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Comparison of a Conventional Antimicrobial Susceptibility Assay to an Oligonucleotide Chip System for Detection of Drug Resistance in *Mycobacterium tuberculosis* Isolates

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An oligonucleotide chip (Combichip Mycobacteria chip) detecting specific mutations in the *rpoB*, *katG*, and *inhA* genes of *Mycobacterium tuberculosis* was compared with conventional antimicrobial susceptibility results. The probes detecting drug resistance were as follows: 7 wild-type and 13 mutant probes for rifampin and 2 wild-type and 3 mutant probes for isoniazid. Target DNA of *M. tuberculosis* was amplified by PCR, followed by hybridization and scanning. Direct sequencing was performed to verify the results of the oligonucleotide chip. One-hundred seven of 115 rifampin-resistant strains (93%) had mutations in the *rpoB* gene. Eighty-five of 119 isoniazid-resistant strains (71%) had mutations in the *katG* gene or *inhA* gene. The diagnostic oligonucleotide chip with mutation-specific probes is a reliable and useful tool for the rapid and accurate diagnosis of resistance against rifampin and isoniazid in *M. tuberculosis* isolates.

In the world's population at large, it is estimated that there are 7.5 million cases of tuberculosis, with 2.5 million deaths per year, making tuberculosis the foremost cause of death due to infection. Both primary resistance and acquired resistance of *Mycobacterium tuberculosis* to antimicrobial agents are a worldwide problem (4, 5, 10).

Rifampin (RIF) and isoniazid (INH) are the most important drugs in the treatment of tuberculosis, and resistance to these antibiotics often results in incurable tuberculosis. Because of the prolonged turnaround time for conventional susceptibility testing, patients infected with drug-resistant tuberculosis may be inadequately treated and thus facilitate the transmission of resistant strains (26). Laboratory diagnosis of tuberculosis and subsequent evaluation of drug resistance of the isolated bacterium by routine methods takes 6 to 9 weeks (14, 18, 20). At the same time, rapid evaluation of drug resistance is significant for choosing effective drug therapy and preventing the propagation of drug-resistant strains. Therefore, development of new rapid methods for evaluation of drug resistance is a challenge facing a tuberculosis control program.

RIF resistance of *M. tuberculosis* strains recovered worldwide is mainly due to mutations in an 81-bp region (rifampin resistance-determining region [RRDR]) of the *rpoB* gene encoding the beta subunit of RNA polymerase (7, 15, 17). Unlike RIF resistance, INH resistance is apparently controlled by a more complex genetic system that involves several genes (1). However, extensive studies have demonstrated that INH resistance is most frequently associated with a specific mutation in *katG* (codon 315), a gene that encodes the catalase-peroxidase enzyme in *M. tuberculosis* (19). Also, *inhA* (enoyl-ACP-reductase), an enzyme involved in mycolic acid biosynthesis, was identified as a main target of INH, and mutations in the regulatory region of the *inhA* gene have been linked to INH resistance (13, 19, 22).

The oligonucleotide chip allows thousands of specific DNA sequences to be detected simultaneously and is already being applied to gene expression profiling, genotyping, mutation detection, and gene discovery. The oligonucleotide chip is an efficient approach for parallel analysis of a large number of specific sequences (9).

In this study, we evaluate the clinical efficacy of the oligonucleotide chip technology and compared this oligonucleotide chip with standard drug susceptibility testing for the detection of RIF- or INH-resistant *M. tuberculosis* strains from clinical specimens.

MATERIALS AND METHODS

***M. tuberculosis* isolates and DNA extraction.** Two hundred forty-three clinical isolates with known susceptibility patterns were collected from Pusan National University Hospital in Busan, Korea. Their antimicrobial susceptibility was determined by the Löwenstein-Jensen (L-J) proportion method at the Korean Institute of Tuberculosis. One-hundred twenty-one isolates were both RIF and INH susceptible, 3 were RIF resistant, 7 were INH resistant, and 112 were both RIF and INH resistant. Chromosomal DNA was prepared from *M. tuberculosis* grown on L-J medium and extracted using the InstaGene matrix (Bio-Rad,

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TABLE 1. Biotin-labeled primer for PCR at oligonucleotide chip reaction

Drug and primer[a]	Sequence (5′→3′)
Rifampin	
rpoB 4F	TCGCCGCGATCAAGGAGT
rpoB 3R	TGCACGTCGCGGACCTCCA
Isoniazid	
kat 1F	AAGAGCTCGTATGGCACCGG
kat 4R	AGCGCCAGCAGGGCTCTTC
inhA F	ACCCCAGTGCGAAAGTTCCC
inhA R	GGTAACCAGGACTGAACGGG

[a] F, forward; R, reverse.

