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# Detection of Multidrug Resistance in *Mycobacterium tuberculosis*[∇]

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**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.

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

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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.
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[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 $A_{240}$ ($\varepsilon_{240}$ = 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*

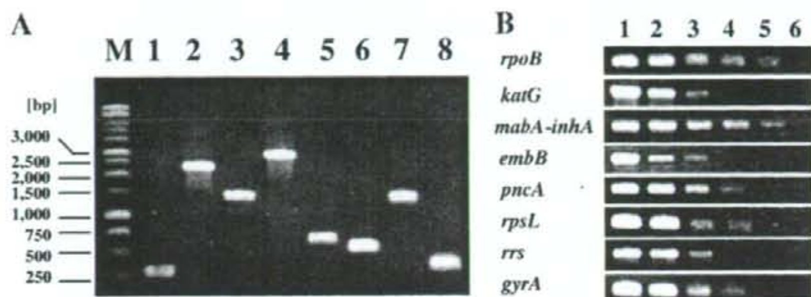


FIG. 1. (A) Amplification products from two-temperature PCR of *M. tuberculosis* H37Rv. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide. Lane M, 1-kbp ladder as a molecular size marker; lane 1, *rpoB*; lane 2, *katG*; lane 3, *mabA-inhA* locus; lane 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 | |

*Continued on following page*

TABLE 4—*Continued*

| Gene | No. of isolates ($n$ = 138) | Isolate origin | Changes | | % Resistant (no. of isolates displaying resistance) | Other mutations |
|---|---|---|---|---|---|---|
| | | | Nucleotide | Amino acid (silent mutation) | | |
| | 4 | Japan | GAG→GCG | E378A | 0 | |
| | 1 | Japan | GTG→TTG | V492L* | 0 | |
| | 1 | Poland | CAG→CGG | Q497R | 100 | |
| | 2 | Japan | GCC→ACC | A680T* | 0 | |
| | 3 | Japan | GCC→GTC | A1007V* | 0 | |
| | 1 | Japan | GAC→AAC | D1024N | 0 | |
| | 2 | Japan | CTG→CTA and GAG→GCG | (L355L) and E378A | 0 | |
| | 1 | Japan | AAT→TAT and ATG→ATA | N296Y* and M306I | 100 | |
| | 2 | Japan | ATG→CTG, GAG→GCG, and CCC→CCA | M306L, E378A, and (P1075P) | 100 | |
| *pncA* | 89 | Japan | None | None | 0 | |
| | 30 | Poland | None | None | 0 | |
| | 2 | Japan | TCC→TCT | (S65S) | 0 | |
| | 1 | Japan | GCG→GAG | A3E* | 100 | |
| | 1 | Poland | CAG→CCG | Q10P | 100 | |
| | 1 | Japan | GAC→GCC | D12A | 100 | |
| | 2 | Japan | CAC→CAA | H51Q | 100 | |
| | 1 | Poland | CAC→CAG | H51Q | 100 | |
| | 3 | Japan | CCG→CTG | P54L* | 100 | |
| | 1 | Japan | TGC→TGG | C72W* | 100 | |
| | 2 | Japan | GGT→AGT | G132S | 100 | |
| | 2 | Japan | ATT→ACT | I133T | 100 | |
| | 1 | Poland | CGC→AGC | R148S | 100 | |
| | 1 | Japan | ATG→GTG | M175V* | 100 | |
| | 1 | Japan | GAC→AAC | D53N* | 100 | |
| *gyrA* | 7 | Japan | GAG→CAG | E21Q* | 0 | |
| | 11 | Poland | GAG→CAG | E21Q* | 0 | |
| | 95 | Japan | GAG→CAG and AGC→ACC | E21Q* and S95T | 0 | |
| | 22 | Poland | GAG→CAG and AGC→ACC | E21Q* and S95T | 0 | |
| | 1 | Japan | GAG→CAG, GAC→GGC, and AGC→ACC | E21Q*, D94G, and S95T | 100 | |
| | 1 | Japan | GAG→CAG, GCG→GTG, and AGC→ACC | E21Q*, A90V, and S95T | 100 | |
| | 1 | Japan | GAG→CAG, GCG→GTG, GAC→GCC, and AGC→ACC | E21Q*, A90V, D94A, and S95T | 100 | |
| *rpsL* | 87 | Japan | AAA→AAG | (K121K) | 11.5 (10) | |
| | 33 | Poland | AAA→AAG | (K121K) | 6.1 (2) | |
| | 2 | Japan | AAA→AAG | (K121K) | 100 | 516C→T in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,061C insertion* in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,400A→G in *rrs* |
| | 1 | Japan | AAA→AAG | (K121K) | 100 | 1,400A→G and 1539A→G in *rrs* |
| | 13 | Japan | AAG→AGG and AAA→AAG | K43R and (K121K) | 100 | |
| *rrs* | 33 | Poland | None | | 6.1 (2) | (K121K) in *rpsL* |
| | 87 | Japan | None | | 11.5 (10) | (K121K) in *rpsL* |
| | 13 | Japan | None | | 100 | K43R and (K121K) in *rpsL* |
| | 2 | Japan | 516C→T | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,061C insertion* | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,400A→G | | 100 | (K121K) in *rpsL* |
| | 1 | Japan | 1,400A→G and 1539A→G | | 100 | (K121K) in *rpsL* |

[a] *, mutation not previously reported.

[b] The codon numbering system of RpoB initially described by Telenti et al. (31) was used. The codon numbers of RpoB are designated on the basis of alignment of translated *E. coli rpoB* sequence with a portion of translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons.

[c] Nucleotide numbering based on nucleotide position relative to *mabA* start codon.

and H37Ra were positive for PZase activity (data not shown). The remaining 17 *M. tuberculosis* clinical isolates and *M. bovis* BCG were negative for PZase activity. All PZase-positive bacilli were sensitive to PZA, and all PZase-negative bacilli were resistant to PZA. These data were consistent with previously published results (15, 39).

**(v) STR resistance and *rpsL* and *rrs*.** STR resistance is related to mutations in *rpsL* and *rrs* (17, 19, 39). In the present

