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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 (≥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.

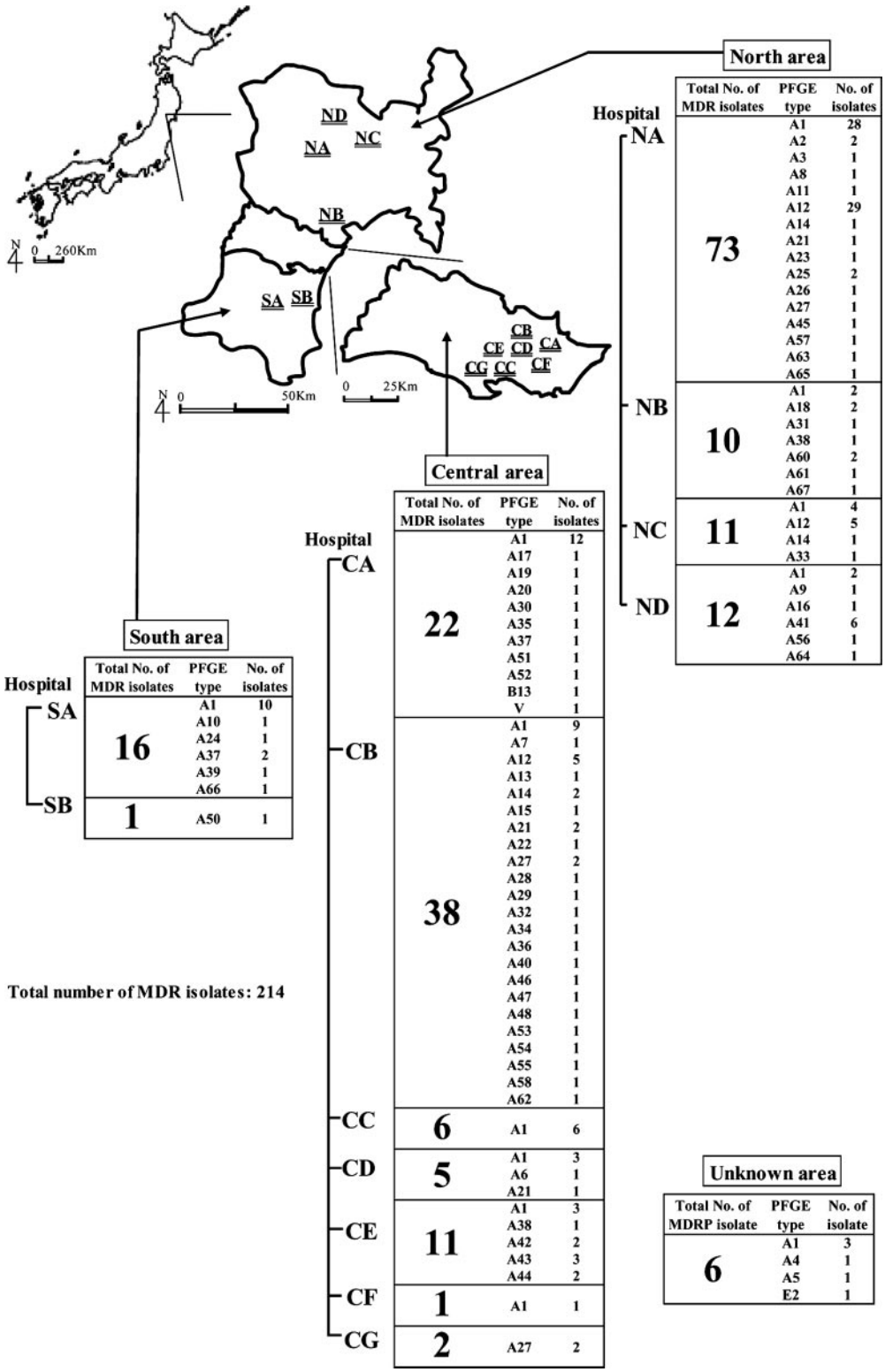

FIG. 1. Distribution of 214 isolates of MDR *P. aeruginosa* among 13 hospitals in Japan. Double capital letters indicate the locations of the hospitals that participated in this MDR *P. aeruginosa* survey.

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

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

Continued on facing page

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).

