切替　照雄 倉辻　忠俊	地域支援ネットワーク構築の新しい流れ：地域ネットワークの現状と将来展望	医学のあゆみ	218	1079 － 1082	2006
倉辻忠俊	ICT：組織としての対応を理解する	臨床医	31	1409 － 1410	2005
芦田信之、竹村匡正、牧本清子、切替照雄	院内感染予防における全国報告事業の展開：事例報告ネットワークシステムの構築	甲子園大学紀要	32	7－14	2004
芦田信之、竹村匡正、牧本清子、切替照雄	院内感染専門知識検索支援システムの構築				2004

Ⅲ. 研究成果の刊行物・別冊

JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2007, p. 979–989 Vol. 45, No. 3
0095-1137/07/$08.00+0 doi:10.1128/JCM.01772-06

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

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Received 28 August 2006/Returned for modification 23 October 2006/Accepted 6 November 2006

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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▽ Published ahead of print on 22 November 2006.

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.

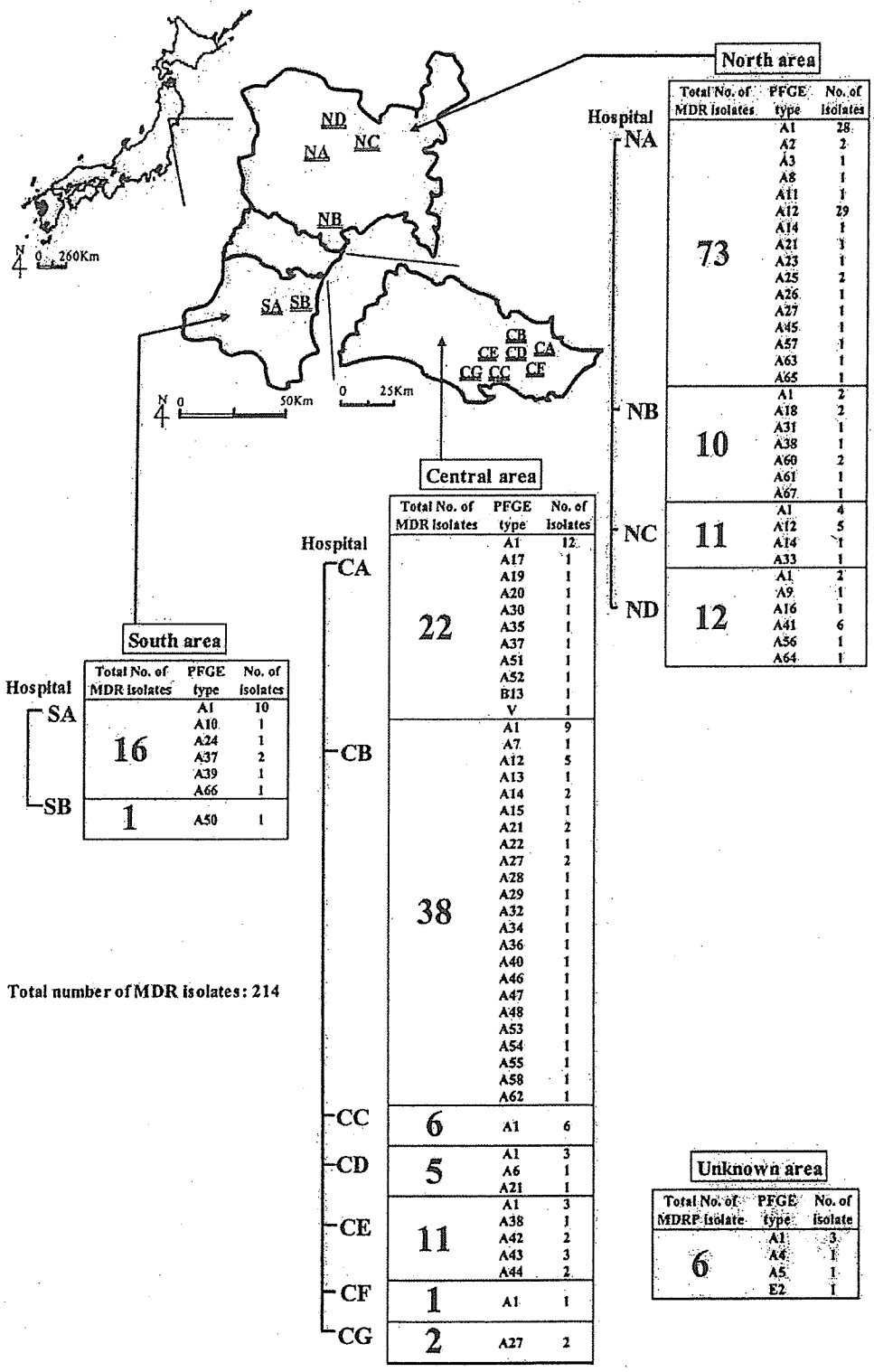

FIG. 1. Distribution of 214 isolates of MDR *P. aeruginosa* among 13 hospitals in Japan. Double capital letters indicate the locations of the hospitals that participated in this MDR *P. aeruginosa* survey.

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).

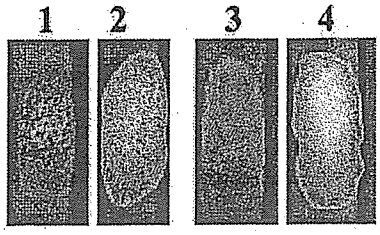

FIG. 2. Slide agglutination test with AAC(6')-Iae antibody-conjugated beads. Lane 1, AAC(6')-Iae positive control; lane 2, *P. aeruginosa* IMCJ2.S1 positive control; lane 3, 50 mM HEPES buffer negative control as solvent of AAC(6')-Iae; lane 4, *P. aeruginosa* ATCC 27853 negative control.

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. 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

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.

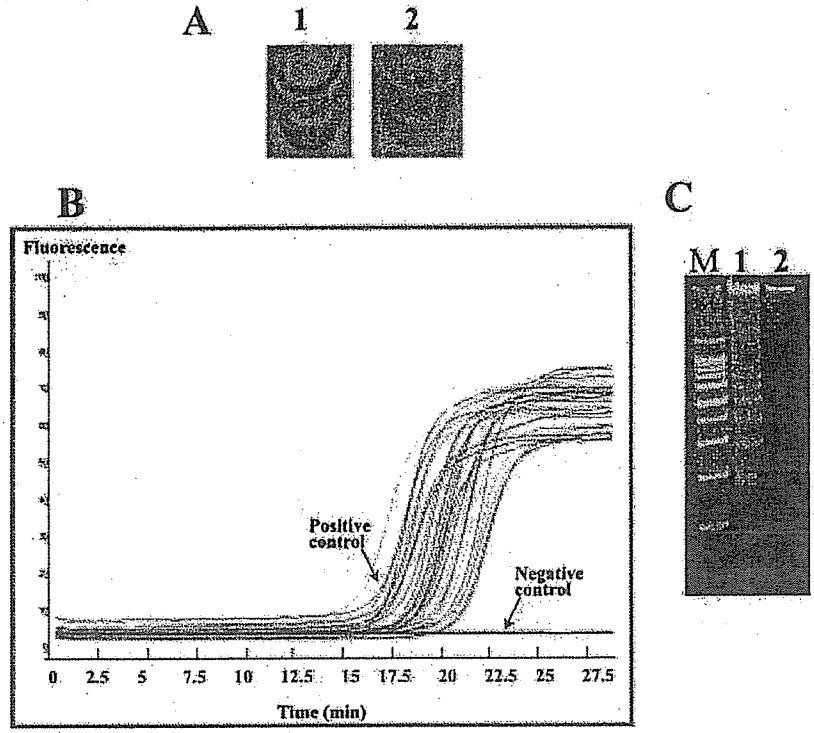

FIG. 3. LAMP assay to detect MDR *P. aeruginosa* isolates possessing the *aac(6')-Iae* gene encoding the aminoglycoside acetyltransferase AAC(6')-Iae. *P. aeruginosa* IMCJ2.S1 and ATCC 27853 were used as positive and negative controls, respectively. (A) Visual inspection analysis of LAMP products. Lane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

