute of Infectious Diseases, Tokyo, Japan). *Escherichia coli* strain TOP10 (Invitrogen Corp., Carlsbad, CA) was used as the host for recombinant plasmids.

Serotyping. The O serotypes of the isolates were determined with a slide agglutination test kit containing three polyvalent antisera and 14 monovalent antisera (Denka Seiken Co., Tokyo, Japan). The kit was not in conformity with the International Antigenic Typing Scheme (IATS) (26) and was not applicable to some O types in the IATS. Therefore, we applied the standard classification of O types from A to N proposed by the Serotyping Committee for the Japan *Pseudomonas aeruginosa* Society (12).

Antimicrobial susceptibility. We obtained AMK and IPM from Banyu Pharmaceutical Co. (Tokyo, Japan), arbekacin [1-*N*-(*S*)-4-amino-2-hydroxybutyl dibekacin; ABK] from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), aztreonam (AZL) from Eizai (Tokyo, Japan), CAZ from GlaxoSmithKline K. K. (Tokyo, Japan), CIP and OFX from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), doripenem (DRPM) from Shionogi & Co., Ltd. (Osaka, Japan), gentamicin (GEN) and streptomycin (STR) from Nacalai Tesque, Inc. (Kyoto, Japan), meropenem (MEM) from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), piperacillin (PIP) and piperacillin-tazobactam (TZP) from Tomiyama Pure Chemical Industries, Ltd. (Tokyo, Japan), and polymyxin B (PL-B) from Sigma-Aldrich (St. Louis, MO). Arbekacin is an aminoglycoside antibiotic and has been used for the treatment of methicillin-resistant *Staphylococcus aureus* infections in Japan (51). Values for MICs at which 50% of isolates were inhibited (MIC_{50}) and MIC_{90} were determined by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI, formally NCCLS; standard M7-A6) (4) except for ABK, PL-B, and STR, for which breakpoints ($\geq$4 μg/ml) were obtained from the published data (16, 30, 46).

Screening for MBL-producing *P. aeruginosa*. *P. aeruginosa* isolates were screened for the presence of MBL by a double-disk synergy test with disks containing sodium mercaptoacetic acid, according to the method of Arakawa et al. (2).

Immunologic detection of AAC(6')-Iae. To detect AAC(6')-Iae produced by *P. aeruginosa*, we developed a new method with AAC(6')-Iae antibody-conjugated beads. Recombinant AAC(6')-Iae was purified as reported previously (46) and used for immunization of Japanese white rabbits. Antibody against AAC(6')-Iae was affinity purified from rabbit antisera with an *N*-hydroxysuccinimide–Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) conjugated to recombinant AAC(6')-Iae. Purified antibody was coupled to Polybead carboxylated microspheres (2.022 μm in diameter; Polysciences, Inc., Warrington, PA) according to the manufacturer's instructions. Antibody-conjugated beads were suspended at 2.5% (vol/vol) in 0.1 M phosphate buffer (pH 7.4) containing 0.1% sodium azide. Agglutination tests were performed with *P. aeruginosa* isolates grown on *N*-acetyl-L-cysteine agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Bacterial cells suspended in distilled water were mixed with the antibody-conjugated beads. To confirm the specificity of the agglutination test, *P. aeruginosa* isolates were analyzed by conventional Western blotting with AAC(6')-Iae antibody.

PCR of class 1 integrons. Class 1 integrons responsible for multidrug resistance in *P. aeruginosa* (21, 34, 46) were detected and characterized by PCR as described previously (24). Primer pairs designed to amplify the gene cassette of In113 (46) and three primer pairs specific for bla_{IMP-1}, bla_{IMP-2}, and bla_{VIM-2} (47) were used. Positive controls were *P. aeruginosa* IMCJ2.S1 for class 1 integron In113, *P. aeruginosa* M207 for bla_{IMP-1}, *P. aeruginosa* NCB326 for bla_{IMP-2}, and *A. baumannii* NCB0211-439 for bla_{VIM-2}. PCR was performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). Genomic DNA was extracted as described by Sambrook et al. (44). When unexpected sizes of PCR products were obtained, the PCR products were cloned into cloning vector pCRII (Invitrogen Corp.) for DNA sequencing.