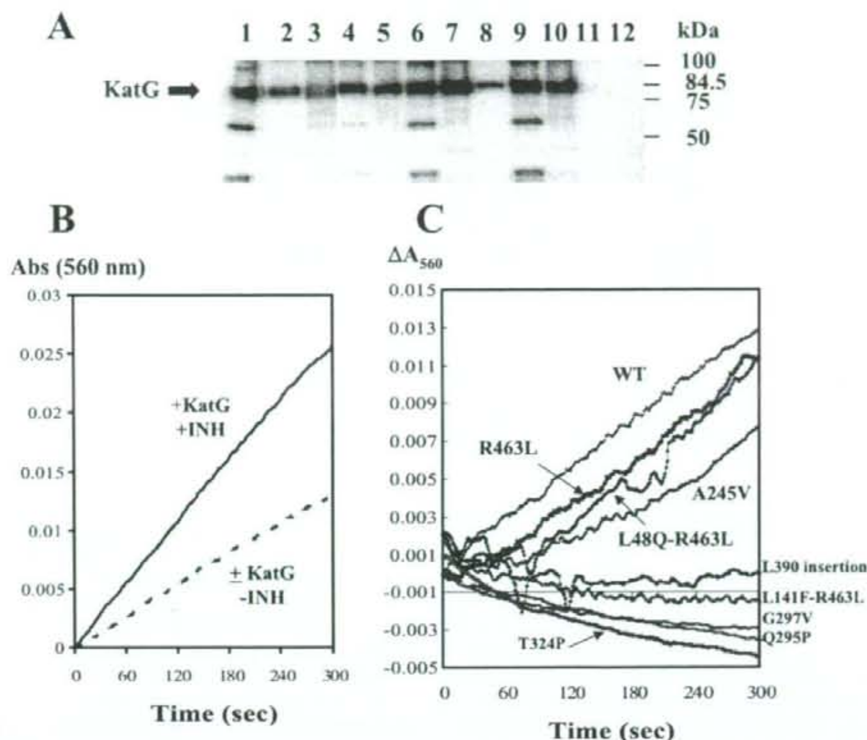


FIG. 2. (A) Western blot analysis of whole-cell protein extracts from KatG-deficient *E. coli* strain UM262 complemented with a control plasmid, pCRT7/NT, or recombinant plasmids expressing various KatG mutants. Lane 1, wild-type KatG; lane 2, $KatG^{Q295P}$; lane 3, $KatG^{T324P}$; lane 4, $KatG^{L48Q\text{-}R463L}$; lane 5, $KatG^{R463L}$; lane 6, $KatG^{G297V}$; lane 7, $KatG^{A245V}$; lane 8, $KatG^{L390\ insertion}$; lane 9, $KatG^{L141F\text{-}R463L}$; lane 10, purified KatG protein; lane 11, control plasmid pCRT7/NT; and lane 12, *E. coli* strain UM262. The positions of molecular mass markers are shown. (B) Time course of NBT reduction with and without the addition of 2.4 mM INH with wild-type KatG. Abs, absorbance. (C) Time course of net INH-dependent NBT reduction by wild-type KatG (WT) and eight KatG mutants. For each KatG sample tested, NBT reduction in the absence of INH was subtracted from that in the presence of INH to obtain the net INH-dependent NBT reduction over time. The concentration of KatG was determined by enzyme-linked immunosorbent assay.

study, all isolates, regardless of STR resistance status, had a silent mutation (K121K) in *rpsL*, and, therefore, the K121K mutation is not associated with STR resistance.

We found a novel insertional mutation in *rrs*. The insertion is the likely cause of STR resistance, because the isolate with the mutation was resistant to STR.

**(vi) FQ resistance and *gyrA*.** Mutations in the FQ resistance-determining region (QRDR) in *gyrA* are responsible for resistance in at least 96% of FQ-resistant *M. tuberculosis* isolates (17, 30, 39). In the present study, we found one novel mutation, E21Q, in *gyrA*, and all isolates tested, except the H37Rv strain, contained this mutation. However, some isolates were susceptible to FQs and others were resistant. Therefore, it is not clear that this mutation is associated with resistance to FQs. E21Q is located upstream of the QRDR.

**Catalase and INH oxidation activities of recombinant KatG mutants.** KatG, catalase-peroxidase, converts INH to its biologically active form (38). Some mutations in *katG* reduce or eliminate the enzymatic activity that is associated with INH resistance (9, 39). To measure catalase and INH oxidation activities, we expressed wild-type KatG and the A245V, Q295P, G297V, T324P, L48Q-R463L, L141F-R463L, R463L, and L390 insertion mutants of KatG in *katG*-deficient *E. coli* (Fig. 2A and Table 5). The catalase activities of these mutants were determined at various $H_2O_2$ concentrations. The $k_{cat}$, $K_m$, and $k_{cat}/K_m$ ratio values are shown in Table 5. Catalase activity was not detected for the $KatG^{Q295P}$, $KatG^{G297V}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, or $KatG^{L390\ insertion}$ mutants. The $k_{cat}$ of the $KatG^{L48Q\text{-}R463L}$ mutant was 26% lower than that of wild-type KatG. In contrast, the $KatG^{A245V}$ and $KatG^{R463L}$ mutants showed activities similar to that of wild-type KatG.

The INH oxidation activities of the mutants were determined in the presence of $H_2O_2$ by monitoring the free radical generation in the NBT reduction reaction. When wild-type KatG was tested in this assay, there was a significant background activity of NBT reduction in the absence of INH, whereas the NBT reduction was increased significantly in the presence of INH (Fig. 2B). We subtracted the background activity to obtain the net INH oxidation/NBT reduction. The net values are shown in Fig. 2C. The $KatG^{Q295P}$, $KatG^{G297V}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, and $KatG^{L390\ insertion}$ mutants did not show enhanced activity, whereas wild-type KatG and

TABLE 5. Catalase activity of wild-type and mutant KatG, KatG-mediated INH-converting activity, and INH susceptibility

| Recombinant KatG[a] | Catalase activity | | | KatG-mediated INH-converting activity[b] | INH susceptibility[c] |
|---|---|---|---|---|---|
| | $k_{cat}$ ($S^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ratio (mutant/wild type) | | |
| Wild type | 2,403 ± 440 | 60.1 ± 9.5 | 1.00 | + | S |
| A245V* | 2,666 ± 530 | 62.8 ± 10.8 | 1.06 | + | S |
| Q295P* | ND[d] | ND | NA[e] | − | R |
| G297V* | 188 ± 36 | 68.7 ± 7.0 | 0.07 | − | R |
| T324P* | ND | ND | NA | − | R |
| L48Q*-R463L | 1,776 ± 310 | 64.8 ± 12.5 | 0.69 | + | R |
| L141F*-R463L | ND | ND | NA | − | R |
| R463L | 2543 ± 450 | 64.0 ± 11.0 | 1.01 | + | S |
| L390 (CTA) insertion* | ND | ND | NA | − | R |

[a] *, novel mutation.
[b] Time courses of net KatG-mediated INH conversion are shown in Fig. 2C.
[c] INH susceptibilities shown are those of *M. tuberculosis* H37Rv strains having the *katG* gene of the wild type and those of clinical strains having the *katG* gene with the respective mutation(s). S, susceptible; R, resistant.
[d] ND, not detected.
[e] NA, not applicable.

the $KatG^{L48Q-R463L}$, $KatG^{A245V}$, and $KatG^{R463L}$ mutants of KatG showed significant enhancement of activity in the presence of INH.

Collectively, these results for the enzymatic activities of KatG mutants indicate that the Q295P, G297V, T324P, L141F, and L390 insertion mutants cause loss of enzymatic activity, whereas the A245V and R463L mutants have no effect on the enzymatic activity. The L48Q mutation has little effect on enzymatic activity; however, there was no isolate that carried only the L48Q mutation in KatG in the present study. These mutations and enzymatic activities, except for those of the L48Q-R463L mutant, correlated well with INH susceptibility (Table 5). The L48Q-R463L mutant also carried the −15C→T transition upstream of *mabA* (Table 4). Therefore, the INH resistance of this mutant is likely due to the −15C→T mutation, which is known to be related to INH resistance (10, 22, 39).