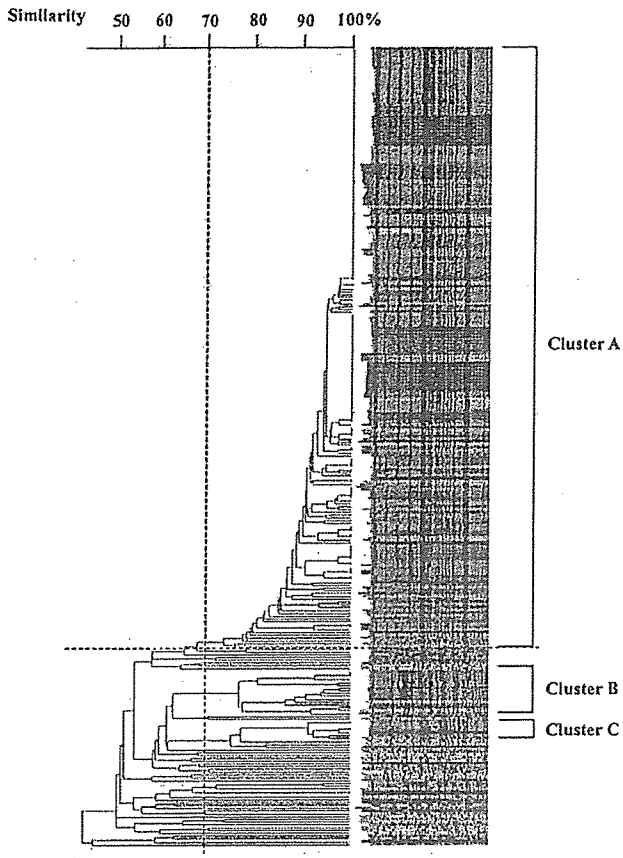

FIG. 4. Cluster analysis based on the PFGE patterns of 284 clinical isolates of *P. aeruginosa* from the 13 hospitals in the present study. Clustering was carried out with Molecular Analyst FingerprintingPlus software, version 1.6, as described in Materials and Methods.

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.

ACKNOWLEDGMENTS

We thank M. Nakano (Jichi Medical School, Japan) for comments on the manuscript and Y. Arakawa and N. Shibata (National Institute of Infectious Diseases, Japan) for providing *P. aeruginosa* M207 possessing bla_{IMP-1}, *P. aeruginosa* NCB326 possessing bla_{IMP-2}, and *Acinetobacter baumannii* NCB0211-439 possessing bla_{VIM-2}. We also thank H. Oikawa, T. Suzuki, I. Kurokawa, T. Sato, N. Onodera, K. Hiratsuka, M. Chiba, M. Utagawa, H. Mikami, M. Tachiya, K. Sasaki, S. Nakanowatari, M. Sasaki, Y. Abe, M. Koseki, N. Yaguchi, T. Nakamura, Y. Sato, and Y. Suzuki for providing clinical isolates of *P. aeruginosa*. Doripemen was kindly provided by Shionogi & Co., Ltd., Osaka, Japan.

This study was supported by Health Sciences Research grants from the Ministry of Health, Labor, and Welfare of Japan (H16-JRYO-IPPAN-011 and H18-SHINKO-11).