LAMP assay of *aac(6')-Iae*. The LAMP assay amplifies DNA with high specificity under isothermal conditions (31). To identify *P. aeruginosa* isolates possessing *aac(6')-Iae*, we designed four primers (FIP, 5'-CAA TAC AAA TGT TTT CGG CGC TAC GTC ACT CCA AAA GGC TAC-3'; BIP, 5'-TAA ACG ATG AAT TGT GTG GTT GGG TTG GAT GTA GTT CCC AAG TT-3'; F3, 5'-TCA CAC ATA AAT TTC GAT TCT TG-3'; and B3, 5'-ACC AAA TCC CTT ATT TTG ATG TT-3') for the LAMP assay. To extract DNA from *P. aeruginosa* isolates, a colony on *N*-acetyl-L-cysteine agar medium was suspended in 100 μl distilled water and boiled for 5 min. The bacterial suspension was then centrifuged at 12,000 × *g* for 2 min, and DNA in the supernatant was used for the LAMP assay. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The LAMP reaction mixture (12.5 μl), supplemented with 1.6 μM FIP and BIP primers, 0.2 μM F3 and B3 primers, 2× reaction mixture (6.25 μl), 4 U *Bst* DNA polymerase, 8 μg monomeric cyanine (YO-PRO-1), and 1.0 μl DNA sample, was incubated at 63°C for 45 min in a real-time thermal cycling system (Roter-Gene 2000; Corbett Research, Mortlake, New South Wales, Australia). Amplified DNA was monitored at 510 nm during the incubation. Alternatively, 25 μl of the reaction mixture was incubated at 63°C for 45 min on a block incubator (Advanced Science and Technology Enterprise Corp., Tokyo, Japan). After incubation, 10 μl of 1/100-diluted SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME) was added to the reaction mixture. A change in color from orange to green indicated positive amplification.

PCR of QRDRs. The *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions (QRDRs) were amplified by PCR with primers from and according to the methods described previously (1, 11, 20, 28). PCR products were sequenced with the same primers.

DNA sequencing. DNA sequences determined by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems), and deduced protein sequences were subjected to homology searches in the DNA Data Bank of Japan (DDBJ), GenBank, and EMBL databases with FASTA and BLAST.

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was prepared by the procedure of Grundmann et al. (10) and digested overnight with 10 U SpeI (Takara Bio, Inc., Shiga, Japan). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm for 20 h. The obtained fingerprinting patterns, normalized to the molecular weight markers, were analyzed by the unweighted-pair-group method with Molecular Analyst Fingerprinting Plus software, version 1.6 (Bio-Rad Laboratories, Inc.), to obtain average linkage-based dendrograms.

Statistical analysis. Results of a PCR assay, a LAMP assay, and an agglutination test were analyzed by chi-square test. A P value of <0.01 was considered statistically significant.

RESULTS

Distribution of MDR *P. aeruginosa* among hospitals. Nineteen hospitals and one clinical laboratory center from a single prefecture (population size, 2,360,000) participated in this study. MDR *P. aeruginosa* was isolated from 13 hospitals (Fig. 1). A total of 214 MDR *P. aeruginosa* isolates were obtained; 73 (34%), 38 (18%), and 22 (10%) were obtained from hospitals NA, CB, and CA, respectively, indicating that the spread of MDR *P. aeruginosa* was relatively limited. Seventy non-MDR *P. aeruginosa* isolates from the same hospitals were used for comparative analysis.

Serotyping. Ten serotypes were identified (Table 1): 222 were O11, 14 were O1, 10 were O10, 8 were B, 7 were M, 5 were O4, 4 were O3, 4 were O6, and 1 each was O9 and C. Six additional isolates showed agglutination with polyvalent antiserum but not with any of the monovalent antisera, i.e., they were nontypeable. A total of 212 of the 214 MDR *P. aeruginosa* isolates (99%) were serotype O11, whereas 70 of the non-MDR isolates were of a variety of serotypes, including O1, O3, O4, O6, O9, O10, O11, B, C, and M. These results indicated that serotype O11 was predominant for MDR *P. aeruginosa* in this prefecture.