Hercules, CA) according to the manufacturer's protocol. Briefly, a 30-μl sample was suspended in 200 μl of InstaGene matrix and vortexed, followed by heating at 56°C for 15 min. The samples were vortexed again, heated at 100°C for 8 min, and then centrifuged to pellet the matrix. Four microliters of each of the sample DNAs was used as a template for PCR.

Preparation of target DNA for hybridization. Biotin-labeled primers were used in the PCR to amplify target DNA for hybridization of the oligonucleotide chip (Table 1) (BioBasic, Seoul, Korea). In a total volume of 25 μl, the PCR mixtures contained 500 mM KCl, 100 mM Tris HCl (pH 9.0), 1% Triton X-100, 200 μM of deoxynucleoside triphosphates (dATP, dGTP, dTTP, and dCTP), 10 pmol of each of the members of the primer pair, 1.5 mM of $MgCl_2$, 1 U *Taq* DNA polymerase (QIAGEN, Inc., Valencia, CA), and 4 μl of template DNA. After heating the mixture to 95°C for 3 min, amplification was carried out in sequential 35 cycles with denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 1 min. The last extension step was performed by incubation at 72°C for 30 s. The DNA fragments were routinely examined on agarose gels stained with ethidium bromide.

Design of oligonucleotide probes. The sequences of *rpoB*, *katG*, and *inhA* genes in *M. tuberculosis* were obtained from GenBank. According to the multiple alignment analysis data using CLUSTALW, probes for the wild type and mutants were designed to meet the following parameters. The oligonucleotide probes were between 14 and 20 nucleotides long, and the position of the potential mismatch in sequences should be close to the center of the probe. The 5′ end of each probe was modified by adding poly(T) and an amino link group to enable covalent immobilization on the aldehyde-coated glass surface (16).

Detection of mutation by oligonucleotide chip. Four microliters of PCR aliquots and Cy5-labeled streptavidin (Amersham Phamacia Biotech, Inc., Little Chalfont, United Kingdom) were applied to the oligonucleotide chip slide. The slide was covered with a coverglass to prevent evaporation of the sample, followed by hybridization at 40°C for 30 min, washing with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature, washing with 0.2× SSC, and drying by centrifugation to completely remove any remaining solution. The oligonucleotide arrays were scanned with a GenePix 4000A array scanner (Axon Instruments, Inc., Sunnyvale, CA). The fluorescent image of the probes was obtained at 635 nm (Cy5). The fluorescent signal from each spot was measured by CombiView software (GeneIn, Inc., Busan, Korea) for the analysis of the diagnostic oligonucleotide chip (16) for detection of mycobacterial drug resistance (CombiChip Mycobacteria chip).

Sequencing. The results of the oligonucleotide chip were confirmed by comparing PCR and direct sequencing. For sequencing of the *rpoB*, *katG*, and *inhA* genes of *M. tuberculosis*, the primers of the same sequences without biotin described in Table 1 were used to amplify each target region. PCR was performed with a PTC-100 thermal cycler system (Bio-Rad Laboratories, Inc., Waltham, MA), and the PCR products were purified using a QIAquick PCR purification kit (QIAGEN). The sequences of PCR products were determined with using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Inc., Foster City, CA).

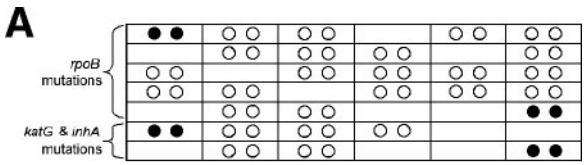
RESULTS

Selection of oligonucleotide probes. Several oligonucleotide probes for each wild-type and mutant strain were designed. To select suitable probes with high discriminatory ability, each type of designed oligonucleotide probe was immobilized on the glass slide and hybridized with DNAs extracted from *M. tuber-*