REFERENCES

1. Akasaka, T., M. Tanaka, A. Yamaguchi, and K. Sato. 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. 45: 2263–2268.
2. Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto. 2000. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. 38:40–43.
3. Bingen, E., S. Bonacorsi, P. Rohrlich, M. Duval, S. Lhopital, N. Brahimi, E. Vilmer, and R. V. Goering. 1996. Molecular epidemiology provides evidence of genotypic heterogeneity of multidrug-resistant *Pseudomonas aeruginosa* serotype O:12 outbreak isolates from a pediatric hospital. J. Clin. Microbiol. 34:3226–3229.
4. Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M07-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
5. Deplano, A., O. Denis, L. Poirel, D. Hocquet, C. Nonhoff, B. Byl, P. Nordmann, J. L. Vincent, and M. J. Struelens. 2005. Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J. Clin. Microbiol. 43:1198–1204.
6. Doring, G., S. Jansen, H. Noll, H. Grupp, F. Frank, K. Botzenhart, K. Magdorf, and U. Wahn. 1996. Distribution and transmission of *Pseudomonas aeruginosa* and *Burkholderia cepacia* in a hospital ward. Pediatr. Pulmonol. 21:90–100.
7. Enomoto, Y., T. Yoshikawa, M. Ihira, S. Akimoto, F. Miyake, C. Usui, S. Suga, K. Suzuki, T. Kawana, Y. Nishiyama, and Y. Asano. 2005. Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. J. Clin. Microbiol. 43:951–955.
8. Farmer, J. J., III, R. A. Weinstein, C. H. Zierdt, and C. D. Brokopp. 1982. Hospital outbreaks caused by *Pseudomonas aeruginosa*: importance of serogroup O11. J. Clin. Microbiol. 16:266–270.
9. Grattard, F., O. G. Gaudin, B. Pozzetto, A. Ros, and A. D. Mbida. 1993. Genotypic homogeneity of nosocomial *Pseudomonas aeruginosa* O12 strains demonstrated by analysis of protein profiles, DNA fingerprints and rRNA gene restriction patterns. Eur. J. Clin. Microbiol. Infect. Dis. 12:57–61.
10. Grundmann, H., C. Schneider, D. Hartung, F. D. Daschner, and T. L. Pitt. 1995. Discriminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. J. Clin. Microbiol. 33:528–534.
11. Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plesiat. 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. 47:1887–1894.
12. Homma, J. Y. 1982. Designation of the thirteen O-group antigens of *Pseudomonas aeruginosa*; an amendment for the tentative proposal in 1976. Jpn. J. Exp. Med. 52:317–320.
13. Hong, T. C., Q. L. Mai, D. V. Cuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, and K. Morita. 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42:1956–1961.
14. Hsueh, P. R., L. J. Teng, P. C. Yang, Y. C. Chen, S. W. Ho, and K. T. Luh. 1998. Persistence of a multidrug-resistant *Pseudomonas aeruginosa* clone in an intensive care burn unit. J. Clin. Microbiol. 36:1347–1351.
15. Iwamoto, T., T. Sonobe, and K. Hayashi. 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. 41:2616–2622.
16. Jones, R. N., T. R. Anderegg, J. M. Swenson, and The Quality Control Working Group. 2005. Quality control guidelines for testing gram-negative control strains with polymyxin B and colistin (polymyxin E) by standardized methods. J. Clin. Microbiol. 43:925–927.
17. Kamachi, K., H. Toyoizumi-Ajisaka, K. Toda, S. C. Soeung, S. Sarath, Y. Nareth, Y. Horiuchi, K. Kojima, M. Takahashi, and Y. Arakawa. 2006. Development and evaluation of a loop-mediated isothermal amplification method for rapid diagnosis of *Bordetella pertussis* infection. J. Clin. Microbiol. 44:1899–1902.
18. Kato, H., T. Yokoyama, and Y. Arakawa. 2005. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. J. Clin. Microbiol. 43:6108–6112.
19. Kimura, S., J. Alba, K. Shiroto, R. Sano, Y. Niki, S. Maesaki, K. Akizawa, M. Kaku, Y. Watanuki, Y. Ishii, and K. Yamaguchi. 2005. Clonal diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in geographically diverse regions of Japan. J. Clin. Microbiol. 43:458–461.
20. Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan. 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. 38:1944–1952.
21. Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J. M. Frere, and G. M. Rossolini. 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. 43:890–901.
22. Lee, J. K., Y. S. Lee, Y. K. Park, and B. S. Kim. 2005. Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents 25:290–295.
23. Legakis, N. J., N. Koukoubanis, K. Malliara, D. Michalitsianos, and J. Papavassiliou. 1987. Importance of carbenicillin and gentamicin cross-resistant serotype 0:12 *Pseudomonas aeruginosa* in six Athens hospitals. Eur. J. Clin. Microbiol. 6:300–303.
24. Levesque, C., L. Piche, C. Larose, and P. H. Roy. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. 39:185–191.
25. Liebert, C. A., R. M. Hall, and A. O. Summers. 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63:507–522.
26. Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan. 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. 33:256–264.
27. Maruyama, F., T. Kenzaka, N. Yamaguchi, K. Tani, and M. Nasu. 2003. Detection of bacteria carrying the *stx2* gene by in situ loop-mediated isothermal amplification. Appl. Environ. Microbiol. 69:5023–5028.
28. Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau. 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 43:62–66.
29. Naas, T., L. Poirel, and P. Nordmann. 1999. Molecular characterisation of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, aadA6, in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta 1489:445–451.
30. Nakamura, A., M. Hosoda, T. Kato, Y. Yamada, M. Itoh, K. Kanazawa, and H. Nouda. 2000. Combined effects of meropenem and aminoglycosides on *Pseudomonas aeruginosa* in vitro. J. Antimicrob. Chemother. 46:901–904.
31. Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:E63.
32. Ohtsuka, K., K. Yanagawa, K. Takatori, and Y. Hara-Kudo. 2005. Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. Appl. Environ. Microbiol. 71:6730–6735.
33. Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato. 1994. Molecular characterization of an enterobacterial metallo-β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. 38:71–78.
34. Pagani, L., C. Colinon, R. Migliavacca, M. Labonia, J. D. Docquier, E. Nucleo, M. Spalla, M. Li Bergoli, and G. M. Rossolini. 2005. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-beta-lactamase. J. Clin. Microbiol. 43:3824–3828.
35. Partridge, S. R., G. D. Recchia, C. Scaramuzzi, C. M. Collis, H. W. Stokes, and R. M. Hall. 2000. Definition of the attI1 site of class 1 integrons. Microbiology 146:2855–2864.
36. Pellegrino, F. L. P. C., L. M. Teixeira, M. da Glória Siqueira Carvalho, S. Aranha Nouer, M. Pinto De Oliveira, J. L. Mello Sampaio, A. D'Ávila Freitas, A. L. P. Ferreira, E. de Lourdes Teixeira Amorim, L. W. Riley, and B. M. Moreira. 2002. Occurrence of a multidrug-resistant *Pseudomonas aeruginosa* clone in different hospitals in Rio de Janeiro, Brazil. J. Clin. Microbiol. 40:2420–2424.
37. Pirnay, J. P., D. De Vos, C. Cochez, F. Bilocq, J. Pirson, M. Struelens, L. Duinslaeger, P. Cornelis, M. Zizi, and A. Vanderkelen. 2003. Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. J. Clin. Microbiol. 41:1192–1202.
38. Pitt, T. L., D. M. Livermore, D. Pitcher, A. C. Vatopoulos, and N. J. Legakis. 1989. Multiresistant serotype O 12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe. Epidemiol. Infect. 103:565–576.
39. Pournaras, S., M. Maniati, E. Petinaki, L. S. Tzouvelekis, A. Tsakris, N. J. Legakis, and A. N. Maniatis. 2003. Hospital outbreak of multiple clones of

Pseudomonas aeruginosa carrying the unrelated metallo-beta-lactamase gene variants bla_{VIM-2} and bla_{VIM-4}. J. Antimicrob. Chemother. 51:1409–1414.
40. Ratnam, S., K. Hogan, S. B. March, and R. W. Butler. 1986. Whirlpool-associated folliculitis caused by *Pseudomonas aeruginosa*: report of an outbreak and review. J. Clin. Microbiol. 23:655–659.
41. Richard, P., R. Le Floch, C. Chamoux, M. Pannier, E. Espaze, and H. Richet. 1994. *Pseudomonas aeruginosa* outbreak in a burn unit: role of antimicrobials in the emergence of multiply resistant strains. J. Infect. Dis. 170:377–383.
42. Saiman, L., and J. Siegel. 2004. Infection control in cystic fibrosis. Clin. Microbiol. Rev. 17:57–71.
43. Saito, R., Y. Misawa, K. Moriya, K. Koike, K. Ubukata, and N. Okamura. 2005. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*. J. Med. Microbiol. 54:1037–1041.
44. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, NY.
45. Seki, M., Y. Yamashita, H. Torigoe, H. Tsuda, S. Sato, and M. Maeno. 2005. Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. J. Clin. Microbiol. 43:1581–1586.
46. Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, T. Fujino, I. Kobayashi, K. Morita, Y. Kikuchi, T. Kuratsuji, and T. Kirikae. 2005. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. 49:3734–3742.
47. Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa. 2003. PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol. 41: 5407–5413.
48. Stanislavsky, E. S., and J. S. Lam. 1997. *Pseudomonas aeruginosa* antigens as potential vaccines. FEMS Microbiol. Rev. 21:243–277.
49. Stokes, H. W., and R. M. Hall. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. 3:1669–1683.
50. Tassios, P. T., V. Gennimata, A. N. Maniatis, C. Fock, N. J. Legakis, and The Greek *Pseudomonas aeruginosa* Study Group. 1998. Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O:11. J. Clin. Microbiol. 36:897–901.
51. Tsuchizaki, N., K. Ishino, F. Saito, J. Ishikawa, M. Nakajima, and K. Hotta. 2006. Trends of arbekacin-resistant MRSA strains in Japanese hospitals (1979 to 2000). J. Antibiot. 59:229–233.
52. Tsuji, A., I. Kobayashi, T. Oguri, M. Inoue, E. Yabuuchi, and S. Goto. 2005. An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *Pseudomonas aeruginosa* isolated at medical institutes nationwide in Japan. J. Infect. Chemother. 11:64–70.
53. Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann. 2005. Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. 18:306–325.
54. Zimakoff, J., N. Hoiby, K. Rosendal, and J. P. Guilbert. 1983. Epidemiology of *Pseudomonas aeruginosa* infection and the role of contamination of the environment in a cystic fibrosis clinic. J. Hosp. Infect. 4:31–40.