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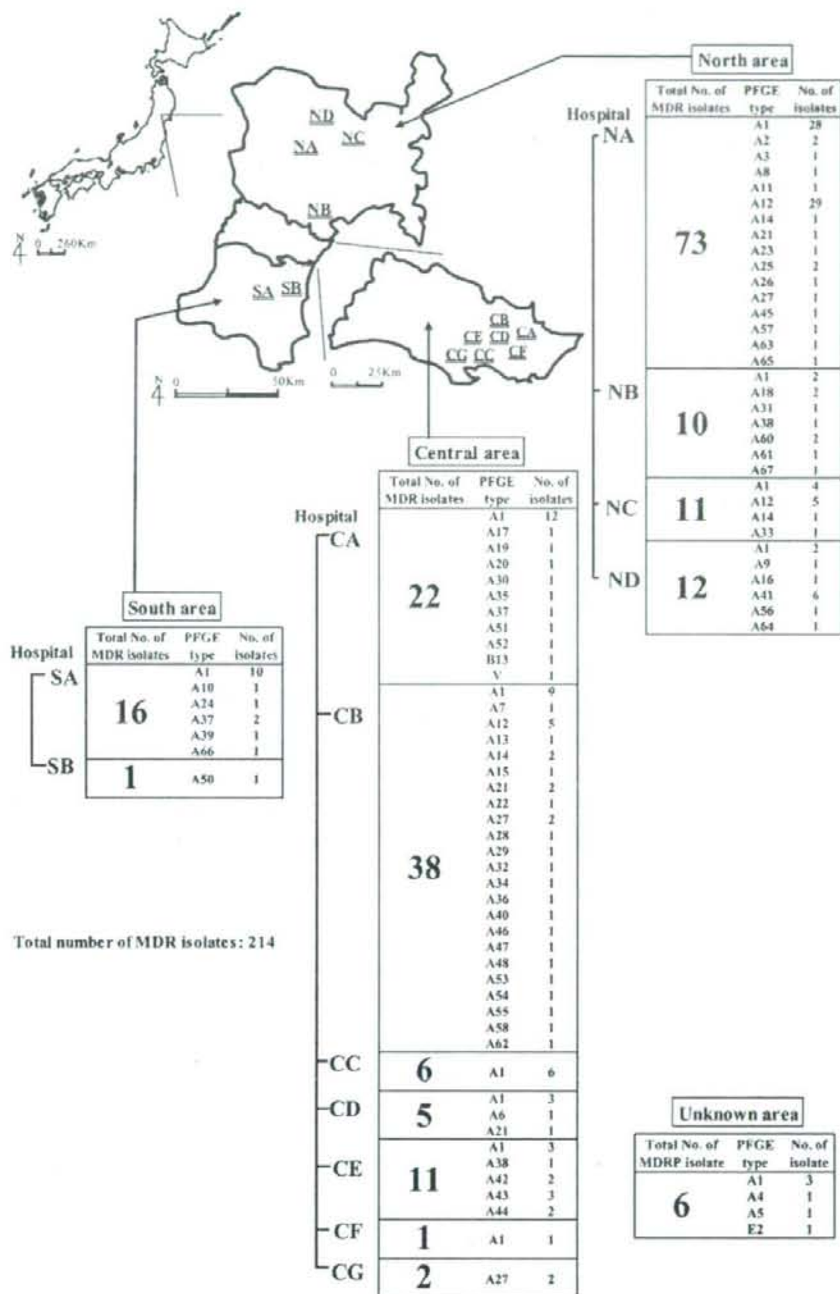

FIG. 1. Distribution of 214 isolates of MDR *P. aeruginosa* among 13 hospitals in Japan. Double capital letters indicate the locations of the hospitals that participated in this MDR *P. aeruginosa* survey.