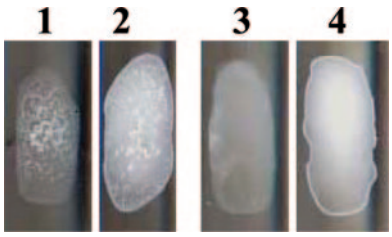

FIG. 2. Slide agglutination test with AAC(6′)-Iae antibody-conjugated beads. Lane 1, AAC(6′)-Iae positive control; lane 2, *P. aeruginosa* IMCJ2.S1 positive control; lane 3, 50 mM HEPES buffer negative control as solvent of AAC(6′)-Iae; lane 4, *P. aeruginosa* ATCC 27853 negative control.

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. aeruginos*a (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

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 ($P < 0.0001$).

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.

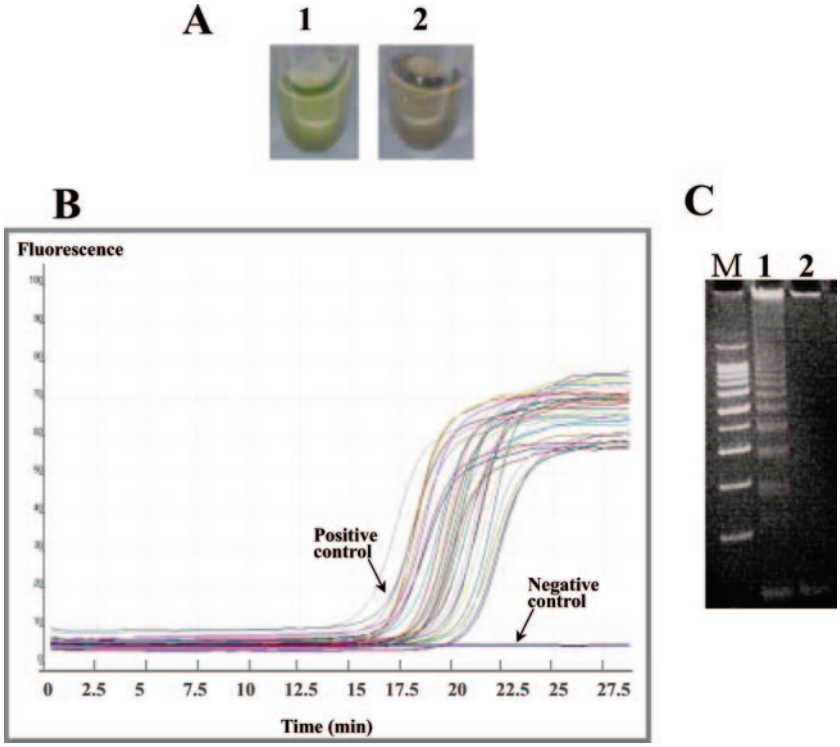

FIG. 3. LAMP assay to detect MDR *P. aeruginosa* isolates possessing the *aac(6′)-Iae* gene encoding the aminoglycoside acetyltransferase AAC(6′)-Iae. *P. aeruginosa* IMCJ2.S1 and ATCC 27853 were used as positive and negative controls, respectively. (A) Visual inspection analysis of LAMP products. Lane 1, *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853. (B) Real-time amplification monitoring of *aac(6′)-Iae*-specific LAMP. The amplification signal was detected at an average of 18 min, as indicated by the continuous increase in fluorescence. Increased fluorescence was not observed in the negative control. (C) Acrylamide gel electrophoresis of LAMP product. Lane 1, LAMP product of the 204-bp target sequence of the *aac(6′)-Iae* gene of *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853 negative control; lane M, 1-kbp ladder.

resistance associated with these mutations remains to be determined.

Analysis of the *aac(6′)-Iae* gene by the LAMP method. To detect *aac(6′)-Iae*, we developed a gene-specific LAMP assay. The index strain IMCJ2.S1 was used to standardize the method. Visual inspection showed that the LAMP assay successfully amplified the target sequence of the *aac(6′)-Iae* gene of *P. aeruginosa* IMCJ2.S1 (Fig. 3A). Real-time kinetics of the LAMP reaction showed that the amplification signal could be detected on average by 18 min; fluorescence increased in the positive samples, following a sigmoid curve (Fig. 3B). Agarose gel electrophoresis of the LAMP products (Fig. 3C) showed a ladder-like pattern on the gel due to the formation of a mixture of stem-loop DNAs of various stem lengths, which are characteristic of LAMP products.

