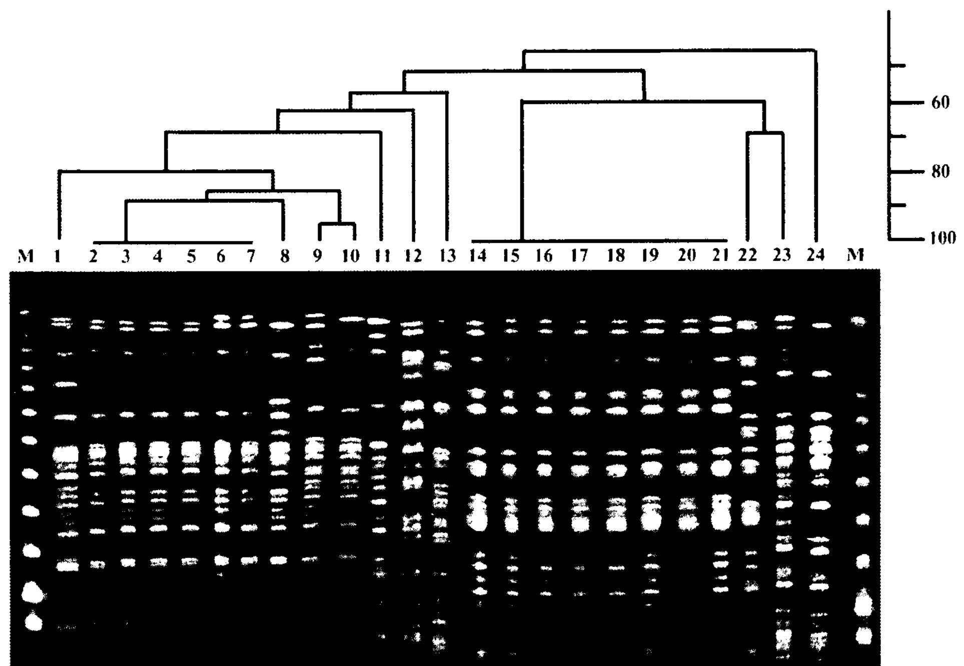
Fig. 2. Pulsed-field gel electrophoresis (PFGE) patterns and dendrogram for *P. aeruginosa* isolates from clinical and environmental sources. The isolates corresponding to *each lane* are listed in Table 1



resistant to IPM and CPFX, but sensitive to AMK, were obtained in ward 5N. One isolate (IMCJ324) was from a sink in a lavatory, and two (IMCJ325 and IMCJ331) were obtained on different days from the surfaces of a urine volume-measuring device in a room for the handling of urine. As mentioned above, PFGE analysis revealed that three of the MDR *P. aeruginosa* isolates and two of three isolates of the IPM- and CPFX-resistant but AMK-sensitive *P. aeruginosa* were causative pathogens of the outbreaks. The data pointed to environmental contamination by drug-resistant *P. aeruginosa* in the bathing room, the lavatory, and the rooms for the handling of urine, which may have been sources of the pathogens during the outbreaks.

During the two outbreaks, we took the following steps: (a) environmental surface monitoring as described, (b) active surveillance for drug-resistant *P. aeruginosa* obtained from the samples of patients, (c) strict isolation of infected patients or carriers of MDR *P. aeruginosa*, (d) rigorous contact precautions, especially during the handling of urine and urinary catheters, and (e) disinfection with 70% alcohol on the surfaces of apparatuses contaminated by MDR or drug-resistant *P. aeruginosa* and in rooms where urine is handled. As a result, the outbreaks were contained. Each patient with MDR *P. aeruginosa* was isolated in a single room during the two outbreaks. However, if a single room is not available, an area in a ward that is separate from other patients could be used for patients with MDR *P. aeruginosa*. Inadequate use of antimicrobial agents against *P. aeruginosa* was not found in the wards where the outbreaks occurred, although prior-approval programs, including pre-approved indications for antibiotics against *P. aeruginosa*, such as carbapenems and aminoglycosides, were not performed, but were in the planning stage in this hospital.