Antimicrobial susceptibility tests. Most of the MDR *P. aeruginosa* isolates were resistant to all antimicrobials tested, except for GEN and PL-B (Tables 1 and 2). Rates of drug resistance were as follows: AMK, 100%; ABK, 91.6%; AZL, 99.5%; CAZ, 100%; CIP, 100%; DRPM, 99.1%; GEN, 57.5%; IPM, 100%; MEM, 100%; OFX, 100%; PIP, 100%; PL-B, 28%; STR, 100%; TZP, 100%. Rates of drug resistance among the non-MDR isolates were less than 63%, except that for

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TABLE 1. Phenotypic and genotypic characterization of 284 clinical isolates of *P. aeruginosa*

No. of isolates	Susceptibility to:									Serotype	Gene cassette(s) of the class 1 integron	PFGE type(s)
	β-Lactams					Amino-glycosides		FQs[a]				
	PIP	TZP	CAZ	IPM	MEM	AMK	GEN	CIP	OFX			
MDR-*P. aeruginosa*[b]												
120	R	R	R	R	R	R	R	R	R	O11	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1, A2, A4, A5, A7, A8, A9, A10, A12, A14, A15, A16, A18, A20, A21, A24, A25, A27, A28, A30, A31, A32, A33, A38, A41, A42, A43, A44, A45, A46, A48, A51, A54, A56, A62, A64, E2
85	R	R	R	R	R	R	S	R	R	O11	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1, A2, A6, A11, A12, A13, A17, A18, A19, A21, A22, A23, A25, A26, A27, A34, A35, A36, A37, A39, A40, A41, A44, A47, A52, A53, A55, A58, A60, A61, A63, A65, A66, A67
1	R	R	R	R	R	R	R	R	R	O1	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1
2	R	R	R	R	R	R	S	R	R	O1	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A38, A50
1	R	R	R	R	R	R	R	R	R	M	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A57
3	R	R	R	R	R	R	S	R	R	M	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A3, A29, A37
1	R	R	R	R	R	R	R	R	R	O10	*aac(6')-31-like1*	B13
1	R	S	R	R	R	R	S	R	R	O1		V
Non-MDR-*P. aeruginosa*												
1	R	S	S	S	R	R	S	R	R	O11		A49
1	S	S	S	S	S	S	S	R	R	O11		A59
1	R	R	S	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B1
1	S	S	R	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B1
1	S	S	S	R	R	S	S	R	R	O1	*aac(6')-31-like2*	B1
1	R	S	S	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B2
1	S	S	S	R	R	S	R	R	R	O1	*aac(6')-31*	B6
1	S	S	S	R	R	S	R	R	R	O1	*aac(6')-31-like1*	B8
1	R	S	S	R	R	S	S	R	R	O1	*aac(6')-31-like1*	B7
1	S	S	S	S	S	S	S	R	R	O6	*aac(6')-31-like1*	B3
1	S	S	S	S	S	S	S	R	R	O10	*aac(6')-31-like1*	B4
1	S	S	S	R	R	S	R	R	R	O10	*aac(6')-31-like1*	B5
1	S	S	S	R	R	S	S	R	R	O10	*aac(6')-31-like1*	B9
1	S	S	S	R	S	S	R	R	R	O10	*aac(6')-31*	B12
1	R	S	S	R	S	S	S	R	R	O10	*aac(6')-31-like1*	B14
1	S	S	S	S	S	S	S	R	R	NT[c]	*aac(6')-31*	B10
1	R	S	S	R	R	S	S	R	R	M	*aac(6')-31-like1*	B11
2	R	R	R	R	R	S	S	R	R	NT		C1
1	R	R	R	R	R	S	S	R	R	O3		C2
2	R	R	R	R	R	S	S	R	R	O3		C4
1	S	S	S	R	R	S	S	R	R	O1		C3
1	S	S	R	R	R	S	S	R	R	O1		C7
1	R	R	R	R	R	S	S	R	R	B		C5
1	S	S	S	S	S	S	S	S	S	B		C6
1	R	R	R	R	R	S	S	R	R	O11		C8
1	S	S	S	S	S	S	S	S	R	O4		D1
1	S	S	S	S	S	S	S	R	R	O4		D2
1	S	S	S	S	S	S	S	R	S	O11		D3
1	S	S	S	R	R	S	S	R	R	O11		E1
1	R	S	S	R	R	S	S	R	R	M		F1
1	S	S	S	R	S	S	S	R	R	O4		F2
1	R	S	S	R	R	S	S	S	R	O11		G1
1	R	S	S	S	R	S	S	R	R	O11		G2
1	R	S	R	R	R	S	S	R	R	O11		H1
1	R	R	R	S	S	S	S	S	S	B		H2
2	S	S	S	R	R	S	S	S	S	O10		I
1	S	S	S	S	S	S	S	S	S	O4		J1
1	S	S	S	S	S	S	S	S	S	O3		J2
1	S	S	S	S	S	S	S	S	S	NT		K1
1	S	S	S	S	S	S	S	S	S	O6		K2
1	R	R	R	S	S	S	S	S	R	O9		L1
1	S	S	S	S	S	S	S	R	R	B		L2
1	R	S	S	S	S	S	R	R	R	O11	*aac(6')-31-like3*, *aadA6*, *orfD*	M
1	R	R	R	R	R	S	R	R	R	B	bla_{IMP-1}, *aadA1*	N
1	R	S	S	S	S	S	S	S	S	O1		O
1	R	S	R	R	R	S	S	S	S	O6		P
1	S	S	S	S	S	S	S	S	S	C		Q
1	R	R	S	R	R	S	S	S	R	O10		R
1	S	S	S	S	S	S	S	S	S	O4		S