**Sequencing of *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG.** PCR products amplified from *rpoB*, *katG*, *mabA-inhA*, *embB*, *pncA*, *rpsL*, *rrs*, and *gyrA* of *M. bovis* BCG were sequenced with the same sequencing primers as those for *M. tuberculosis* (Table 2). When the nucleotide sequences of BCG were compared with those of *M. bovis* AF2122/97 (GenBank accession no. NC_002945) (8), the sequences were identical. When the sequences of *M. bovis* BCG were compared with those of *M. tuberculosis* H37Rv, the sequences of *rpoB*, the promoter region of the *mabA-inhA* operon, and *rrs* were identical. The R463L (CGG→CTG at nt positions 1387 to 1389) and silent P29P (CCC→CCA at nt positions 85 to 87) mutations were found in *katG* of BCG. E378A (GAG→GCG at nt positions 1159 to 1161) in *embB*, H57D (CAC→GAC at nt positions 169 to 171) in *pncA*, K121K (AAA→AAG at nt positions 361 to 363 [silent]) in *rpsL*, and S95T (AGC→ACC at nt positions 283 to 285) in *gyrA* were identified in *M. bovis* BCG.

**Correlation between drug susceptibility and mutations in BCG.** We next compared the sequences of BCG and *M. tuberculosis* H37Rv and found four mutations, R463L in *katG*, E378A in *embB*, H57D in *pncA*, and S95T in *gyrA*, that caused amino acid substitutions in *M. bovis* BCG. R463L in *katG* is known not to be associated with INH resistance in *M. tuberculosis* (33) and may not be associated with INH resistance in *M. bovis*. E378A in *embB* is not associated with EMB resistance in *M. tuberculosis* (32, 39). H57D in *pncA* was reported previously and is characteristic of PZA resistance in *M. bovis* (39). S95T in *gyrA* is not associated with FQ resistance in *M. tuberculosis* (39). Therefore, in *M. bovis*, mutations except H57D in *pncA* may be polymorphisms not associated with drug resistance.

**Detection and sequencing of drug resistance-related genes of *M. tuberculosis* in sputa from tubercular patients.** We tested a total of 10 sputa from 10 tuberculosis-diagnosed patients. These patients had been received treatment with antitubercular drugs. Of these samples, six were positive for acid-fast bacilli (AFB; >101/field in two samples, 26 to 50/field in two samples, 1/field in one sample, and 1/several fields in one sample) under microscopic observation, and four were negative. Five of the six samples that were positive for AFB were positive for all eight genes tested by PCR. One sample which was positive for AFB (one/several fields) was positive for five genes (*rpoB*, *pncA*, *rpsL*, *rrs*, and *gyrA*) by PCR. The four AFB-negative samples yielded no PCR products and were negative by culture, suggesting that tuberculosis was not active in these patients who had received treatment.

PCR products (a total of 45) were subjected to DNA sequencing. No mutations were identified in 38 of the PCR products. The remaining seven PCR products contained nine mutations. The seven PCR products were obtained from one sputum sample. The sputum sample was cultured, and after several weeks, an isolate of *M. tuberculosis* was obtained and analyzed. We conducted PCR analysis of this isolate and detected the same nine mutations. This isolate was resistant to RIF, INH, EMB, STR, FQs, and PZA. These results indicate that our DNA sequencing-based method can be used to detect MDR strains of *M. tuberculosis* in sputa obtained from clinical tuberculosis patients.

## DISCUSSION

Our novel DNA sequencing-based method described here is useful for detection and diagnosis of drug-resistant strains of *M. tuberculosis*, especially MDR strains. Our method has

several advantages. First, it allows simultaneous detection of mutations in eight genes associated with resistance to six antituberculosis drugs. Second, the entire assay from DNA extraction to the DNA sequencing can be completed within 1 working day. Third, this method is sensitive enough to detect 1 ng of genomic DNA (i.e., $3 \times 10^5$ *M. tuberculosis* cells). We found that this method worked well even in positive sputum containing few bacilli, as determined by acid-fast staining. Fourth, our DNA sequencing-based method allows detection of both novel and known mutations. In the present study, we identified 25 novel mutations by PCR-based analysis.

This strategy does have a few disadvantages. The DNA sequencer and sequencing are costly, and the procedure is somewhat complicated. However, this issue may be addressed if DNA sequencing costs are reduced by new sequencing methods and equipment. Also, this strategy may not be able to detect very low numbers of bacilli in sputa. We did not have the opportunity to test AFB smear-negative but culture-positive samples. Among the smear- and culture-positive samples we tested, one sample with small numbers of bacilli (one/several fields) was negative for three of the eight genes tested, indicating that the sensitivity of this method is limited. However, the sensitivity and accuracy of our method are comparable to those of traditional drug susceptibility tests, and this is sufficient for use in the clinical setting.

The method described here was excellent for diagnosis of RIF-resistant *M. tuberculosis* isolates with 100% specificity, sensitivity, and test efficiency. RIF interferes with the synthesis of mRNA by binding to the β-subunit of RNA polymerase (RpoB) in bacterial cells (39). The RIF-binding site is a pocket in the upper wall of the main channel for double-stranded DNA entry just upstream of the polymerase catalytic center. The various RIF-resistant mutations are clustered around this pocket (39). Mutations in *rpoB* have been found in 95% to 100% of clinical RIF-resistant isolates of *M. tuberculosis* (39). Most of the mutations found in the 28 RIF-resistant isolates tested here were located between nucleotides 1276 and 1356 (codons 507 to 533) of *rpoB*, which is the 81-bp core region of this gene (Table 4) (31, 39). Two other mutations, V146F and E562A, were located outside of the 81-bp core region. Isolates with V146F were reported to show low-level resistance to RIF (MIC, ≤4 μg/ml) (39). It is not known whether the E562A mutation is involved in resistance because the isolate with this mutation also had another mutation in the 81-bp core region (39). The V146F mutation could not be detected by the DNA sequencing method described here. Although we were able to detect the E562A mutation by our sequencing method, we did not find this mutation in any of 138 isolates tested in the present study.

Our sequencing method is applicable for diagnosis of INH-resistant isolates with 89.5% sensitivity, 100% specificity, and 97.1% test efficiency (Table 6). The sensitivity of the two-temperature PCR for *katG* was lower than that of the PCR for *rpoB* or *mabA-inhA* (Fig. 1B), and, therefore, we will need to increase the sensitivity to detect *katG* mutations to assess INH resistance. The mode of INH action is one of the most complicated among all antibiotics. INH is a prodrug that requires activation of the bacterial catalase-peroxidase enzyme (KatG) (38) to generate a range of reactive radicals, which then affect multiple systems, including cell wall mycolic acid synthesis and lipid peroxidation and NAD metabolism, and cause DNA damage (39). Deficient efflux of INH radicals and defective antioxidative defenses may underlie the susceptibility of *M. tuberculosis* to INH (39). Mutations in *katG* are among the most frequently detected mutations in INH-resistant clinical isolates. Mutations in *inhA* and its promoter region, which is located upstream of the *mabA-inhA* operon, are also common (16, 17, 39). Our sequencing method should identify a majority of INH-resistant isolates. We are able to detect mutations in *katG* and the region upstream of *mabA* in 90% (34/38) of INH-resistant isolates. Ten different mutations (L48Q, L141F, M257T, Q295P, G297V, S315T, S315N, T324P, R463L, and V708P) were detected in *katG* of INH-resistant isolates, and 3 mutations (A65T, A245V, and V725A) were identified in INH-susceptible isolates. The L48Q, A65T, L141F, A245V, M257T, Q295P, G297V, T324P, V708P, V725A, and L390 insertion mutations are novel (Table 4).