However, sporadic MDR *P. aeruginosa* will continue to be isolated from inpatients who may bring the pathogens into the hospital. In fact, we have reported outbreaks of MDR *P. aeruginosa* in hospitals in a prefecture in Japan.[3] Analysis indicated that the *P. aeruginosa* IMCJ.S1 strain responsible for the outbreaks underwent clonal expansion.[3] The PFGE patterns of MDR *P. aeruginosa* isolates from the first outbreak described herein had 80% similarity to the pattern of *P. aeruginosa* IMCJ.S1. In addition, MDR isolates from the sporadic cases in the present study (patients 10, 11, and 12) had close similarity to the IMCJ.S1 and the strains associated with the first outbreak. These results suggest that some dominant MDR *P. aeruginosa* strains may be prevalent in Japan.

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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.

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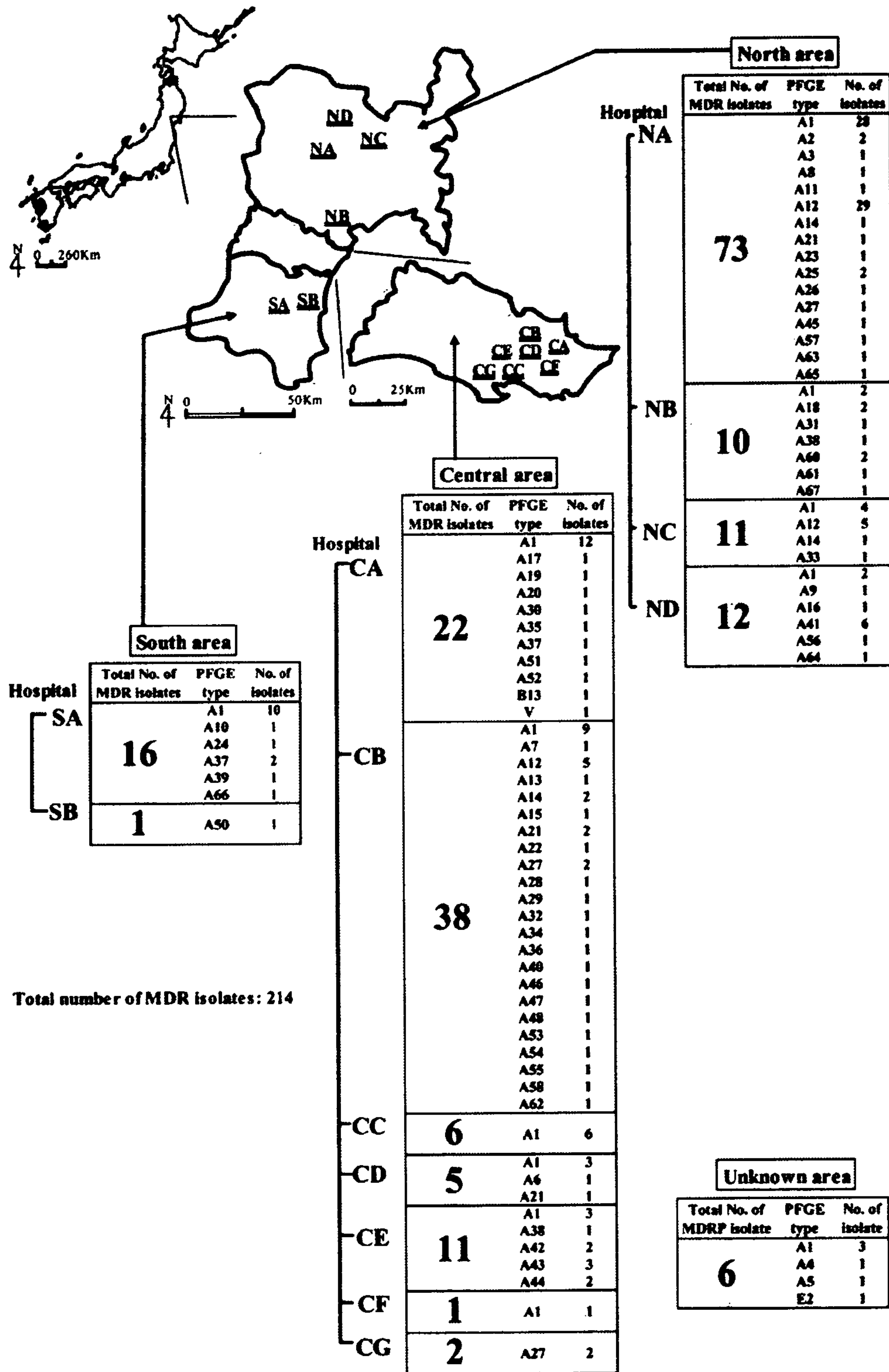