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2007, p. 179–192 Vol. 45, No. 1
0095-1137/07/$08.00+0 doi:10.1128/JCM.00750-06

Detection of Multidrug Resistance in *Mycobacterium tuberculosis*▽

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Received 8 April 2006/Returned for modification 3 July 2006/Accepted 9 October 2006

We developed a DNA sequencing-based method to detect mutations in the genome of drug-resistant *Mycobacterium tuberculosis*. Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of the genome. Eight genome regions associated with drug resistance, including *rpoB* for rifampin (RIF), *katG* and the *mabA* (*fabG1*)-*inhA* promoter for isoniazid (INH), *embB* for ethambutol (EMB), *pncA* for pyrazinamide (PZA), *rpsL* and *rrs* for streptomycin (STR), and *gyrA* for levofloxacin, were amplified simultaneously by PCR, and the DNA sequences were determined. It took 6.5 h to complete all procedures. Among the 138 clinical isolates tested, 55 were resistant to at least one drug. Thirty-four of 38 INH-resistant isolates (89.5%), 28 of 28 RIF-resistant isolates (100%), 15 of 18 EMB-resistant isolates (83.3%), 18 of 30 STR-resistant isolates (60%), and 17 of 17 PZA-resistant isolates (100%) had mutations related to specific drug resistance. Eighteen of these mutations had not been reported previously. These novel mutations include one in *rpoB*, eight in *katG*, one in the *mabA-inhA* regulatory region, two in *embB*, five in *pncA*, and one in *rrs*. *Escherichia coli* isolates expressing individually five of the eight *katG* mutations showed loss of catalase and INH oxidation activities, and isolates carrying any of the five *pncA* mutations showed no pyrazinamidase activity, indicating that these mutations are associated with INH and PZA resistance, respectively. Our sequencing-based method was also useful for testing sputa from tuberculosis patients and for screening of mutations in *Mycobacterium bovis*. In conclusion, our new method is useful for rapid detection of multiple-drug-resistant *M. tuberculosis* and for identifying novel mutations in drug-resistant *M. tuberculosis*.

The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis*, especially multidrug-resistant (MDR) strains, are serious threats to the control of tuberculosis and comprise an increasing public health problem (40). Patients infected with MDR strains, which are defined as strains resistant to both rifampin (RIF) and isoniazid (INH), are difficult to cure and are more likely to remain sources of infection for a longer period of time than are patients with drug-susceptible strains (40).

It is essential that rapid drug susceptibility tests be developed to prevent the spread of MDR *M. tuberculosis*. The time necessary for culture of specimens was reduced by the radiometric BACTEC 460TB system (BD Biosciences, Sparks, MD), the nonradiometric ESP II system (Trek Diagnostics, Westlake, OH), and other rapid broth methods, such as BACTEC MGIT 960 SIRE (BD Biosciences) (20). These drug susceptibility tests, however, still require 1 to 2 weeks for final determination and reporting to the clinician (23). Additional reductions in the detection period are needed.

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▽ Published ahead of print on 15 November 2006.

Drug resistance in *M. tuberculosis* is caused by mutations in relatively restricted regions of the genome (17, 39). Mutations associated with drug resistance occur in *rpoB* for RIF, *katG* and the promoter region of the *mabA* (*fabG1*)-*inhA* operon for INH, *embB* for ethambutol (EMB), *pncA* for pyrazinamide (PZA), *rpsL* and *rrs* for streptomycin (STR), and *gyrA* for fluoroquinolones (FQs) such as ofloxacin (OFX) and levofloxacin (LVX) (17, 39). For example, 96% to 100% of RIF-resistant *M. tuberculosis* isolates have at least 1 mutation in *rpoB*, which encodes the RNA polymerase β-subunit (17, 31, 39). Of INH-resistant isolates, 42% to 58% have at least 1 mutation in *katG*, which encodes catalase-peroxidase, and 21% to 34% carry at least 1 mutation in the promoter of *mabA*, a synonym for *fabG1* (10), which encodes a 3-ketoacyl reductase (3, 17, 38, 39). Of EMB-resistant isolates, 47% to 65% have at least one mutation in *embB*, which encodes arabinosyltransferase (17, 32, 39). Seventy-two to 97% of PZA-resistant isolates have at least one mutation in *pncA*, which encodes pyrazinamidase (17, 26, 39). Of STR-resistant isolates, 52% to 59% and 8% to 21% have mutations in *rpsL*, which encodes ribosomal protein S12, and *rrs*, which encodes 16S rRNA, respectively (17, 19, 39). Of FQ-resistant isolates, 75% to 94% have mutations in *gyrA*, which encodes the A subunit of DNA gyrase (17, 30, 39).

Various molecular methods have been used to identify the

TABLE 1. Bacterial strains used in the present study

Strain	Characteristic(s) or susceptibility pattern	No. of isolates
M. tuberculosis IMCJ	Clinical strains isolated in Japan	105
	Rif^r Inh^r Emb^r Pza^r Str^r Ofx^r	3
	Rif^r Inh^r Emb^r Pza^r Str^r	3
	Rif^r Inh^r Pza^r Str^r	3
	Rif^r Inh^r Emb^r Str^r	4
	Rif^r Inh^r Emb^r Pza^r	2
	Inh^r Emb^r Str^r	2
	Rif^r Inh^r	2
	Inh^r Str^r	4
	Pza^r Str^r	1
	Rif^r Str^r	2
	Rif^r	2
	Inh^r	5
	Pza^r	2
	Str^r	6
	Susceptible to all drugs tested	64
M. tuberculosis P	Clinical strains isolated in Poland	33
	Rif^r Inh^r Emb^r Pza^r	1
	Rif^r Inh^r Emb^r	2
	Rif^r Inh^r Pza^r	1
	Rif^r Inh^r	2
	Inh^r Str^r	1
	Rif^r	1
	Inh^r	3
	Emb^r	1
	Pza^r	1
	Str^r	1
	Susceptible to all drugs tested	19
M. tuberculosis H37Rv (ATCC[a] 27294)	Susceptible to all drugs tested	1
M. tuberculosis H37Ra (ATCC 25177)	Susceptible to all drugs tested	1
M. avium ATCC 25291	ND[g]	1
M. bovis BCG[b] (Japanese strain 172)	Pza^r	1
M. chelonae JCM[c] 6390 (ATCC 14472)	ND	1
M. fortuitum RIMD[d] 1317004 (ATCC 6841)	ND	1
M. gastri GTC[e] 610 (ATCC 15754)	ND	1
M. intracellulare JCM 6384 (ATCC 13950)	ND	1
M. kansasii JCM 6379 (ATCC 124878)	ND	1
M. marinum GTC 616 (ATCC 927)	ND	1
M. nonchromogenicum JCM 6364 (ATCC 19530)	ND	1
M. phlei RIMD 1326001 (ATCC19249)	ND	1
M. scrofulaceum JCM 6381 (ATCC 19981)	ND	1
M. simiae GTC 620 (ATCC 25275)	ND	1
M. smegmatis ATCC 19420	ND	1
M. szulgai JCM 6383 (ATCC 35799)	ND	1
M. terrae GTC 623 (ATCC 15755)	ND	1
Escherichia coli ATCC 8739	ND	1
Haemophilis influenzae IID[f] 984 (ATCC 9334)	ND	1
Klebsiella pneumoniae IID5209 (ATCC 15380)	ND	1
Legionella pneumophila GTC 745	ND	1
Mycoplasma pneumoniae IID 817	ND	1
Pseudomonas aeruginosa ATCC 27853	ND	1
Rhodococcus equi ATCC 33710	ND	1
Staphylococcus aureus N315	ND	1
Streptococcus pneumoniae GTC 261	ND	1

[a] American Type Culture Collection, Rockville, MD.
[b] Japan BCG Laboratory, Tokyo, Japan.
[c] Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan.
[d] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
[e] Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan.
[f] Institute of Medical Science, University of Tokyo, Tokyo, Japan.
[g] ND, not determined.

mutations in *rpoB*, *katG*, *rpsL*, *rrs*, *embB*, *pncA*, *gyrA*, and other genes (7, 17). Among these methods, DNA sequencing is the most direct and reliable for detection of both known and novel mutations. The conventional methods, nevertheless, are not applicable for analysis of strains that may have multiple mutations in genes related to drug resistance because different PCR conditions are required for amplification of each target region. We describe here a new PCR-based method for simultaneous detection of mutations in eight genes responsible for resistance to six antitubercular drugs.