To date, several mutations in *katG* in MDR isolates have been reported (9, 16, 17, 38, 39). Rouse et al. (24) reported previously that codons 104 and 108 encode amino acids located near the catalytic site of KatG and that the residues encoded by codons 270, 275, and 315 participate in binding the heme group

TABLE 6. The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing[a]

| Drug susceptibility test result[b] | No. of isolates (*n* = 138) | | % Sensitivity[c] | % Specificity[d] | % Test efficiency[e] |
|---|---|---|---|---|---|
| | Mutation positive | Mutation negative | | | |
| RIF | | | | | |
| Resistant | 28 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 110 | | | |
| INH | | | | | |
| Resistant | 34 | 4 | 89.5 | 100 | 97.1 |
| Susceptible | 0 | 100 | | | |
| EMB | | | | | |
| Resistant | 15 | 3 | 83.3 | 100 | 97.8 |
| Susceptible | 0 | 120 | | | |
| PZA | | | | | |
| Resistant | 17 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 121 | | | |
| STR | | | | | |
| Resistant | 18 | 12 | 60.0 | 100 | 91.3 |
| Susceptible | 0 | 108 | | | |
| OFX | | | | | |
| Resistant | 3 | 0 | 100 | 100 | 100 |
| Susceptible | 0 | 135 | | | |

[a] The diagnostic performance of the DNA sequencing-based method in comparison with drug susceptibility testing was determined after resolution of polymorphisms.

[b] Drug susceptibility for antituberculosis agents except for PZA was determined by the agar proportion method according to NCCLS (now CLSI) guidelines, and that for PZA was determined by PZase activity.

[c] Sensitivity: no. of drug-resistant isolates with mutations/(no. of drug-resistant isolates with mutations + no. of drug-resistant isolates without mutations).

[d] Specificity: no. of drug-susceptible isolates without mutations/(no. of drug-susceptible isolates with mutations + no. of drug-susceptible isolates without mutations).

[e] Test efficiency: (no. of drug-resistant isolates with mutations + no. of drug-susceptible isolates without mutations)/no. of all isolates tested.

of KatG (24). Mutations in these regions, therefore, are thought to cause loss of KatG enzymatic function (24). Yu et al. (36) reported that residue W321 of KatG was important for substrate binding and that residue Y229 was critical to protect the catalase activity of KatG (37). We compared the catalase and INH oxidation activities of eight KatG mutants identified in this study with those of the wild type. Although we were able to express all of the KatG mutants (Fig. 2A), we were unable to detect the catalase and INH oxidation activities of four KatG mutants, $KatG^{Q295P}$, $KatG^{T324P}$, $KatG^{L141F\text{-}R463L}$, and $KatG^{L390\ insertion}$ (Table 5 and Fig. 2C). The specific effects of these mutations on KatG function need to be analyzed further. The lack of activity or lower activity of these mutants, however, correlated quite well with the INH-resistant phenotype of their respective *M. tuberculosis* isolates. The enzymatic activity of $KatG^{L48Q\text{-}R463L}$ was not correlated with INH susceptibility (Table 5), and this mutation had little effect on the measured activities (Table 5 and Fig. 2C). The isolate carrying the L48Q-R463L mutations also had the −15C→T transition upstream of *mabA* (Table 4), which is known to be associated with INH resistance (10, 22, 39). The $KatG^{A245V}$ and $KatG^{R463L}$ mutants showed activities similar to those of wild-type KatG, and these results are consistent with the INH-susceptibility phenotypes of their respective isolates. Therefore, we concluded that the L48Q, A245V, and R463L mutations are merely polymorphisms that do not influence INH resistance.

We found mutations in the region upstream of *mabA* or in the regulatory region of the *mabA-inhA* operon in 12 of 38 INH-resistant isolates. Five of these isolates had no other mutations within *katG* (Table 4). Our present results support those of Morris et al. (16), who examined the *inhA* locus for sequence polymorphisms by single-strand conformation polymorphism analysis and DNA sequencing of 42 INH-resistant isolates. They found no alterations in the coding portion of *inhA*, but five isolates had mutations in the regulatory region of the *mabA-inhA* operon (16).

Mutations in *kasA*, which encodes β-ketoacyl ACP synthase (11), and *ndh*, which encodes NADH dehydrogenase (12), have been found in a small proportion of clinical isolates, and we plan to modify our sequencing method to analyze *ndh* and *kasA*.

Our method was sufficient for diagnosis of EMB-resistant isolates, although a limited portion (80%) of *embB* was sequenced. EMB inhibits polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (39). Three homologues of arabinosyltransferases, EmbC, EmbA, and EmbB, have been proposed to be the targets of EMB (32, 39). Mutations in *embB* are found in 47% to 69% of EMB-resistant isolates of *M. tuberculosis* (28, 32). Most EMB-resistant isolates with *embB* mutations exhibited high-level resistance (2, 27). The 35% of EMB-resistant isolates that do not have *embB* mutations showed decreased resistance to EMB (2). We were able to detect a majority (15/18 isolates) of EMB-resistant isolates with our sequencing-based analysis. In addition, we identified two novel mutations, D354A and N296Y, in EMB-resistant isolates in the present study.

*pncA* is known to be associated with PZA resistance (17, 39). In the present study, we sequenced the complete open reading frame of *pncA* and its promoter region. PZA enters the organism through passive diffusion and is converted to pyrazinoic acid by cytoplasmic PZase. Despite recent progress, the targets of pyrazinoic acid are still not known (39). All PZA-resistant *M. tuberculosis* isolates tested in the present study contained at least one mutation within *pncA* and showed no PZase activity (Table 4). Our results are consistent with those of previous studies that showed 72% to 95% of PZA-resistant clinical isolates of *M. tuberculosis* carried *pncA* mutations (25). All of the *pncA* mutations identified in the present study of PZA-resistant isolates caused amino acid substitutions. Among these mutations, 5A3E, D53N, P54L, C72W, and M175V were novel. The *pncA* mutations were highly diverse and scattered across the gene.

STR, an aminoglycoside, inhibits initiation of mRNA translation. The site of action is the small 30S subunit of the ribosome, especially ribosomal protein S12 and the 16S rRNA (17). *M. tuberculosis* becomes resistant when targets of STR in the ribosomes are mutated. The principal site of mutation is the *rpsL* gene, which encodes ribosomal protein S12 (6, 19, 27). The most frequently observed mutation in *rpsL* was K43R. In the present study, 13 of 30 STR-resistant isolates tested had the K43R mutation. Mutation of the *rrs* gene is also associated with STR resistance in *M. tuberculosis*. *M. tuberculosis* has only a single copy of the *rrs* gene, which encodes the 16S rRNA. Thus, the loops of 16S rRNA that interact with the S12 protein constitute an easily selected mutation site. Such *rrs* mutations are clustered in the highly conserved 530 loop and in the adjacent 915 region (6). In addition, a 1400A→G mutation of *rrs* was identified in both amikacin- and kanamycin-resistant clinical isolates of *M. tuberculosis* (1, 29). These isolates were resistant to STR, indicating that this mutation may contribute to STR resistance (1, 29). In the present study, one STR-resistant isolate had two mutations, 1400A→G and 1539A→G. Because the STR resistance of the isolate can be explained by the 1400A→G mutation, it is unclear whether the 1539A→G mutation is associated with STR resistance.