FIG. 1. Distribution of 214 isolates of MDR *P. aeruginosa* among 13 hospitals in Japan. Double capital letters indicate the locations of the hospitals that participated in this MDR *P. aeruginosa* survey.

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					Aminoglycosides		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		FOs[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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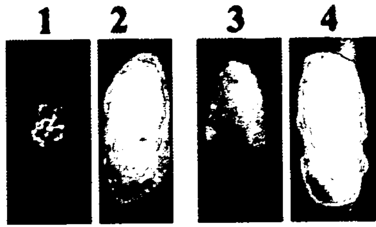

FIG. 2. Slide agglutination test with AAC(6′)-Iae antibody-conjugated beads. Lane 1, AAC(6′)-Iae positive control; lane 2, *P. aeruginosa* IMCJ2.S1 positive control; lane 3, 50 mM HEPES buffer negative control as solvent of AAC(6′)-Iae; lane 4, *P. aeruginosa* ATCC 27853 negative control.

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.

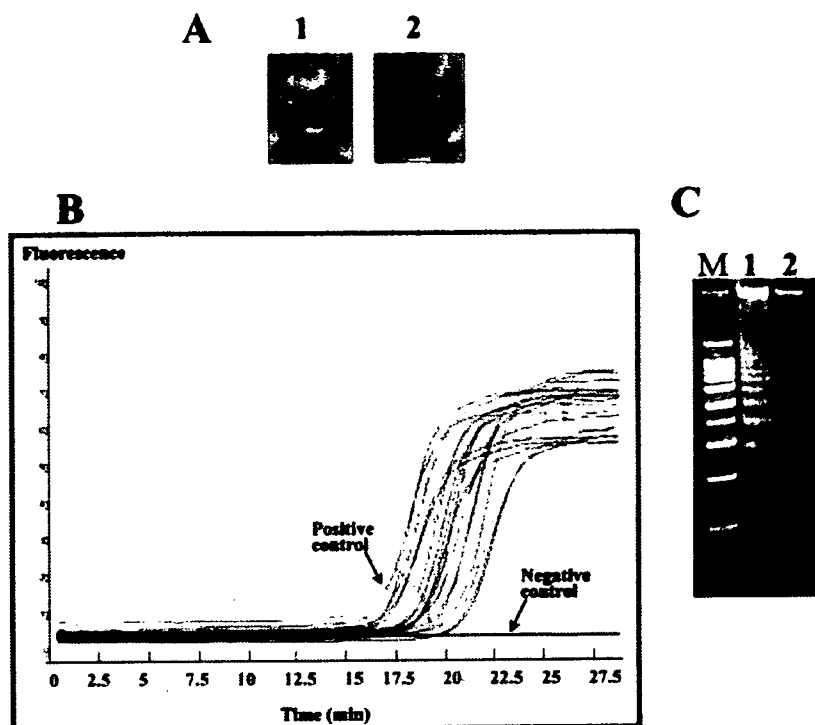

FIG. 3. LAMP assay to detect MDR *P. aeruginosa* isolates possessing the *aac(6')-Iae* gene encoding the aminoglycoside acetyltransferase AAC(6')-Iae. *P. aeruginosa* IMCJ2.S1 and ATCC 27853 were used as positive and negative controls, respectively. (A) Visual inspection analysis of LAMP products. Lane 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-