MATERIALS AND METHODS

Bacterial strains and plasmids. One hundred five and 33 clinical isolates of *M. tuberculosis* were obtained from patients with pulmonary tuberculosis in Japan and Poland, respectively. All of the bacterial strains used in this study, except for those used in cloning experiments, are listed in Table 1. *Escherichia coli* UM262 (*recA katG*::Tn*10 pro leu rpsL hsdM hsdR endI lacY*) (13) was provided by Barbara L. Triggs-Raine (University of Manitoba, Manitoba, Canada) and was used as a host for the expression of *katG* derived from *M. tuberculosis* clinical isolates and H37Rv, an *M. tuberculosis* reference strain. *E. coli* TOP10 (Invitrogen, Carlsbad, CA) and BL21-AI (Invitrogen) were used as hosts for cloning and protein overexpression studies, respectively. pCRT7/NT (Invitrogen) was used as a cloning and protein expression vector.

Drug susceptibility test. All clinical isolates of *M. tuberculosis*, *M. tuberculosis* strains H37Rv and H37Ra, and *Mycobacterium bovis* BCG Japanese strain 172 were tested for drug susceptibility. Strains were analyzed by three different methods. Two methods were agar proportion methods: the Middlebrook 7H10 agar medium method recommended by the United States Public Health Service (20) and the egg-based Ogawa medium method recommended by the Japanese Society for Tuberculosis (Vit Spectrum-SR; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan). The third method was a rapid broth method (BD BACTEC MGIT 960 SIRE, BD Biosciences, Sparks, MD) (20). The proportion method with 7H10 agar medium was used to assess susceptibilities to RIF, INH, EMB, STR, and OFX. Ogawa medium was used to test susceptibilities to RIF, INH, EMB, SM, and LVX. The broth method was applied to assess susceptibilities to RIF, INH, EMB, STR, and PZA. All isolates were tested by all three methods.

Assay for PZase activity. Pyrazinamidase (PZase) activity was determined as described previously (34). *M. tuberculosis* strain H37Rv, which is susceptible to PZA and positive for PZase, was used as a positive control for the assay. *M. bovis* strain BCG, which is resistant to PZA and negative for PZase, was used as a negative control. Each test tube was read and classified by three independent observers. There were no discrepancies between the classifications for any of the isolates tested.

DNA extraction. Genomic DNAs from bacteria were extracted as described previously (21).

Clinical samples. Six samples of mycobacterial staining-positive sputa from six patients with relapsed active tuberculosis and four samples of staining-negative sputa from four patients who had been treated previously with antitubercular drugs, were treated with *N*-acetyl-L-cysteine–NaOH solution according to the procedure of the BBL MycoPrep mycobacterial system digestion/decontamination kit (BD Diagnostic Systems, Franklin Lakes, NJ). Each sample was resuspended in 1.5 ml phosphate buffer. One milliliter of the suspension was transferred to a 1.5-ml tube for PCR. The remaining suspension was inoculated into Ogawa medium and MGIT 960 broth and cultured for mycobacterial examination. The 1 ml of suspension for PCR was centrifuged for 15 min at 13,000 × *g*, and the supernatant was removed with a pipette. Tris-EDTA (TE) buffer (500 μl) was added to resuspend the sediment, and the solution was again centrifuged for 15 min at 13,000 × *g*. The sediment was resuspended in 100 μl of a 10% solution of Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) in distilled water. The sample was resuspended by vortexing and incubated at 45°C for 45 min followed by incubation at 100°C for 10 min. The sample was vortexed again, allowed to cool, and centrifuged at 12,000 × *g* for 5 min to clarify the supernatant, which was transferred to another 1.5-ml tube and used for PCR.

DNA sequencing of drug resistance-related genes. Eight pairs of PCR primers were designed to amplify simultaneously regions of eight genes associated with resistance to six antituberculosis drugs. Sixteen primers were designed to determine the DNA sequences of the amplicons. The sequences of oligonucleotide primers for PCR, PR1 to PR16, and for DNA sequencing, PR17 to PR32, and the regions analyzed are listed in Table 2.

A two-temperature PCR consisting of 30 cycles of 95°C for 1 s for denaturation and 68°C for 30 s for annealing and elongation was performed with a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA) for DNA amplification, according to the instructions in the manufacturer's manual. Each PCR primer pair in TE (1.0 μl of 10 μM) listed in Table 2 was added to an individual reaction tube (0.2 ml Thermo-Tube; Advanced Biotechnologies, Epsom, Surrey, United Kingdom). Hence, eight reaction tubes containing the different primer pairs were prepared. Forty-nine microliters of a solution containing 1.0 μl DNA template, 1.25 U *Z-Taq* polymerase (Takara Bio, Ohtsu, Shiga, Japan), 4 μl of 2.5 mM each deoxynucleotide triphosphate, and 5 μl of 10× Z *Taq* PCR buffer (Takara Bio) was added to each reaction tube. The *Z-Taq* polymerase offers unmatched PCR productivity, with a processing speed five times faster than those of other commercially available *Taq* polymerases, which allowed us to reduce the annealing and elongation times.

PCR products were purified with MicroSpin S-300 HR columns (Amersham Biosciences, Uppsala, Sweden) or a DyeEx 2.0 spin kit (QIAGEN K.K., Tokyo, Japan) and used as templates for DNA sequencing. PCR products were sequenced with the appropriate gene-specific primers (Table 2). Sequencing was performed with an ABI PRISM BigDye terminator cycle sequencing ready reaction kit for a 96-well format (Applied Biosystems). Five microliters of premixed reagents from the kit (Terminator Ready Reaction mix; Applied Biosystems) and 13.5 μl of 1× reaction buffer were added to each tube containing 100 ng of templates and 5 pmol sequencing primer and mixed with a pipette. Amplification conditions were 25 cycles of 96°C for 10 s for denaturation, 50°C for 5 s for annealing, and 60°C for 4 min for elongation. It took 2.5 h to complete the entire reaction. Centri-Sep spin columns (Applied Biosystems) were used to remove unincorporated reagents and primers. Purified products were dried in a vacuum centrifuge, resuspended in Hi-Di formamide (Applied Biosystems), heated for 2 min at 95°C for denaturation, immediately cooled on ice, and loaded into a 96-well plate (MicroAmp 96-well reaction plate; Applied Biosystems). The purified samples were then analyzed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems). DNA sequences were collected and edited with Data Collection version 1.01 and Sequencing Analysis version 3.7 software (Applied Biosystems) and compared with those of *M. tuberculosis* H37Rv (GenBank accession no. NC_000962) with Genetyx-WIN (version 5; Software Development Co., Tokyo, Japan). The codon numbers of *rpoB* were designated on the basis of alignment of the *E. coli rpoB* sequence with a portion of the *M. tuberculosis* H37Rv sequence and are not the positions of the actual *M. tuberculosis rpoB* codons (14, 31).

Cloning of *katG*. The coding regions of *katG* from six INH-resistant clinical isolates of *M. tuberculosis*, two INH-susceptible isolates, and the H37Rv strain were cloned. *katG* was amplified by PCR with 2.5 U of Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) and primers PR3 and PR4 (Table 2). The PCR products were ligated into the pCRT7/NT vector downstream of the region encoding a His_6 tag.

KatG enzyme assays. For expression of *M. tuberculosis* KatG, *katG*-deficient *E. coli* UM262 (13) cells were transformed with pCRT7/NT carrying cloned *katG* genes derived from eight clinical isolates and the H37Rv strain. KatG-mediated catalase activity was assayed spectrophotometrically by monitoring the decrease in H_2O_2 concentration at A_{240} (ε_{240} = 0.0436 mM^{-1} cm^{-1}) as described previously (18).