FQs are active in vitro against *M. tuberculosis* isolates (5) and are increasingly being used in combination with other agents to treat tuberculosis. The principal mechanism of resistance to FQs identified in other bacterial species is alteration of the target proteins DNA gyrase and topoisomerase IV. DNA gyrase is composed of two A and two B subunits, which are encoded by *gyrA* and *gyrB*, respectively (39). Mutations in *gyrA* are associated with high-level resistance of *M. tuberculosis* to FQs (39). *gyrB* mutations associated with resistance have only been identified in laboratory mutants of *M. tuberculosis* (39). Mutations associated with FQ resistance occur within a relatively restricted region of *gyrA*. We identified three mutations, A90V, D94GA, and D94G, in FQ-resistant isolates. We also identified a polymorphism, S95T, that is not associated with FQ resistance. The G88C, D89G, S91P, and D94A, -N, -H, or -Y mutations in *gyrA* have also been found in FQ-resistant isolates (4, 30). These mutations are presumed to be located in the FQ-binding region (4, 30).

Some researchers have described mutations that caused amino acid substitutions but not drug resistance (30, 33, 39). In the present study, we identified several novel mutations that cause amino acid substitutions but do not confer drug resistance. Except for these mutations and silent mutations, the drug resistance profiles of the isolates tested correlated quite

well with the various mutations that we identified (Table 6). The sensitivities of the DNA sequencing-based method (i.e., the ability to detect true drug resistance) were 100%, 89.5%, 83.3%, 100%, 60%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. The specificities (i.e., the ability to detect true drug susceptibility) were 100% for all drugs tested. The test efficiencies (i.e., the ability to give the correct answer in all samples tested) were 100%, 97.1%, 97.8%, 100%, 91.3%, and 100% for the RIF-, INH-, EMB-, PZA-, STR-, and OFX-resistant strains, respectively. These results indicate that our DNA sequencing-based method is effective for detection of MDR strains. However, when novel mutations in drug resistance-related genes are detected by our method, it is essential to also perform drug susceptibility testing, because novel mutations are not necessarily associated with drug resistance. Of the 25 novel mutations we detected, we cloned 7 novel mutations in KatG. Significant information could be gained if all novel mutations were cloned. For practical purposes, it would be helpful to know the phenotypic manifestations of specific mutations.

In conclusion, we have shown the usefulness of our DNA sequencing strategy for drug susceptibility screening of various targets. Most MDR *M. tuberculosis* strains, which are defined as those strains resistant to both RIF and INH, are resistant to other antitubercular drugs. Our new sequencing-based method can rapidly and efficiently assess MDR of *M. tuberculosis*. The method can also be used to detect MDR *M. tuberculosis* in sputa from patients. Further studies will focus on the clinical application of this method for diagnosis of drug-resistant *M. tuberculosis*.

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# Development and Evaluation of a Line Probe Assay for Rapid Identification of *pncA* Mutations in Pyrazinamide-Resistant *Mycobacterium tuberculosis* Strains[▽]

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**Resistance of *Mycobacterium tuberculosis* to pyrazinamide (PZA) derives mainly from mutations in the *pncA* gene. We developed a reverse hybridization-based line probe assay with oligonucleotide probes designed to detect mutations in *pncA*. The detection of PZA resistance was evaluated in 258 clinical isolates of *M. tuberculosis*. The sensitivity and specificity of PZA resistance obtained by this new assay were both 100%, consistent with the results of conventional PZA susceptibility testing. This assay can be used with sputa from tuberculosis patients. It appears to be reliable and widely applicable and, given its simplicity and rapid performance, will be a valuable tool for diagnostic use.**

Pyrazinamide (PZA) is an important first-line antituberculosis drug used clinically for short-course chemotherapy because of its effectiveness against semidormant bacilli sequestered within macrophages (6, 10). The intracellular sterilizing activity of PZA allows the treatment period to be reduced to 6 months, whereas 9 months of treatment is required when PZA is not used (19). PZA is a prodrug. It requires conversion to pyrazinoic acid by bacterial pyrazinamidase (PZase) to affect tuberculosis bacilli (7, 17). Recent reports have established that mutations in the PZase gene (*pncA*) lead to the loss of PZase activity and constitute the primary mechanism of PZA resistance in *Mycobacterium tuberculosis* (11, 21, 22).

The time required for in vitro drug susceptibility testing of *M. tuberculosis* is constrained by the organism's relatively slow growth. Conventional drug susceptibility testing takes 7 to 28 days, depending on the culture system used (15). For most antituberculosis drugs, conventional methods produce reliable results, although PZA susceptibility testing with such methods is impaired by the poor bacterial growth under acidic conditions (7, 9). However, new culture methods were developed recently to resolve this problem (2, 13).

Previously, we described a DNA sequencing-based method to detect mutations in the genome of drug-resistant strains, including PZA-resistant *M. tuberculosis* (18). However, the use of this method in ordinary-scale clinical laboratories can be difficult. Therefore, we developed and describe here a new hybridization-based line probe assay (LiPA) for the rapid detection of *pncA* mutations in *M. tuberculosis* that is easily applied to clinical use. This assay can be used to evaluate PZA resistance, particularly in multidrug-resistant organisms, analyze PZA-resistant genes, and identify epidemic strains.

## MATERIALS AND METHODS

**Bacterial strains.** Two hundred twenty-five clinical isolates of *M. tuberculosis* were obtained from patients with pulmonary tuberculosis in Japan, and 33 were obtained from patients in Poland. The other bacterial strains used in this study are listed in Table 1.

**Clinical samples.** Fifty-three sputum samples were collected from patients with tuberculosis or suspected tuberculosis. These samples were treated with an *N*-acetyl-L-cysteine–NaOH solution according to the procedure provided with the BBL MycoPrep Mycobacterial System Digestion/Decontamination kit (BD Diagnostic Systems, Franklin Lakes, NJ). Each sample was suspended in 1.5 ml of phosphate buffer. One milliliter of the suspension was transferred into a 1.5-ml tube. The remaining suspension was inoculated onto Ogawa medium and into MGIT 960 broth (BD BACTEC MGIT 960; BD Biosciences) and cultured for mycobacterial examination. One milliliter of the suspension was centrifuged for 15 min at 13,000 × *g*, and the supernatant was removed with a pipette. Tris-EDTA (TE) buffer (100 μl) was added to the sediment, and the solution was again centrifuged for 15 min at 13,000 × *g*. The sediment was suspended in 50 μl of TE buffer (50 μl), resuspended by vortexing, and incubated at 95°C for 30 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 into another 1.5-ml tube. Each aliquot of the supernatant (5 μl) was used for each of the LiPA or Cobas Amplicor assays (Roche Diagnostic Systems, Basel, Switzerland).