A total of 284 isolates, including 214 MDR *P. aeruginosa* isolates, were tested by the LAMP assay (Table 3). A total of 212 isolates were positive by the LAMP assay (Table 3). The results of the LAMP assay were in complete concordance with the PCR data, indicating that the PCR can be replaced by the LAMP method for detection of *aac(6′)-Iae*-carrying *P. aeruginosa*. These results, together with ones of the agglutination test (Table 3), indicate that multidrug resistance was strongly associated with the presence of *aac(6′)-Iae* and AAC(6′)-Iae production in the *P. aeruginosa* isolates ($P < 0.0001$).

Genotyping by PFGE. The 284 isolates, including 214 MDR isolates, were typed by PFGE. One hundred thirty-three different PFGE types, designated from A1 to AL, were distinguished (Table 1). Fourteen types, A1, A2, A12, A14, A18, A21, A25, A27, A37, A41, A42, A43, A44, and A60, were identified in more than 2 isolates (Fig. 1), and type A1, which represented 83 of the isolates (29%), was the most prevalent and widely disseminated (Fig. 1), suggesting prefecture-wide clonal dissemination. Types A1, A12, A14, A21, A27, A37, and A38 were identified at two or more hospitals. Cluster analysis of the PFGE restriction patterns showed three large clusters, A, B, and C, sharing ≥70% similarity (Fig. 4). Of the 214 MDR isolates, 211 belonged to cluster A, comprising types A1 to A67, indicating that multidrug resistance was associated with one genotype, cluster A (Fig. 4 and Table 3). Fifteen isolates belonged to cluster B comprising types B1 to B14, and 10 isolates belonged to cluster C, comprising types C1 to C8. The PFGE patterns of the 35 non-MDR isolates varied greatly.

DISCUSSION

A clonal expansion of *P. aeruginosa* resistant to three antibiotics, carbapenems, amikacin, and fluoroquinolones, has been reported (4, 14, 36, 37, 46). However, previous surveillance studies in Japan have not shown clonal expansion involving multiple hospitals (19, 52). The present study showed clonal expansion of MDR *P. aeruginosa* in hospitals in the Tohoku area of Japan. To our knowledge, this is the first description of a large-scale, community-wide outbreak of nos-

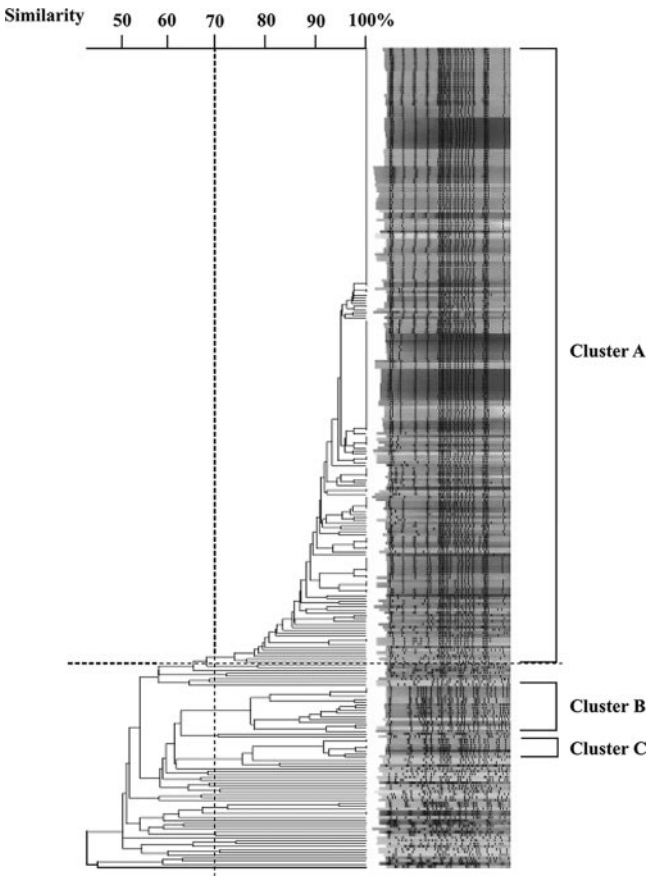

FIG. 4. Cluster analysis based on the PFGE patterns of 284 clinical isolates of *P. aeruginosa* from the 13 hospitals in the present study. Clustering was carried out with Molecular Analyst FingerprintingPlus software, version 1.6, as described in Materials and Methods.

ocomial infection caused by a single *P. aeruginosa* clone with high-level resistance to a large number of antibiotics. The routes of transmission of the MDR *P. aeruginosa* clone remain unclear. *P. aeruginosa* that can be recovered from the hospital environment could be a possible source of nosocomial infection (6, 42, 54). Patient-to-patient transmission has been documented among patients with cystic fibrosis (5, 42, 54). Catheter-associated urinary tract infections appeared widespread among the hospitals in our study; the majority of the isolates (approximately 70%) were obtained from urine specimens, and approximately 80% of these were from patients with urinary catheters.