Assay for free-radical formation from INH oxidation. Rates of KatG-mediated free-radical formation from INH oxidation in the presence of H_2O_2 were monitored spectrophotometrically by following the reduction of nitroblue tetrazolium (NBT) as described previously (35).

Purification of KatG. *M. tuberculosis* KatG from strain H37Rv was overexpressed in *E. coli* BL21-AI cells and purified with chelating Sepharose (Ni Sepharose 6 Fast Flow; Amersham Biosciences) loaded with Ni^{2+} in a column. The purity of the KatG protein was more than 95% by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme-linked immunosorbent assay and Western blotting. Purified His-tagged KatG was used as an antigen to raise polyclonal antibodies in a male Japanese white rabbit. Antiserum against KatG was used for Western blotting and enzyme-linked immunosorbent assay.

Data analysis. The correlations between mutation data from the DNA sequence-based assays and data from conventional culture methods with drugs or PZase activities were assessed by the index of test efficiency. The efficiency of a test was defined as the percentage of times that the test gave the correct answer compared to the total number of tests.

RESULTS

Drug susceptibility patterns. The susceptibility patterns of the 138 clinical isolates for six drugs, RIF, INH, EMB, PZA, STR, and OFX, are shown in Table 1. Among the 138 clinical isolates, 55 were resistant to at least one drug and 23 were MDR strains displaying resistance to both INH and RIF. Twenty of the 23 MDR strains were resistant to at least one other drug in addition to INH and RIF. Eighty-three clinical isolates and 2 laboratory strains, H37Rv and H37Ra, were susceptible to all of the drugs tested. The BCG strain

TABLE 2. Primers used to detect MDR tuberculosis

Target gene[a]	Primer set (direction)	Nucleotide sequence	Positions[b]	Product size (bp)
PCR primers				
rpoB	PR1 (forward)	5′-CCGCGATCAAGGAGTTCTTC-3′	1256–1275	315
	PR2 (reverse)	5′-ACACGATCTCGTCGCTAACC-3′	1570–1551	
katG	PR3 (forward)	5′-GTGCCCGAGCAACACCCACCCATTACAGAAAC-3′	1–32	2,223
	PR4 (reverse)	5′-TCAGCGCACGTCGAACCTGTCGAG-3′	2223–2200	
mabA promoter	PR5 (forward)	5′-ACATACCTGCTGCGCAATTC-3′	−217 to −198	1,362
	PR6 (reverse)	5′-GCATACGAATACGCCGAGAT-3′	1145–1126	
embB	PR7 (forward)	5′-CCGACCACGCTGAAACTGCTGGCGAT-3′	640–665	2,748
	PR8 (reverse)	5′-GCCTGGTGCATACCGAGCAGCATAG-3′	3387–3303	
pncA	PR9 (forward)	5′-GGCGTCATGGACCCTATATC-3′	−80 to −61	670
	PR10 (reverse)	5′-CAACAGTTCATCCCGGTTC-3′	590–572	
rpsL	PR11 (forward)	5′-CCAACCATCCAGCAGCTGGT-3′	4–23	572
	PR12 (reverse)	5′-GTCGAGAGCCCGCTTGAGGG-3′	575–556	
rrs (16S RNA)	PR13 (forward)	5′-AAACCTCTTTCACCATCGAC-3′	428–447	1,329
	PR14 (reverse)	5′-GTATCCATTGATGCTCGCAA-3′	1756–1737	
gyrA	PR15 (forward)	5′-GATGACAGACACGACGTTGC-3′	−1–19	398
	PR16 (reverse)	5′-GGGCTTCGGTGTACCTCAT-3′	397–379	
Sequencing primers				
rpoB	PR17	5′-TACGGCGTTTCGATGAAC-3′ (complementary strand)	1529–1512	
katG	PR18	5′-ACGTAGATCAGCCCCATCTG-3′ (complementary strand)	689–670	
	PR19	5′-GAGCCCGATGAGGTCTATTG-3′	574–593	
	PR20	5′-CCGATCTATGAGCGGATCAC-3′	1162–1181	
	PR21	5′-GAACAAACCGACGTGGAATC-3′	1729–1748	
mabA promoter	PR22	5′-ACATACCTGCTGCGCAATTC-3′	−217 to −198	
embB	PR23	5′-ACGCTGAAACTGCTGGCGAT-3′	646–665	
	PR24	5′-GTCATCCTGACCGTGGTGTT-3′	1462–1481	
	PR25	5′-GGTGGGCAGGATGAGGTAGT-3′ (complementary strand)	1596–1577	
	PR26	5′-CACAATCTTTTTCGCCCTGT-3′	2007–2026	
	PR27	5′-GCGTGGTATCTCCTGCCTAAG-3′	2581–2601	
pncA	PR28	5′-GGCGTCATGGACCCTATATC-3′	−80 to −61	
rpsL	PR29	5′-CCAACCATCCAGCAGCTGGT-3′	4–23	
rrs (16S RNA)	PR30	5′-CAGGTAAGGTTCTTCGCGTTG-3′ (complementary strand)	979–959	
	PR31	5′-GTTCGGATCGGGGTCTGCAA-3′	1291–1310	
gyrA	PR32	5′-GATGACAGACACGACGTTGC-3′	−1–19	

[a] The complete sequences of target genes in *M. tuberculosis* H37Rv are in the GenBank database under accession no. NC_000962.
[b] Numbering based on nucleotide position relative to the initiation codon of each gene.

was sensitive to all drugs tested except PZA. When the results of the proportion method were compared with those of the Vit Spectrum-SR and BD BACTEC MGIT 960 SIRE methods, there was full agreement for all drugs. However, there were some differences in the degrees of susceptibility to INH between the methods. One hundred isolates were susceptible to 0.2 μg/ml of INH when they were assessed with the solid media, whereas only 6 of these isolates were resistant to 0.1 μg/ml of INH when they were tested by the broth method.

Two-temperature PCR. We optimized a two-temperature PCR strategy to amplify regions of eight drug resistance-related genes in *M. tuberculosis*. The target regions varied in length from 315 to 2,748 bp (Table 2). Genomic DNA (approximately 100 ng) from *M. tuberculosis* strain H37Rv was amplified with eight primer pairs simultaneously. The entire procedure, including PCR, took less than 60 min. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide (Fig. 1A). Genomic DNAs from strain H37Ra and 138 clinical isolates of *M. tuberculosis*

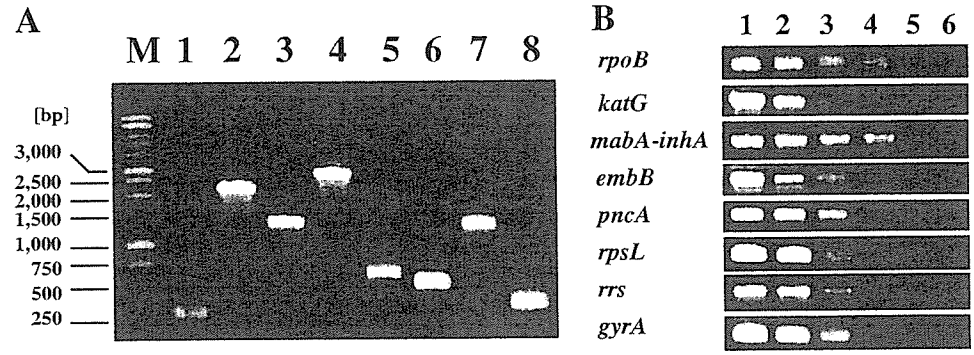

FIG. 1. (A) Amplification products from two-temperature PCR of *M. tuberculosis* H37Rv. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Lane M, 1-kbp ladder as a molecular size marker; lane 1, *rpoB*; lane 2, *katG*; lane 3, *mabA-inhA* locus; lane 4, *embB*; lane 5, *pncA*; lane 6, *rpsL*; lane 7, *rrs*; and lane 8, *gyrA*. (B) Determination of the sensitivity of two-temperature PCR with serially diluted *M. tuberculosis* H37Rv DNA as a template. Experiments were repeated twice with similar results. Lane 1, 100 ng of template DNA; lane 2, 10 ng; lane 3, 1 ng; lane 4, 100 pg; lane 5, 10 pg; lane 6, 1 pg.

were then amplified by PCR. Each PCR yielded a single band of the expected length (data not shown). These results indicate that the PCR is reliable for use in clinical isolates of *M. tuberculosis*.