**PZA susceptibility testing and assay for PZase activity.** All clinical isolates of *M. tuberculosis* and *M. tuberculosis* strains H37Rv and H37Ra were tested for PZA susceptibility. Susceptibility to PZA was assessed by the broth method

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TABLE 1. Species specificity of the LiPA for detecting *M. tuberculosis pncA*

| Species | Strain[a] | Nested PCR result[b] | Hybridization signal with probes: | |
|---|---|---|---|---|
| | | | 1–24 | 25–47 |
| *M. tuberculosis* | H37Rv (ATCC 27294) | + | All positive | All positive |
| *M. tuberculosis* | H37Ra (ATCC 25177) | + | All positive | All positive |
| *M. bovis* | BCG Japanese strain 172[c] | + | Δ16[d] | All positive |
| *M. avium* | ATCC 25291 | −* | All negative | All negative |
| *M. fortuitum* | RIMD 1317004 (ATCC 6841) | −* | All negative | All negative |
| *M. gastri* | GTC 610 (ATCC 15754) | −* | All negative | All negative |
| *M. intracellulare* | JCM 6384 (ATCC 13950) | −* | All negative | All negative |
| *M. kansasii* | JCM 6379 (ATCC 124878) | − | All negative | All negative |
| *M. marinum* | GTC 616 (ATCC 927) | −* | All negative | All negative |
| *M. nonchromogenicum* | JCM 6364 (ATCC 19530) | −* | All negative | All negative |
| *M. phlei* | RIMD 1326001 (ATCC 19249) | − | All negative | All negative |
| *M. scrofulaceum* | JCM 6381 (ATCC 19981) | − | All negative | All negative |
| *M. simiae* | GTC 620 (ATCC 25275) | − | All negative | All negative |
| *M. smegmatis* | ATCC 19420 | − | All negative | All negative |
| *M. szulgai* | JCM 6383 (ATCC 35799) | − | All negative | All negative |
| *M. terrae* | GTC 623 (ATCC 15755) | − | All negative | All negative |
| *Escherichia coli* | ATCC 8739 | − | All negative | All negative |
| *Haemophilus influenzae* | IID 984 (ATCC 9334) | − | All negative | All negative |
| *Klebsiella pneumoniae* | IID5209 (ATCC 15380) | − | All negative | All negative |
| *Legionella pneumophila* | GTC 745 | − | All negative | All negative |
| *Mycoplasma pneumoniae* | IID 817 | − | All negative | All negative |
| *Pseudomonas aeruginosa* | ATCC 27853 | − | All negative | All negative |
| *Rhodococcus equi* | ATCC 33710 | − | All negative | All negative |
| *Staphylococcus aureus* | N315 | − | All negative | All negative |
| *Streptococcus pneumoniae* | GTC 261 | − | All negative | All negative |

[a] ATCC, American Type Culture Collection, Manassas, VA; RIMD, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; GTC, Gifu Type Culture Collection, Department of Microbiology-Bioinformatics, Regeneration and Advanced Medical Science, Gifu University, Graduate School of Medicine, Bacterial Genetic Resources, Gifu, Japan; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Saitama, Japan; IID, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

[b] Approximately 100 ng of genomic DNA was used in the first PCR. Amplification results were determined by agarose gel electrophoresis. +, presence of amplification product of the expected; −, absence of amplification products; −*, presence of amplification products, but the sizes of the products were different from that of *M. tuberculosis*.

[c] From Japan BCG Laboratory, Tokyo, Japan.

[d] Absence of hybridization signal with one of the probes (probe 16).

(MGIT 960). Nontuberculous *Mycobacterium* spp. were also tested for PZA susceptibility with the MGIT 960 method. However, standard methods for susceptibility testing are not available for nontuberculous *Mycobacterium* spp. (5). PZase activity was determined as described previously (20). In brief, a heavy loopful of mycobacterial culture freshly grown on Ogawa medium was inoculated onto 5 ml of Middlebrook 7H11 agar supplemented with pyrazinecarboxamide (0.812 mM; Wako Pure Chemical Industries, Osaka, Japan), sodium pyruvate (18.18 mM; Nacalai Tesque, Kyoto, Japan), and glycerol (0.5%, vol/vol; Nacalai) in a glass tube with a screw cap. After incubation at 37°C for 4 days, 1 ml of freshly prepared ferrous ammonium sulfate solution (25.5 mM; Sigma Chemical, St. Louis, MO) was added to each tube, and the presence of a pink band was assessed. *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.

**DNA extraction.** Two different methods were applied to extract genomic DNA. One method was described previously (12). The other method was performed as follows. Mycobacterial cells and other bacterial cells were collected from Ogawa medium and broth medium, respectively. A loopful of cells was suspended in 0.5 ml 1× TE buffer and inactivated at 100°C for 10 min. Cellular debris was pelleted at 13,000 × *g* for 5 min, and the supernatant with genomic DNA was used for PCR. Mycobacterial DNA in sputa was extracted with a cell lysis solution contained in a diagnosis kit (Amplicor respiratory specimen preparation kit; Roche Molecular Systems, Inc., Branchburg, NJ) or extracted by heating at 95°C for 30 min followed by freezing and thawing.

**Preparation of oligonucleotide probes and strips.** Forty-seven oligonucleotide probes were designed to cover the entire *pncA* gene of wild-type H37Rv (Fig. 1). Two oligonucleotide probes were designed to compensate for silent mutations of C to T at nucleotide positions 180 and 195. A total of 49 probes were synthesized. These probes were immobilized on two strips. One strip contained 24 probes (probes 1 to 24) plus two probes to compensate for silent mutations. The other contained 23 probes (probes 25 to 47).

**LiPA.** The LiPA described here was developed on the basis of the same principle as that of the commercially available INNO-LiPA Rif. TB kit (Innogenetics, Ghent, Belgium) for the detection of rifampin resistance (14). The LiPA was conducted as described previously (14). In brief, biotinylated PCR products from test samples were hybridized to the immobilized probes and washed under strict conditions (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] buffer containing 0.1% sodium dodecyl sulfate at 62°C). The presence or absence of bands on all strips was judged independently by three different observers. The classifications by the three observers were identical. Genomic DNA from the PZA-susceptible H37Rv strain was used as a positive control. Results for all samples were compared to those for the positive control. DNA from *M. tuberculosis* H37Rv was diluted into TE buffer (final concentrations, 4.84 pg/μl, 484 fg/μl, 242 fg/μl, 48.4 fg/μl, 24.2 fg/μl, and 2.42 fg/μl), and 1 μl of each solution was used to determine the sensitivity of the LiPA.

**PCR and DNA sequencing.** Unless otherwise indicated, approximately 2 to 5 ng of genomic DNA was used for the amplification of a 670-bp fragment that includes the complete open reading frame of the *pncA* gene. To increase the sensitivity, nested PCR was performed with unlabeled external primers PR9-1 (5'-GGC GTC ATG GAC CCT ATA TCT G-3') and PR10-1 (5'-CTT GCG GCG AGC GCT C-3') for the first PCR and biotin-labeled internal primers IP-F (5'-GCT GCG GTA GGC AAA CTG C-3') and IP-R (5'-CCA ACA GTT CAT CCC GGT TCG-3') for the second PCR. The amplification conditions for the first and second PCRs were the same and consisted of 5 min of denaturation at 95°C followed by 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. In some experiments, only the second PCR was done. Sequencing of *pncA* and its promoter region (nucleotides −80 to 572 relative to the initiation codon) was performed as described previously (18) for *M. tuberculosis* H37Rv and H37Ra, *Mycobacterium bovis* BCG, and all 258 clinical *M. tuberculosis* isolates tested regardless of the LiPA results.