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TABLE 1—*Continued*

No. of isolates	Susceptibility to:									Serotype	Gene cassette(s) of the class 1 integron	PFGE type(s)
	β-Lactams					Amino-glycosides		FQs[a]				
	PIP	TZP	CAZ	IPM	MEM	AMK	GEN	CIP	OFX			
1	S	S	S	S	S	S	S	S	S	O11		T
1	S	S	S	S	S	S	S	S	S	O11		U
1	S	S	S	S	S	S	S	S	S	O11		W
1	S	S	S	S	S	S	S	S	S	O11		Z
1	S	S	S	S	S	S	S	S	S	O11		AA
1	S	S	S	S	S	S	S	S	S	O11		AJ
1	S	S	S	S	S	S	S	S	S	M		X
1	S	S	R	S	S	S	S	S	S	O1		Y
1	S	S	S	S	S	S	S	S	S	O10		AB
1	R	S	R	S	S	S	S	S	R	B		AC
1	S	S	S	S	S	S	S	S	S	O6		AD
1	R	R	R	S	S	R	S	S	S	O11		AE
1	S	S	S	S	S	R	R	S	S	O11		AF
1	R	R	S	S	S	S	S	S	S	NT		AG
1	R	S	S	S	S	S	S	S	S	B		AH
1	S	S	S	R	S	S	S	S	R	O1		AI
1	S	S	S	S	S	S	S	S	S	B		AK
1	S	S	S	S	S	S	S	S	S	NT		AL

[a] FQs, fluoroquinolones.
[b] Numbers of MDR isolates showing a respective PFGE type are shown in Fig. 1.
[c] NT, nontypeable.

STR, which was 98.6%. MIC_{50} and MIC_{90} values for MDR isolates were high, except those for ABK, GEN, and PL-B, and MIC_{50} and MIC_{90} values for non-MDR isolates were low, except those for AMK.

MBL production. MBL confers bacterial resistance to all β-lactams except AZL (53). Of the 284 isolates, 213 (75%) produced MBL and all except one were MDR isolates.

AAC(6′)-Iae production. AAC(6′)-Iae was first identified in MDR *P. aeruginosa* strain IMCJ2.S1 (46). We developed a slide agglutination test with AAC(6′)-Iae antibody-conjugated beads. *P. aeruginosa* IMCJ2.S1 showed a positive result within 30 s (Fig. 2, lane 2), whereas AAC(6′)-Iae-negative *P. aeruginosa* strain ATCC 27853 did not (Fig. 2, lane 4). Two hundred seventeen isolates were positive for the production of AAC(6′)-Iae in this test (Table 3). The results of the slide agglutination test were in complete agreement with Western blotting data obtained with AAC(6′)-Iae antibody (data not shown).