Sensitivity and specificity of two-temperature PCR. To determine the sensitivity of the two-temperature PCR for the target DNA, 100, 10, and 1 ng and 100, 10, and 1 pg of genomic DNA from *M. tuberculosis* H37Rv were amplified with the PCR assay. As shown in Fig. 1B, the limits of detection for *mabA-inhA* and *rpoB*, for *gyrA*, *pncA*, and *rpsL*, and for *embB*, *katG*, and *rrs* were 10 pg, 100 pg, and 1 ng of DNA, respectively.

To determine the species specificity of the PCR, genomic DNA (100 ng) was isolated from various species of bacteria, including *Mycobacterium* spp. and additional pathogenic bacterial species listed in Table 1. DNA was amplified with the PCR primer pairs shown in Table 2. The PCR patterns for *M. bovis* BCG were identical to those of *M. tuberculosis* (Table 3). Some mycobacterial species were positive for the PCR with primer pairs for *rpsL*, *rrs*, and *gyrA*; however, all mycobacterial species except *M. tuberculosis* and *M. bovis* were negative for *rpoB*-, *katG*-, *mabA*-, *embB*-, and *pncA*-specific PCR products (Table 3). Nonmycobacterial strains tested were negative for all eight gene targets.

Sequencing of *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. tuberculosis*. PCR products were purified and then sequenced with the 16 sequencing primers listed in Table 2. Sequencing yielded 8.8 kb of sequence for each *M. tuberculosis* strain. Sequences were obtained for regions of the *rpoB*, *katG*, *mabA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* genes and the promoters of *mabA-inhA* and *pncA*. The mutations identified in the 138 clinical isolates of *M. tuberculosis* are shown in Table 4.

(i) *rpoB*. We sequenced a 240-bp fragment containing the "81-bp core region" of *rpoB*. One hundred nine isolates had no mutations in *rpoB*. The remaining 25 each had a single point mutation, and 4 isolates each had two point mutations. All of the detected mutations resulted in amino acid substitutions. Of these mutations, two, S450L (TCG→TTG at nucleotide [nt] positions 1348 to 1350) and S509R (AGC→AGG at nt positions 1525 to 1527) were novel (Table 4).

(ii) *katG*. Thirty-seven isolates had no mutations in *katG*, whereas 81 isolates each had a single point mutation, 11 isolates had two point mutations each, 1 isolate had three point mutations, and 2 isolates had a 3-bp insertion each. One mutation was a silent mutation (CTG→TTG at nt positions 1957 to 1959 [L653L]), and all others caused amino acid substitutions. We identified 10 novel point mutations and the novel L390 insertion.

(iii) *mabA-inhA* locus. We found no mutations in the *mabA* gene and the regulatory region of *mabA-inhA* in 126 isolates. Ten isolates had a C-to-T transition −15 bp upstream of the *mabA* initiation codon, 1 isolate had a T-to-A transition 8 bp upstream of the initiation codon, and 1 isolate had a T-to-A transition 5 bp upstream of the initiation codon. The T-to-A transition −5 bp upstream of the initiation codon was novel.

(iv) *embB*. We found no mutations in *embB* in 107 isolates. Twenty-six isolates each had a single point mutation, 3 each had two point mutations, and 2 each had three point mutations. Several isolates had silent mutations (D345D, D534D, L355L, and P1075P). Five of these point mutations were novel.

(v) *pncA*. One hundred nineteen isolates had no mutations in *pncA* or the *pncA* regulatory region. Nineteen isolates each had a single point mutation. Five of these mutations were novel.

(vi) *gyrA*. All isolates tested contained the E21Q mutation of *gyrA*. Eighteen isolates each had one point mutation, 117 isolates each had two point mutations, 2 isolates each had three point mutations, and 1 isolate carried four mutations.

(vii) *rpsL*. All isolates carried the K121K mutation of *rpsL*. One hundred twenty-five isolates each had a single point mutation, and the remaining 13 isolates each had two point mutations.

(viii) *rrs*. One hundred thirty-three isolates had no mutations in *rrs*. Two isolates had a C-to-T transition at nt position 516. One isolate had an A-to-G transition at nt position 1400. One isolate had two point mutations, an A-to-G transition at nt position 1400 and an A-to-G transition at nt position 1539. One isolate had an insertion of a cytosine at position 1061 of *rrs*.

TABLE 3. Species specificity of two-temperature PCR

Bacterium	Origin	Results of PCR with various primer pairs[g]							
		rpoB	*katG*	*mabA-inhA*	*embB*	*pncA*	*rpsL*	*rrs*	*gyrA*
M. tuberculosis IMCJ	105 clinical isolates from Japan	+	+	+	+	+	+	+	+
M. tuberculosis P	33 clinical isolates from Poland	+	+	+	+	+	+	+	+
M. tuberculosis H37Rv	ATCC[a] 27294	+	+	+	+	+	+	+	+
M. tuberculosis H37Ra	ATCC 25177	+	+	+	+	+	+	+	+
M. bovis BCG Japanese strain 172	Japan BCG Laboratory[b]	+	+	+	+	+	+	+	+
M. avium	ATCC 25291	−	−	−	−	−	+	+	−
M. gastri	GTC[c] 610 (ATCC 15754)	−	−	−	−	−	+	+	+
M. intracellulare	JCM[d] 6384 (ATCC 13950)	−	−	−	−	−	+	+	−
M. kansassii	JCM 6379 (ATCC 124878)	−	−	−	−	−	+	+	−
M. marinum	GTC 616 (ATCC 927)	−	−	−	−	−	+	+	+
M. simiae	GTC 620 (ATCC 25275)	−	−	−	−	−	−	−	+
M. scrofulaceum	JCM 6381 (ATCC 19981)	−	−	−	−	−	−	+	−
M. szulgai	JCM 6383 (ATCC 35799)	−	−	−	−	−	+	+	+
M. nonchromogenicum	JCM 6364 (ATCC 19530)	−	−	−	−	−	+	−	−
M. terrae	GTC 623 (ATCC 15755)	−	−	−	−	−	−	−	−
M. chelonae	JCM 6390 (ATCC 14472)	−	−	−	−	−	+	−	−
M. fortuitum	RIMD[e] 1317004 (ATCC 6841)	−	−	−	−	−	+	−	−
M. phlei	RIMD 1326001 (ATCC 19249)	−	−	−	−	−	−	−	−
M. smegmatis	ATCC 19420	−	−	−	−	−	+	−	−
Escherichia coli	ATCC 8739	−	−	−	−	−	−	−	−
Haemophilus influenzae	IID[f] 984 (ATCC 9334)	−	−	−	−	−	−	−	−
Klebsiella pneumoniae	IID5209 (ATCC 15380)	−	−	−	−	−	−	−	−
Legionella pneumophila	GTC 745	−	−	−	−	−	−	−	−
Mycoplasma pneumoniae	IID 817	−	−	−	−	−	−	−	−
Pseudomonas aeruginosa	ATCC 27853	−	−	−	−	−	−	−	−
Rhodococcus equi	ATCC 33710	−	−	−	−	−	−	−	−
Staphylococcus aureus	N315	−	−	−	−	−	−	−	−
Streptococcus pneumoniae	GTC 261	−	−	−	−	−	−	−	−

[a] American Type Culture Collection, Rockville, MD.
[b] Japan BCG Laboratory, Tokyo, Japan.
[c] Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan.
[d] Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan.
[e] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.
[f] Institute of Medical Science, University of Tokyo, Tokyo, Japan.
[g] Amplification results were determined by agarose gel electrophoresis. Symbols: +, presence of amplification products; −, absence of amplification products.