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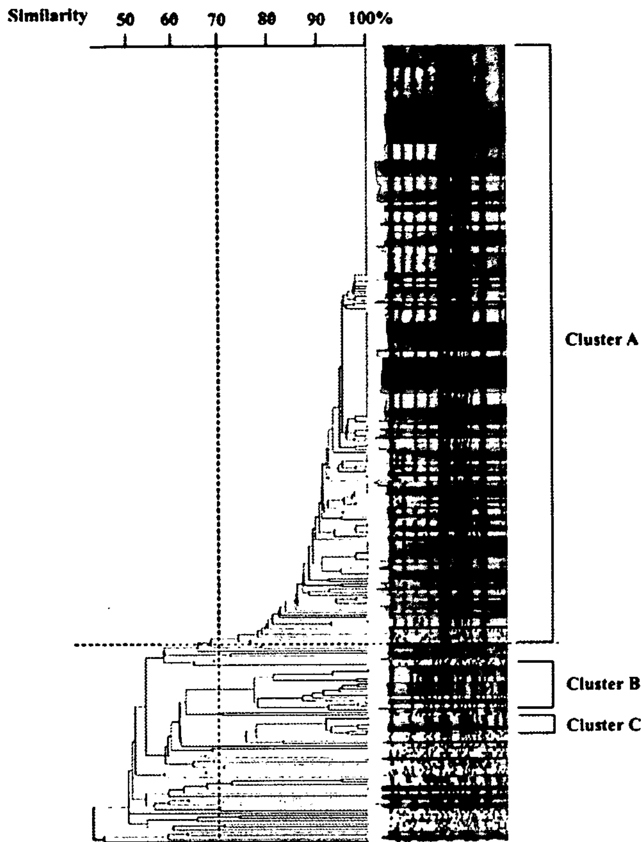

FIG. 4. Cluster analysis based on the PFGE patterns of 284 clinical isolates of *P. aeruginosa* from the 13 hospitals in the present study. Clustering was carried out with Molecular Analyst FingerprintingPlus software, version 1.6, as described in Materials and Methods.

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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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

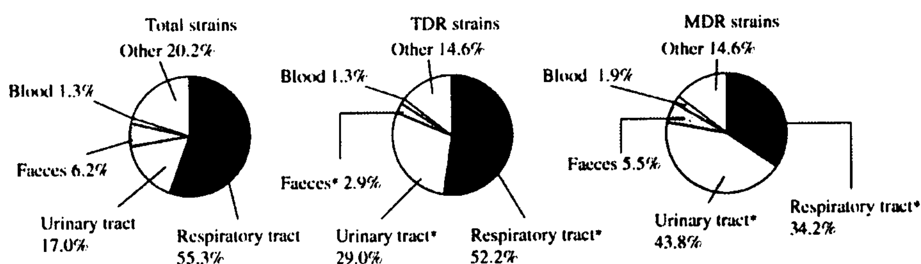
The tissue sources and percentages of the total *P. aeruginosa* strains isolated at the 339 medical facilities for the study periods and those of TDR and MDR strains are shown in Figure 1a. The percentages for each year were similar to those for the entire study period (data not shown). These results 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*.

(a) Medical facilities



(b) Clinical laboratories

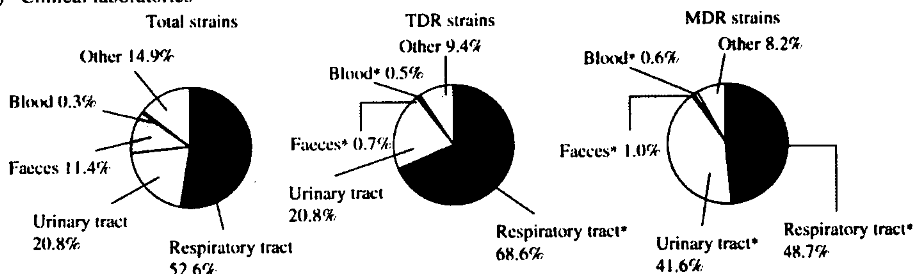


Figure 1. Tissue sources of total *P. aeruginosa* strains, TDR strains and MDR strains isolated during the study period at medical facilities (a) and clinical laboratories (b). An asterisk indicates a significant difference between numbers of TDR and total isolates or between MDR and total isolates.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