Detection of class 1 integrons. PCR assay with primers 5′-cs and 3′-cs (24), which are specific for the 5′ conserved segments (CS) (49) and the 3′ CS (49) of class 1 integrons, respectively, showed that 230 of the 284 isolates were positive. Amplified band sizes ranged from 0.8 kb to 2.5 kb (data not shown). All of these 230 isolates yielded a single band. Of these isolates, 212 yielded a 2.5-kb band, which is the same as that of the class 1 integron In113 (46). Sixteen isolates yielded a 0.8-kb band, and the remaining two yielded a 1.8-kb band and a 1.7-kb band. For the 212 isolates showing a 2.5-kb band, the presence of In113 was confirmed by PCR with specific primers, as described previously. MBL genes bla_{IMP-2} and bla_{VIM-2} are frequently found in Japan and are often associated with integrons (47). Therefore, we screened the 284 MDR *P. aeruginosa* iso-

TABLE 2. MIC_{50} and MIC_{90} values and percent antimicrobial resistance for 284 samples of *P. aeruginosa*

Antimicrobial agent	Breakpoint for resistance (μg/ml)	MDR isolates[a] (n = 214)				Non-MDR isolates (n = 70)			
		% Resistant	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistant	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
PIP	≥128	100	128–>512	>512	>512	41.4	1–>512	64	512
TZP	≥128/4	100	128–>512	512	>512	21.4	0.5–256	32	128
CAZ	≥32	100	32–>512	>512	>512	25.7	1–>512	8	64
IPM	≥16	100	32–>512	256	512	47.1	0.25–>512	8	32
DRPM	≥16	99.1	2–>512	>512	>512	34.3	<0.125–>512	8	32
MEM	≥16	100	32–>512	512	>512	44.3	<0.125–>512	4	32
AZT	≥32	99.5	16–>512	128	128	52.9	0.5–128	32	64
ABK	≥4	91.6	2–16	4	8	24.3	<0.125–16	1	8
AMK	≥32	100	32–256	128	256	2.9	0.25–256	2	16
GEN	≥16	57.5	0.25–>32	16	16	12.9	<0.125–>128	1	16
STR	≥4	100	512–>512	>512	>512	98.6	2–>512	32	128
CIP	≥4	100	16–>128	64	>128	51.4	<0.125–>128	4	64
OFX	≥8	100	32–>128	>128	>128	62.9	<0.125–>128	16	>128
PL-B	≥4	28.0	2–8	2	4	22.9	1–8	2	4

[a] Isolates defined as resistant to three antibiotics, imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 32 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml).

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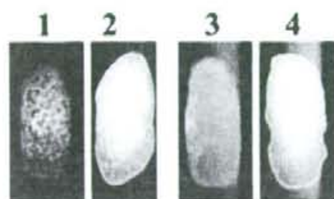

FIG. 2. Slide agglutination test with AAC(6')-Iae antibody-conjugated beads. Lane 1, AAC(6')-Iae positive control; lane 2, *P. aeruginosa* IMCJ2.S1 positive control; lane 3, 50 mM HEPES buffer negative control as solvent of AAC(6')-Iae; lane 4, *P. aeruginosa* ATCC 27853 negative control.

lates for bla_{IMP-2} and bla_{VIM-2} by PCR. None of the 284 isolates were positive for bla_{IMP-2} or bla_{VIM-2}.

The regions between the 5' CS and 3' CS of amplicons of unexpected sizes were sequenced, and the gene cassettes were identified (Table 1). Of 16 isolates showing an 0.8-kb band, three possessed a single gene cassette containing *aac(6')-31*, encoding 6'-*N*-aminoglycoside acetyltransferase type IV (R. E. Mendes, unpublished data; DDBJ/EMBL/GenBank accession no. AJ640197) (Table 1). This gene cassette was 639 nucleotides (nt) and contained a 65-nt 59-base-element (be) site, for site-specific cointegration events (35). Nine isolates possessed an *aac(6')-31-like1* cassette identical to *aac(6')-31*, with the exception of a C-to-T substitution at nt 269 in the coding region. Four isolates possessed an *aac(6')-31-like2* cassette identical to *aac(6')-31*, with the exception of a C-to-A substitution at nt 269. One isolate showing a 1.8-kb band possessed an array of three gene cassettes (Table 1). Of them, the first cassette was an *aac(6')-31-like3* cassette similar to *aac(6')-31* except for T-to-C and A-to-T substitutions at nt 57 and 266, respectively. The second cassette was 855 nt and contained the aminoglycoside adenylyltransferase gene *aadA6* (29) and a 60-nt 59-be site. The third cassette was 320 nt and contained open reading frame *orfD*, of unknown function (29). The *aadA6* and *orfD* cassettes were identical to those of In51 reported previously (29). One isolate showing a 1.7-kb band possessed two gene cassettes of bla_{IMP-1} (33) and *aadA1* (25) (Table 1).