**COBAS Amplicor assays.** COBAS Amplicor assays, including the Amplicor MTB test, Amplicor *M. avium* test, and Amplicor *M. intracellulare* test (Roche

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```
    external primer: PR9-1    internal primer: IP-F
    ggcgtcatggaccctatatctgtggctgccgctgcggtaggcaaactgcccgggcagtc

    probe 5
    probe 2    probe 4
    probe 1    probe 3
    gcccgaacgtatggtggacgtATGcgggcgttgatcatcgtcgacgtgcagaacgacttc
                         METArgAlaLeuIleIleValAspValGlnAsnAspPhe

    probe 7    probe 9
    probe 6    probe 8    probe 10
 40 tgcgagggtggctcgctggcggtaaccggtggcgccgcgctggcccgcgccatcagcgac
    CysGluGlyGlySerLeuAlaValThrGlyGlyAlaAlaLeuAlaArgAlaIleSerAsp

    probe 11    probe 13
    probe 12    probe 14
100 tacctggccgaagcggcggactaccatcacgtcgtggcaaccaaggacttccacatcgac
    TyrLeuAlaGluAlaAlaAspTyrHisHisValValAlaThrLysAspPheHisIleAsp

    probe 17 silent
    probe 15    probe 17
    probe 16 silent    probe 18
    probe 16    probe 19
160 ccgggtgaccacttctccggcacaccggactattcctcgtcgtggccaccgcattgcgtc
    ProGlyAspHisPheSerGlyThrProAspTyrSerSerSerTrpProProHisCysVal

    probe 20    probe 22    probe 24
    probe 21    probe 23
220 agcggtactccgggcgcggacttccatcccagtctggacacgtcggcaatcgaggcggtg
    SerGlyThrProGlyAlaAspPheHisProSerLeuAspThrSerAlaIleGluAlaVal

    probe 26    probe 28
    probe 25    probe 27
280 ttctacaagggtgcctacaccggagcgtacagcggcttcgaaggagtcgacgagaacggc
    PheTyrLysGlyAlaTyrThrGlyAlaTyrSerGlyPheGluGlyValAspGluAsnGly

    probe 30    probe 32
    probe 29    probe 31
340 acgccactgctgaattggctgcggcaacgcggcgtcgatgaggtcgatgtggtcggtatt
    ThrProLeuLeuAsnTrpLeuArgGlnArgGlyValAspGluValAspValValGlyIle

    probe 34    probe 36
    probe 33    probe 35    probe 37
400 gccaccgatcattgtgtgcgccagacggccgaggacgcggtacgcaatggcttggccacc
    Ala ThrAspHisCysValArgGlnThrAlaGluAspAlaValArgAsnGlyLeuAlaThr

    probe 39    probe 42
    probe 38    probe 40    probe 43
    probe 41    probe 44
460 agggtgctggtggacctgacagcgggtgtgtcggccgatacCaccgtcgccgcgctggag
    ArgValLeuValAspLeuThrAlaGlyValSerAlaAspThrThrValAlaAlaLeuGlu

    probe 45
    probe 46
    probe 47    internal
520 gagatgcgcaccgccagcgtcgagttggtttgcagctccTGAtggcaccgccgaaccggg
    GluMetArgThrAlaSerValGluLeuValCysSerSer***