IS*6110* DNA 指紋法少コピー数結核菌による精神病院での集団感染事例

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序　文

今回我々は，山梨県内の精神病院の同一病棟で5年間に4人の結核患者が発病した，という事例を経験した．4例中3例の菌株を得てRFLP (restriction fragment length polymorphism) 解析を施行，IS*6110* 指紋法では三者のパターンが一致したのだが，バンド数は2本であり，本法での感染伝播の確認は困難であった．しかし，他のタイピング法を併用することにより，院内感染伝播の確認が可能であった．文献も含めて考察して行きたい．

症　例

症例1：Index case で，平成12年2月現在50歳女性，躁鬱病，精神発達遅滞で17歳時より山梨県A市の精神病院B病院のC病棟に入院中だった．同時期，発熱と咳を主訴に国立療養所西甲府病院（後の国立病院機構西甲府病院，以下同様）に入院，胸部X線で空洞病変を認め，日本結核病学会の病型・拡がり（以下学会分類）はbII3，検痰で塗抹はガフキー6号，培養（小川培地上，以下同様）は3+，薬剤耐性はなかった．家族歴は祖父が肺結核で死亡，姉も3歳時に結核の治療を受けた．菌株の保存はない．

症例2：平成13年6月現在46歳の女性，統合失調症，精神発達遅滞にて10年前からC病棟入院中で，発熱と胸部異常陰影（学会分類rII2rpl）のため当院に紹介された．検痰で塗抹3+，培養4+，薬剤耐性はなく，胸水中 Adenosine deaminase (ADA) は80.5 IU/L に上昇していた．平成11年に症例1と同室だった．

症例3：平成13年現在78歳男性，統合失調症にて44歳時よりC病棟入院中だったが，健康診断で胸部異常陰影（学会分類bII1）の指摘を受け，検痰で塗抹1+，培養3+（薬剤耐性なし）であり，症例2と同時期に当院に入院した．

症例4：平成16年3月現在53歳の男性，15歳時より統合失調症にてC病棟入院中で，症例1～3と面識があった．平成16年2月中旬より発熱を認め，胸部X線で右下肺野に浸潤影があったが，一般抗生剤投与で改善せず陰影は右肺全体に及び（学会分類bII3），呼吸不全が進行した．3月下旬に肺結核と判明し，当院に転院した．入院時の検痰では塗抹2+，培養3+，薬剤耐性は認めなかった．

以上の症例1～症例4の結核発病の時間経過は，Fig. 1に示す通りである．

RFLP 解析：平成14年の段階でB病院C病棟の院内感染と推定され，結核研究所に症例2，3の菌株を送り，RFLP 解析を依頼した．IS*6110* をプローブとしたDNA指紋法でバンドパターンは一致したが，バンド数が2つに止まったため，解析不能であった．平成17年に今度は国立国際医療センター感染症制御研究部において，症例2～4の菌株のRFLP解析を行った．まずIS*6110* 法で三者のパターンが一致（Fig. 2A），次に $(CGG)_5$[1]をプローブとして解析，同様に三者の指紋法が一致し（Fig. 2B），しかもバンド数は15だった．

Table 1に症例2～4の主要薬剤耐性関連遺伝子上の変異を示す．F1は症例2，F2は症例3，F3は症例4である．既報[2]の方法に準拠し，ダイレクトシークエンス法により，主要抗結核剤5剤を含む7剤に対する耐性遺伝子の変異の有無を解析した．それぞれの菌株より抽出した結核菌ゲノムDNAを鋳型とし，耐性遺伝子に特異的な8組のプライマーペアを用いて，リファンピシン耐性遺伝子 (*rpoB*)，イソニアジド耐性