Resistance to fluoroquinolones. Amino acid alterations to GyrA, GyrB, ParC, and ParE QRDRs of the 284 isolates are listed in Table 4. Amino acid replacement in the QRDR of GyrA (83Thr→Ile or 87Asp→Asn, Gly, or Tyr) was detected in 254 of the 284 isolates (89.4%). Of these 254 isolates, 8 possessed a mutation of GyrA alone. The remaining isolates possessed additional substitutions in GyrA, GyrB, ParC, and ParE. The 83Thr→Ile substitution in GyrA was the predominant replacement (251 of 284 isolates, 88.4%), in agreement with previous data on fluoroquinolone-resistant *P. aeruginosa* isolates (1, 22, 28). A double mutation of GyrA, 83Thr→Ile and 87Asp→Asn or Gly, was detected in nine isolates.

Amino acid replacement in the QRDR of ParC (87Ser→Leu or 91Glu→Lys) was detected in 244 of the 284 isolates (85.9%). All of these 244 isolates possessed additional mutations. The 87Ser→Leu substitution was the predominant replacement (242 of 284 isolates, 85.2%) and has been implicated in fluoroquinolone resistance of *P. aeruginosa* (1, 22, 28). A double mutation of ParC, 87Ser→Leu and 91Glu→Lys, was detected in three isolates. We found an 83Pro→Leu, 85Gly→Asp, and 88Ala→Pro alterations in one isolate each (Table 4).

Amino acid replacement in the QRDR of GyrB (468Glu→Asp) was detected in 70 of the 284 isolates (24.6%). No double mutations in GyrB were detected. Lee et al. (22) recently reported that 468Glu→Asp was a predominant alteration of GyrB, and isolates with this alteration, in addition to GyrA (83Thr→Ile) and ParC (87Ser→Leu) substitutions, showed a high level of resistance to CIP (MIC > 64 μg/ml). Our results were in accordance with their findings. We also found a 458Ala→Thr alteration in four isolates and a 496Ile→Val alteration in one isolate. These alterations are probably not associated with CIP resistance in *P. aeruginosa* because they were found in CIP-susceptible isolates.

Amino acid replacement in the QRDR of ParE (425Ala→Val or 459Glu→Asp or both) was detected in 30 of the 284 isolates (10.6%). All isolates possessed multiple mutations of ParE. Lee et al. (22) speculated that the 459Glu→Asp mutation of ParE is associated with moderate or high-level fluoroquinolone resistance in *P. aeruginosa*. The 425Ala→Val mutation has been reported in fluoroquinolone-resistant isolates of *P. aeruginosa* (1). Other mutations leading to amino acid changes were found at codons 419 (Asp→Asn, 1 isolate), 427 (Gln→Leu, 1 isolate), and 457 (Ser→Alg, 1 isolate). The fluoroquinolone

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TABLE 3. Comparison of PCR, LAMP, and agglutination test results for the detection of MDR *P. aeruginosa* isolates belonging to genotype cluster A[a]

Isolates	No. of isolates with indicated result by:								
	PCR			LAMP			Agglutination test with AAC(6')-Iae antibody-conjugated beads		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
MDR *P. aeruginosa*									
Cluster A	212	0	212	212	0	212	207	5	212
Other	0	2	2	0	2	2	0	2	2
Non-MDR *P. aeruginosa*									
Cluster A	0	2	2	0	2	2	0	2	2
Other	0	68	68	0	68	68	10	58	68
Total	212	72	284	212	72	284	217	65	284

[a] In all tests and combinations, the multidrug resistance of the isolates was positively associated with the positive results of *aac(6')* tests based on chi-square tests ($P < 0.0001$).