Most MDR isolates tested (205 of 214; Table 1) showed a serotype of O11. This was not surprising because these isolates belonged to a single cluster, as revealed by PFGE analysis (Fig. 4). *P. aeruginosa* is categorized into 31 chemotypes, including 20 IATS serotypes and subtypes (48). Thus far, however, particular serotypes, such as serotypes O12 and O11, appear to have been preferentially associated with *P. aeruginosa* outbreaks (9, 23, 38, 41). A clone of *P. aeruginosa* belonging to serogroup O12, which was resistant to both carbenicillin and gentamicin, was predominant in outbreaks involving six hospitals in Athens in 1987 (23). Later, O12 isolates resistant to these two drugs were reported in European countries (9, 38, 41). *P. aeruginosa* O12 resistant to ciprofloxacin and ceftazidime and/or fosfomycin was implicated in hospital outbreaks in France during the period 1993 to 1994 (3). *P. aeruginosa* serotype O11 caused hospital outbreaks in the 1980s in the United States (8) and in 1994 and 1995 in Greece (50). *P. aeruginosa* O11 was implicated in folliculitis caused by the use of whirlpools and hot tubs in the 1970s and 1980s in the United States and Canada (40). More recently, hospital outbreaks caused by MDR *P. aeruginosa* serotype O11 occurred in Belgium (5) and in Japan (46). Different strains of serotype O11 were involved in the above-mentioned outbreaks because their PFGE profiles were quite different. In addition, the Japanese strains produced IMP-1 carbapenemase (46), but the Belgian strains did not (5). It is not known why *P. aeruginosa* strains belonging to particular serotypes of O12 and O11 were involved in these outbreaks.

We analyzed several features including serotype, antimicrobial susceptibility, MBL production, prevalence of *aac(6′)-Iae*, structure of class 1 integrons, resistance to fluoroquinolones, and genotype based on PFGE analysis for MDR *P. aeruginosa* isolates. Results indicated that *aac(6′)-Iae* is a good candidate marker for MDR *P. aeruginosa* infection. To detect the *aac(6′)-Iae* gene and its product, we developed a LAMP-based detection assay and an agglutination assay. LAMP is a nucleic acid amplification method which relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (31). The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. LAMP assays are simple and short and do not require expensive equipment. LAMP assays have been applied to the analysis of various infectious agents such as hepatitis B virus (7), *Mycobacterium tuberculosis* (15), severe acute respiratory syndrome coronavirus (13), *E. coli* O157:H7 (27), *Clostridium difficile* (18), *Bordetella pertussis* (17), *Salmonella enterica* (32), *Mycoplasma pneumoniae* (43), and *Streptococcus pneumoniae* (45). The LAMP assay developed in this study was as sensitive and specific as PCR. Though less sensitive and specific than the LAMP assay, the agglutination assay for AAC(6′)-Iae is sufficiently accurate to detect MDR *P. aeruginosa* (98% of MDR *P. aeruginosa* isolates were positive). The agglutination assay is simpler and cheaper than the LAMP assay and is also useful in detecting MDR *P. aeruginosa* in the clinical setting.

MDR *P. aeruginosa* may have spread across Japan as a result of the increasing use of carbapenems such as IPM, aminoglycosides such as AMK, and fluoroquinolones such as CIP. Nationwide surveillance for MDR *P. aeruginosa* is under way. At the hospital level, monitoring for environmental sources of bacteria, cleaning of contaminated surfaces of treatment rooms and bathrooms, review of infection control measures in the treatment of urine, and avoidance of unnecessary measurements of urine are considered effective in preventing *P. aeruginosa* nosocomial infections. Although the mode of transmission between hospitals is unknown, the movement of infected patients from one hospital to another is a possibility. Thirty-one patients infected with MDR *P. aeruginosa* had been transferred from other hospitals to the hospitals participating in the present study.

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