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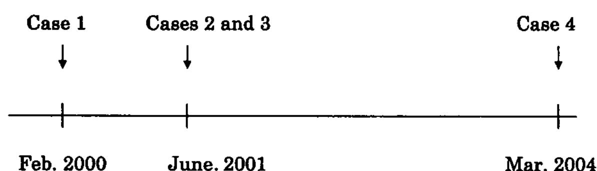
Table 1 Analysis of amino-acid mutations conferring resistance to rifampicin, isoniazid, ethambutol, pyrazinamide, streptomycin, kanamysin, and levofloxacin (2)

Isolate	RFP	INH		EB	PZA	SM, KM		LVFX
	rpoB mutation	*katG* mutation	*inhA* promoter mutation	*embB* mutation	*pncA* mutation	*rpsL* mutation	*rrs* mutation	*gyrA* mutation
F1	WT	WT	WT	Ala1007Val[a] (GCC → GTC)	WT	Lys121Lys[a] (AAA → AAG)	WT	Glu21Gln[a] (GAG → CAG), Ser95Thr[a] (AGC → ACC)
F2	WT	WT	WT	Ala1007Val[a] (GCC → GTC)	WT	Lys121Lys[a] (AAA → AAG)	WT	Glu21Gln[a] (GAG → CAG), Ser95Thr[a] (AGC → ACC)
F3	WT	WT	WT	Ala1007Val[a] (GCC → GTC)	WT	Lys121Lys[a] (AAA → AAG)	WT	Glu21Gln[a] (GAG → CAG), Ser95Thr[a] (AGC → ACC)

Abbreviations: RFP, rifampicin ; INH, isoniazid ; EB, ethambutol ; PZA, pyrazinamide ; SM, streptomycin ; KM, kanamycin ; LVFX, levofloxacin ; A, adenine ; C, cytosine ; G, guanine ; T, tymine ; Ala, alanine ; Val, valine ; Lys, lysine ; Glu, glutamic acid ; Gln, glutamine ; Ser, serine ; Thr, threonine ; WT, wild type ; [a]Natural polymorphism not to be associated with drug resistance.
Isolates : F1, Case 2 ; F2, Case 3 ; F3, Case 4.

Fig. 1 Four cases of pulmonary tuberculosis in a mental hospital in Yamanashi, Japan



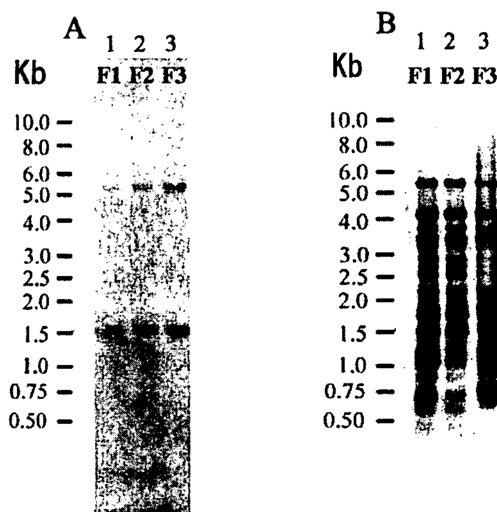
遺伝子（*katG* 及び *inhA*），塩酸エタンブトール耐性遺伝子（*embB*），ピラジナミド耐性遺伝子（*pncA*），硫酸ストレプトマイシン及び硫酸カナマイシン耐性遺伝子（*rpsL* 及び *rrs*），レボフロキサシン耐性遺伝子（*gyrA*）を増幅した．これらの PCR 産物の塩基配列は，ABI3100 オートシークエンサーを用いて決定した．結果としては，*embB*，*rpsL* 及び *gyrA* に変異は認められたが，すべて薬剤耐性とは関連のない変異[2]であり，さらに 3 株の変異はすべて一致した．