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TABLE 4. Amino acid changes in *gyrA*, *gyrB*, *parC*, and *parE* genes in 284 clinical isolates of *P. aeruginosa*

No. of strains (n = 284)	MIC (μg/ml) of:		Replacement in QRDRs[j]									
			GyrA at position:		ParC at position:			GyrB at position:		ParE at position:		
	CIP	OFX	83Thr (ACC)	87Asp (GAC)	87Ser (TCG)	91Glu (GAG)	Other	468Glu (GAG)	Other	425Ala (GCG)	459Glu (GAG)	Other
MDR *P. aeruginosa*												
1	>128	>128	Ile (ATC)	—[a]	Leu (TTG)	—	83Pro→Leu[b]	Asp (GAT)	—	—	Asp (GAT)	—
25	128–>128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	Asp (GAT)	—
1	128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	427Gln→Leu[g]
37	32–128	128–>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	—
1	>128	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	Lys (AAG)	—	—	—	—	—	—
1	16	32	Ile (ATC)	—	Leu (TTG)	—	85Gly→Asp[c]	—	—	—	—	—
147	16–>128	32–>128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	—	—	—
1	32	64	Ile (ATC)	—	—	—	—	—	—	—	—	457Ser→Alg[h]
Non-MDR *P. aeruginosa*												
5	64–>128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	—
4	32–128	64–>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	—	—	—	—	—	—	—
1	128	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	Lys (AAG)	—	—	—	—	—	—
1	>128	>128	Ile (ATC)	Asn (AAC)	—	Lys (AAG)	—	—	—	—	—	—
1	64	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	—	—	—	—	—	Asp (GAT)	—
1	64	128	Ile (ATC)	Gly (GGC)	Leu (TTG)	—	88Ala→Pro[d]	—	—	—	—	—
13	32–64	64–>128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	—	—	—
2	16–32	32–128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	Val (GTG)	—	—
1	16	128	Ile (ATC)	—	Leu (TTG)	—	—	—	458Ala→Thr[e]	—	—	—
1	16	128	Ile (ATC)	—	—	Lys (AAG)	—	—	—	—	—	—
1	16	128	Ile (ATC)	—	—	—	—	—	—	Val (GTG)	—	—
1	8	128	Ile (ATC)	—	—	—	—	—	458Ala→Thr	—	—	419Asp→Asn[i]
1	2	16	—	—	Leu (TTG)	—	—	—	458Ala→Thr	—	—	—
6	<0.25–0.5	1–8	—	—	—	—	—	—	458Ala→Thr	—	—	—
2	<0.25	0.25	—	—	—	—	—	—	496Ile→Val[f]	—	—	—
1	4	64	—	—	—	—	—	Asp (GAT)	—	—	—	—
5	0.5–4	8–16	Ile (ATC)	—	—	—	—	—	—	—	—	—
2	1–2	2–8	—	Tyr (TAC)	—	—	—	—	—	—	—	—
1	<0.25	0.25	—	—	—	—	—	—	—	—	—	—
20	<0.25–16	<0.25–64	—	Asn (AAC)	—	—	—	—	—	—	—	—

[a] —, no amino acid change.
[b] 83Pro→Leu, Pro at position 83 of ParC changed to Leu (CCG→CTG).
[c] 85Gly→Asp, Gly at position 85 of parC changed to Asp (GGC→GAC).
[d] 88Ala→Pro, Ala at position 88 of ParC changed to Pro (GCC→CCC).
[e] 458Ala→Thr, Ala at position 453 of GyrB changed to Thr (GCG→ACG).
[f] 496Ile→Val, Ile at position 496 of GyrB changed to Val (ATG→GTC).
[g] 427Gln→Leu, Gln at position 427 of ParE changed to Leu (CAG→CTG).
[h] 457Ser→Arg, Ser at position 457 of ParE changed to Arg (AGC→AGG).
[i] 419Asp→Asn, Asp at position 419 of ParE changed to Asn (GAC→AAC).
[j] Mutated nucleotides are underlined.