    primer: IP-R    external primer: PR10-1
    atgaactgttggcggcggtggagcgctcgccgcaagcggccgccgcgcacgaccgcgcc
```

FIG. 1. Locations of 49 oligonucleotide probes designed to cover the *pncA* gene of *M. tuberculosis* H37Rv.

Diagnostic Systems), were performed according to the instructions provided by the manufacturer.

## RESULTS AND DISCUSSION

Among the 25 bacterial strains listed in Table 1, three strains, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and *M. bovis* BCG, yielded PCR products of the expected length (approximately 600 bp). The other 13 strains of nontuberculous *Mycobacterium* spp. and nine strains of nonmycobacterial spp. yielded products of different lengths or were not amplified to a level detectable in agarose gels (Table 1). In the LiPA, PCR products from *M. tuberculosis* strains hybridized with all of the *pncA* probes (Table 1). LiPA results for strain H37Rv are shown in Fig. 2 (lane 1). The *M. bovis* product hybridized with all probes except for probe 16 (Table 1 and Fig. 2, lane 22). All other bacteria tested showed no hybridization with any of the probes (data not shown). These data indicate that the LiPA is specific for *M. tuberculosis* and *M. bovis*.

Sensitivity of the LiPA with the nested PCR was 24.2 fg of *M. tuberculosis* DNA, which is equivalent to five copies of the *pncA* gene (data not shown), whereas when only the second PCR was done, the sensitivity of the LiPA was 484 fg, which is equivalent to 1,000 copies of *pncA* (data not shown). These data suggest that nested PCR is needed to yield the higher sensitivity.

Of 258 clinical isolates of *M. tuberculosis* tested with the LiPA, 228 were wild type, and the other 30 showed at least one mutation pattern (Table 2). Representative LiPA patterns are

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FIG. 2. Representative patterns of isolates that had a mutation(s) according to the LiPA for detection of *pncA* mutations. Positions of the oligonucleotides, conjugate control lines, and marker lines are shown. A negative signal is shown by an open triangle. LiPA patterns of *M. tuberculosis* strains are shown in lanes 1 to 21. Lanes: 1, H37Rv; 2, IMCJ(130); 3, IMCJ904III; 4, IMCJ(66); 5, IMCJ(85); 6, IMCJ(67); 7, 13229; 8, IMCJ.M22; 9, P26; 10, 13243; 11, IMCJ850; 12, IMCJ835; 13, IMCJ501; 14, IMCJ844; 15, IMCJ479; 16, IMCJ843; 17, P12; 18, IMCJ901III; 19, IMCJ(80); 20, P10; 21, IMCJ.K1; 22, *M. bovis* BCG Japanese strain 172; 23, no DNA. WT, wild-type *pncA*.

shown in Fig. 2. Hybridization signals visualized as violet bands on the strips were strong and readily discernible, with low background, although there was variability in bands intensities, and some strains yielded less intense bands than others. The 228 isolates hybridized to all probes, as shown by the data for strain H37Rv in Fig. 2 (lane 1). The products of the other 30 *M. tuberculosis* strains containing a mutation(s) did not hybridize to the probes corresponding to the position of the mutation(s) but did hybridize to the others. These results were fully consistent with the DNA sequencing results (Table 2). DNA sequencing confirmed that *pncA* of BCG had a C-to-G point mutation at codon 59, causing histidine to become aspartic acid (Table 2). These data are consistent with our previous findings and those of others (17, 18) showing that *M. bovis* BCG is naturally resistant to PZA. In addition, of the 13 other nontuberculous *Mycobacterium* spp. listed in Table 1, *M. gastri* was susceptible to PZA. All others were resistant to PZA (data not shown).

Regarding the PZA resistance profile, the LiPA yielded results that were 100% in agreement with those obtained by the culture-based susceptibility method (Table 3). In addition, all PZase-positive bacilli were sensitive to PZA, and all PZase-negative bacilli were resistant to PZA (Table 2). These data were consistent with those of previously published reports (18, 22). The LiPA correctly identified PZA susceptibility and resistance in all strains in which a mutation(s) occurred. All of the 30 PZA-resistant *M. tuberculosis* isolates were correctly identified as being PZA resistant by the LiPA, and all of the 228 PZA-susceptible isolates were identified as being PZA susceptible. The specificity, sensitivity, and positive and negative predictive values of the LiPA for the detection of PZA resistance were all 100% (Table 3). In addition, we determined PZase activities of all *Mycobacterium* spp. listed in Table 1 and all clinical isolates of *M. tuberculosis* tested. Strains with PZase activity were sensitive to PZA, and those without PZase activity were resistant to PZA (data not shown).

To examine whether the LiPA can be applied to clinical samples from patients with tuberculosis, sputum samples obtained from 53 patients with suspected tuberculosis were tested (data not shown). All samples were positive for acid-fast staining. Among these 53 samples, 46 were positive for tuberculosis by the Amplicor MTB test and/or mycobacterial culture, and six were positive for *M. avium* by the Amplicor *M. avium* test. The remaining sample was positive for acid-fast staining but PCR and culture negative. In 45 of the 46 samples, *M. tuberculosis pncA* was detected with the LiPA. However, no muta-

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TABLE 2. Identification of *pncA* mutations by LiPA among 258 clinical isolates of *M. tuberculosis* and *M. bovis* BCG

| Strain | LiPA profile[a] | Mutation | | PZA susceptibility | PZase activity |
|---|---|---|---|---|---|
| | | Amino acid change | Variant nucleotides | | |
| IMCJ.K1 | Δ2 | 3 Ala→Glu | GCG→GAG | R | – |
| P10 | Δ4, Δ5 | 10 Gln→Pro | CAG→CCG | R | – |
| IMCJ(80) | Δ4, Δ5 | 12 Asp→Ala | GAC→GCC | R | – |
| IMCJ693 | Δ4, Δ5 | 12 Asp→Ala | GAC→GCC | R | – |
| IMCJ(120) | Δ4, Δ5 | 12 Asp→Ala | GAC→GCC | R | – |
| IMCJ901III | Δ8, Δ9 | 27 Leu→Pro | CTG→CGC | R | – |
| IMCJ918III | Δ8, Δ9 | 27 Leu→Pro | CTG→CGC | R | – |
| P12 | Δ13, Δ14 | 51 His→Gln | CAC→CAG | R | – |
| IMCJ843 | Δ13, Δ14 | 51 His→Gln | CAC→CAA | R | – |
| IMCJ846 | Δ13, Δ14 | 51 His→Gln | CAC→CAA | R | – |
| IMCJ479 | Δ14, Δ15, Δ29 | 53 Asp→Asn frameshift | GAC→AAC, 349 insertion CACTG | R | – |
| IMCJ844 | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ695 | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ838 | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ29 | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ907III | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ908III | Δ15 | 54 Pro→Leu | CCG→CTG | R | – |
| IMCJ501 | Δ19 | 72 Cys→Trp | TGC→TGG | R | – |
| IMCJ(133) | Δ19 | 72 Cys→Trp | TGC→TGG | R | – |
| IMCJ(130) | Δ19 | Frameshift | 218 insertion CGCATT GCCG | R | – |
| IMCJ835 | Δ32 | 132 Gly→Ser | GGT→AGT | R | – |
| IMCJ849 | Δ32 | 132 Gly→Ser | GGT→AGT | R | – |
| IMCJ850 | Δ32 | 133 Ile→Thr | ATT→ACT | R | – |
| IMCJ837 | Δ32 | 133 Ile→Thr | ATT→ACT | R | – |
| IMCJ904III | Δ32 | Frameshift | 386–388 deletion ATG | R | – |
| 13243 | Δ33 | 136 Asp→Tyr | GAT→TAT | R | ND |
| IMCJ(66) | Δ34 | Frameshift | 420 insertion G | R | – |
| P26 | Δ36 | 148 Arg→Ser | CGC→AGC | R | – |
| 13229 | Δ41 | Frameshift | 493 insertion C | R | ND |
| IMCJ.M22 | Δ45 | 175 Met→Val | ATG→GTG | R | – |
| IMCJ(67) | Wild type (Δ16)[b] | (60 Gly→Gly) | (GGC→GGT silent) | S | + |
| IMCJ(134) | Wild type (Δ16) | (60 Gly→Gly) | (GGC→GGT silent) | S | + |
| IMCJ(85) | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ(75) | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ851 | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ839 | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ(90) | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ(96) | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| IMCJ(125) | Wild type (Δ17) | (65 Ser→Ser) | (TCC→TCT silent) | S | + |
| H37Rv | Wild type | No change | No change | S | + |
| BCG[c] | Δ16 | 59 His→Asp | CAC→GAC | R | – |

[a] Δ indicates the negative signal at any of the probes.
[b] Δ in parentheses indicates the negative signal at any of the probes unless the probe to compensate for the silent mutation was used.
[c] *M. bovis* BCG Japanese strain 172.

tion of *pncA* was found in these 45 samples, suggesting that all of the samples contained PZA-sensitive organisms. These results were later confirmed by drug susceptibility testing when *M. tuberculosis* isolates were obtained from the samples. Hybridization was not detected on the strips in the six *M. avium*-positive samples as well as the remaining sample that was positive for acid-fast staining but negative for PCR and culture. All samples were culture positive for mycobacteria. These results indicate that the LiPA is applicable to clinical samples. However, further studies of clinical samples containing PZA-resistant *M. tuberculosis* are necessary.

It appears that nested PCR rarely introduces additional mu-

TABLE 3. Diagnostic performance of the LiPA compared to PZA susceptibility testing

| Result by PZA susceptibility test | No. of *M. tuberculosis* clinical isolates (*n* = 258) | LiPA result | | Sensitivity (%) | Specificity (%) | Predictive value (%) | |
|---|---|---|---|---|---|---|---|
| | | No. resistant | No. susceptible | | | Positive | Negative |
| Resistant | 30 | 30 | 0 | 100 | 100 | 100 | 100 |
| Susceptible | 228 | 0 | 228[a] | | | | |

[a] Includes nine isolates with a silent mutation in *pncA*.



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tations that may lead to false-positive results for the LiPA. *Taq* DNA polymerase is reported to make one error every 120 bases, and it was reported that these errors occur randomly (3). To affect LiPA results, the error must occur very early in the amplification process and at a specific site causing false-positive results in almost all PCR products. The frequency may be $3 \times 10^{-11}$ $[(1/120)^5]$ when there are five copies of the template. In fact, nested PCR is used for other LiPA assays (1, 8) and for single-strand conformation polymorphism analysis (4).

We showed the usefulness of the LiPA for PZA susceptibility testing of *M. tuberculosis*. This assay can detect *M. tuberculosis* in smear-positive sputa from patients. This LiPA can rapidly and efficiently assess the resistance of *M. tuberculosis* to PZA. It is simple, convenient, and highly reliable when run in parallel with a convenient *M. tuberculosis* diagnostic algorithm in laboratories. However, the LiPA has some limitations. First, this assay does not have an internal control. In addition, this assay cannot correctly identify mixed PZA-resistant and -susceptible isolates. This assay cannot detect novel silent mutations; however, it can detect known mutations. Finally, genes other than *pncA* may be associated with PZA resistance. Scorpio et al. (16) previously reported PZase-positive PZA-resistant *M. tuberculosis* strains that were very rare and usually showed a low level of resistance. Nevertheless, our LiPA is a valuable tool for the detection of resistant *M. tuberculosis* strains within one working day and can easily be included in the routine workflow.

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