以上より，同一施設で 5 年間にわたり 4 例の発病がみられ（Fig. 1），院内集団感染の定義にはあてはまらないものの，菌株の同一性より施設内集団感染が強く疑われた．

考 察

症例 2～4 の菌株は RFLP 解析パターンが一致し（Fig. 2），液体培地上で抗結核薬主要 5 剤に対する薬剤耐性は認めなかった．主要薬剤耐性関連遺伝子上の変異は認めず，耐性と関係のない変異もすべて一致した（Table 1）．症例 1 も含め 4 例は 10 年以上精神病院の同じ病棟で生活し，疫学的関連も有する．IS*6110* 結核菌遺伝子タイピングの欠点は，DNA 指紋法でバンド数 5 以下の場合，遺伝子多形性が乏しいため鑑別不能になることで，アフリカ及びアジアの菌株に多い[3)4)]．Burman らは[4)]ヴェトナム等からの移民も含まれるデンバーにおける結核患者の菌株に RFLP 解析を施行し，IS*6110* タイピング法単独でのクラスター形成率は，コピー数 5 以下が 82%，6 以上が 32% であったが，IS*6110* と Polymorphic G-C rich repetitive sequence（PGRS）[5)]を組み合わせてタイピングした場合，コピー数 5 以下が 32%，6 以上が 25% であり，クラスターの疫学的関連性は 78% で認められた．IS*6110* 法少コピー例は，指紋法一致例の 6 割以上が，2 次タイピング法（PGRS）で別菌種とされた計算になる．van

Fig. 2 IS*6110* (A) and (CGG)$_5$ (B) fingerprinting of *Mycobacterium tuberculosis* strains in 3 cases. F1: Case2, F2: Case 3, F3: Case 4. Both types of fingerprinting showed identical patterns for 3 isolates. The copy number of IS*6110* printing was two (A), that of (CGG)$_5$ printing was 15.



IS*6110* typing **(CGG)$_5$typing**

Soolingen[3]は2次タイピングとしてスポリゴタイピング[6]も推奨，使用例の報告も多く見られる．

日本における IS6110 法少数コピー例の頻度に関する報告例として，阿野ら[7]の大阪地区の検討では4.2%であった．高橋[8]が1988年と1992年の北海道から九州までの結核菌株を集め，IS6110 のコピー数の分布を集計した結果からは，5本以下の例の割合はグラフ上10%弱と考えられた．関東が中心で仙台から福岡に渡る HIV（+）患者の結核菌株33株の RFLP 解析では[9]，少コピー例は7株21.2%であった．

大塚らは[1]ヒトの遺伝子上で神経筋疾患患者などの疾患に存在することが知られているトリヌクレオチドリピートに着目し，細菌ゲノム上で同様なリピートが存在するか，検索した．その結果，結核菌に比較的特異的に DNA 塩基 CGG の5回の繰り返しが存在することを見出した．この $(CGG)_5$ は，おもに PPE 及び PE ファミリー遺伝子上に存在し（PGRS も同部位に存在），これを疫学指標とした RFLP 解析が可能である[1]．結核親子感染事例では，$(CGG)_5$ を用いた RFLP 解析により IS6110 法とほぼ一致した結果が得られている[10]．

$(CGG)_5$ を用いた RFLP 解析はとくに IS6110 法でバンド数が少ない菌株のクラスター解析に有用である．日本における HIV（+）患者の結核菌株33株の RFLP 解析では[9]，$(CGG)_5$ 法では菌株のバンド数が8～16であり，少コピー例が7例存在した IS6110 法とは異なり，全例でクラスター解析可能であった．今回の我々の報告においても，$(CGG)_5$ が IS6110 法でのコピー少数例の補助診断法として，PGRS やスポリゴタイピングと同様に有用であることが示された．

謝辞：稿を終えるにあたり，症例2，3の菌株を平成13年～平成17年の長期間保存し，我々の求めに応じてお送り下さった結核予防会結核研究所抗酸菌レファレンスセンター結核菌情報課職員の方々に深謝致します．

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