Correlation between drug susceptibility and mutation(s) in *M. tuberculosis*. (i) RIF resistance and *rpoB*. Mutations in the 81-bp core region of *rpoB* are responsible for resistance in at least 96% of RIF-resistant *M. tuberculosis* isolates (17, 31, 39). In the present study, we identified two novel mutations, S450L (TCG→TTG at nt positions 1348 to 1350) and S509R (AGC→AGG at nt positions 1525 to 1527). S450L was located upstream of the 81-bp core region, and the isolate with S450L was susceptible to RIF, indicating this mutation is not associated with RIF resistance. Isolates with both S509R and H526R (CAC→CGC at nt positions 1576 to 1578) mutations were RIF resistant. However, it is unclear whether S509R is associated with RIF resistance because H526R is known to be associated with RIF resistance (39).

(ii) INH resistance and *katG* and *mabA-inhA*. INH resistance is related to mutation(s) in *katG*, *inhA*, and/or the promoter region of *mabA-inhA* (17, 22, 38, 39). In the present study, we found 11 novel mutations and a CTA insertion at nt position 1170 in *katG*. Among these mutations, Q295P and G297V conferred INH resistance. Two INH-resistant isolates carried the L141F and R463L mutations. R463L is known not to be associated with INH resistance (33, 39), and L141F may confer INH resistance. The A65T, A245V, and V725A mutations did not influence INH susceptibility. Isolates carrying the T324P, L48Q, or M257T mutation and −15C→T upstream of *mabA* were resistant to INH. However, it is unclear whether T324P, L48Q, or M257T is related to INH resistance because the −15C→T upstream of *mabA* is known to confer INH resistance (10, 22, 39).

(iii) EMB resistance and *embB*. EMB resistance is related to mutations in *embB* (17, 32, 39). In the present study, we found five novel mutations in *embB*. Among these, D354A conferred EMB resistance. V492L, A680T, and A1007V were not associated with EMB resistance. An isolate with both N296Y and M306I was resistant to EMB. However, it is unclear whether N296Y is related to EMB resistance because the M306I mutation is known to confer EMB resistance (39).

(iv) PZA resistance and *pncA*. It is known that PZA resistance is related to mutations in *pncA* (17, 26, 39). In the present study, we identified five novel mutations in *pncA*. Among these, A3E, D53N, P54L, C72W, and M175V conferred PZA resistance. It will be necessary to determine whether the PZA activities of various mutants are correlated with PZA susceptibility. We then evaluated the PZase activities of *M. tuberculosis* clinical isolates, strains H37Rv and H37Ra, and *M. bovis* strain BCG. The BCG strain was included as a negative control as described in Materials and Methods. One hundred twenty-one clinical isolates and H37Rv

TABLE 4. Nucleotide and amino acid changes found in 138 clinical isolates of *M. tuberculosis*[a]

Gene	No. of isolates (n = 138)	Isolate origin	Changes		% Resistant (no. of isolates displaying resistance)	Other mutations
			Nucleotide	Amino acid (silent mutation)		
rpoB[b]	83	Japan	None	None	0	
	26	Poland	None	None	0	
	1	Japan	TCG→TTG	S450L*	0	
	1	Poland	CAA→CTA	Q513L	100	
	1	Japan	GAC→GTC	D516V	100	
	1	Japan	TCG→TTG	S522L	100	
	1	Japan	CAC→TAC	H526Y	100	
	2	Japan	CAC→CGC	H526R	100	
	1	Poland	CAC→ACC	H526T	100	
	7	Japan	TCG→TTG	S531L	100	
	5	Japan	TCG→TGG	S531W	100	
	5	Poland	TCG→TTG	S531L	100	
	2	Japan	AGC→AGG and CAC→CGC	S509R* and H526R	100	
	2	Japan	ATG→ATT and GAC→TAC	M515I and D516Y	100	
katG	14	Japan	None	None	0	
	23	Poland	None	None	4.4 (1)	
	1	Japan	CTG→TTG	(L653L)	0	
	1	Japan	GCC→ACC	A65T*	0	
	1	Japan	GCG→CTG	A245V*	0	
	1	Poland	CAG→CCG	Q295P*	100	
	1	Poland	GGC→GTC	G297V*	100	
	4	Japan	AGC→ACC	S315T	100	
	2	Japan	AGC→AAC	S315N	100	
	3	Poland	AGC→ACC	S315T	100	
	1	Japan	GTC→GCC	V725A*	0	
	63	Japan	CGG→CTG	R463L	3.2 (2)	
	2	Japan	CGG→CTG	R463L	100	−15C→T upstream of *mabA*
	1	Poland	ACC→CCC	T324P*	100	−15C→T upstream of *mabA*
	7	Japan	AGC→ACC and CGG→CTG	S315T and R463L	100	
	2	Japan	TTG→TTC and CGG→CTG	L141F* and R463L	100	
	1	Japan	ATG→ACG and CGG→CTG	M257T* and R463L	100	−5T→A* upstream of *mabA*
	1	Japan	CTG→CAG and CGG→CTG	L48Q* and R463L	100	−15C→T upstream of *mabA*
	1	Japan	ATG→ACG, CGG→CTG, and GTC→GCC	M257T*, R463L and V708P*	100	−15C→T upstream of *mabA*
	2	Japan	CTA insertion at position 1170	L390 insertion*	100	
mabA-inhA operon[c]	97	Japan	None	None	0	
	29	Poland	None	None	3.4 (1)	
	2	Japan	−15C→T upstream of *mabA*	None	100	R463L in *katG*
	1	Poland	−15C→T upstream of *mabA*	None	100	T324P* in *katG*
	1	Japan	−15C→T upstream of *mabA*	None	100	L48Q* and R463L in *katG*
	1	Japan	−15C→T upstream of *mabA*	None	100	M257T*, R463L and V708P* in *katG*
	1	Japan	−15C→T upstream of *mabA*	None	100	
	4	Poland	−15C→T upstream of *mabA*	None	100	
	1	Japan	−8T→A upstream of *mabA*	None	100	
	1	Japan	−5T→A* upstream of *mabA*	None	100	M257T* and R463L in *katG*
embB	77	Japan	None	None	0	
	30	Poland	None	None	3.3 (1)	
	2	Japan	GAC→GAT	(D345D)	100 (2)	
	1	Japan	GAC→GAT	(D534D)	0	
	5	Japan	ATG→GTG	M306V	100	
	1	Japan	ATG→ATT	M306I	100	
	1	Japan	ATG→ATC	M306I	100	
	2	Poland	ATG→ATA	M306I	100	
	2	Japan	GAC→GCC	D354A*	100	

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