TABLE 2. Probes used in this study

Drug	Target gene	Probe name[a]	Sequence[b]	Temp (°C)	% GC
Rifampin	*rpoB*	RMP-W511L	5′-T_{15}-CAGCCAGCTGAGCCAATTCA-3′	54	67
		RMP-M511P	5′-T_{15}-AGCCAGCCGAGCCAA-3′	47	55
		RMP-W513Q	5′-T_{15}-CTGAGCCAATTCATG-3′	39	47
		RMP-M513L	5′T_{15}-CTGAGCCTATTCATGG-3′	43	50
		RMP-W516D	5′-T_{15}-TTCATGGACCAGAACA-3′	41	44
		RMP-M516V	5′-T_{15}-TTCATGGTCCAGAACA-3′	41	44
		RMP-M516Y	5′-T_{15}-ATTCATGTACCAGAACA-3′	40	35
		RMP-W522S	5′-T_{15}-CTGTCGGGGTTGAC-3′	43	64
		RMP-M522L	5′-T_{15}-CTGTTGGGGTTGAC-3′	40	57
		RMP-W526H	5′-T_{15}-GTTGACCCACAAGCGCCGA-3′	55	63
		RMP-M526Y	5′-T_{15}-GGTTGACCTACAAGCGC-3′	49	59
		RMP-M526D	5′-T_{15}-GGTTGACCGACAAGCGC-3′	52	65
		RMP-M526R	5′-T_{15}-TTGACCCGCAAGCGC-3′	47	67
		RMP-M526L	5′-T_{15}-TTGACCCTCAAGCGC-3′	45	60
		RMP-M526P	5′-T_{15}-TTGACCCCCAAGCGC-3′	47	67
		RMP-W531S	5′-T_{15}-CGACTGTCGGCGCTG-3′	50	73
		RMP-M531L	5′-T_{15}-CGACTGTTGGCGCTG-3′	47	67
		RMP-M531W	5′-T_{15}-CCGACTGTGAGCGCT-3′	47	67
		RMP-W533L	5′-T_{15}-GGCGCTGGGGCCCGGC-3′	61	94
		RMP-M533P	5′-T_{15}-GTCGGCGCCGGGGCCCG-3′	64	94
Isoniazid	*katG*	INH-W315S	5′-T_{15}-ATCACCAGCGGCATC-3′	45	60
		INH-M315T	5′-T_{15}-ATCACCACCGGCATC-3′	45	60
		INH-M315N	5′-T_{15}-ATCACCAACGGCATC-3′	42	53
	inhA	inhA-W	5′-T_{15}-GGCGAGACGATAGGT-3′	57	60
		inhA-M	5′-T_{15}-GGCGAGATGATAGGT-3′	56	52

[a] W, wild type; M, mutant. Numbers and letters following W or M refer to amino acid types encoded by the codon at the mutation region.
[b] In each sequence, the underlined letter is the position of the potential mismatch.



B

1. Rmix	2. W511L	3. M511P		4. W513Q	5. M513L
	6. W516D	7. M516V	8. M516Y		9. W522S
10. M522L		11. W526H	12. M526Y	13. M526D	14. M526R
15. M526L	16. M526P		17. W531S	18. M531L	19. M531W
	20. W533L	21. M533P			1. Rmix
22. Imix	23. W315S	24. M315T	25. M315N		
	26. WinhAC	27. MinhAT			22. Imix

FIG. 1. Probe layout in the oligonucleotide chip. For an explanation of the probe names, see the text.

culosis of different mutants. The optimal probes to detect wild-type and mutant *rpoB*, *katG*, and *inhA* genes (Table 2 and Fig. 1) were selected. The oligonucleotide chip composed of the selected probes covered the most common mutations, which were 20, 3, and 2 types of *rpoB*, *katG*, and *inhA* gene mutations, respectively. The 25 probes were named as follows. W (M) indicates wild type (mutant). The number or letter following W (M) represents an ordinal codon of the respective gene or the gene itself. The last letter indicates an amino acid encoded by codon. Rmix or Imix indicates all probes' are a mixture of RIF or INH resistance-related genes, respectively. Figure 2A shows an example of the hybridization pattern of RIF- and INH-susceptible *M. tuberculosis* isolates. The signal intensity of probes representing the wild type was significantly higher than those of respective mutants (i.e., probe no. 2 versus 3, 4 versus 5, and so on). Figure 2B shows an example of the hybridization pattern of RIF-resistant and INH-susceptible *M. tuberculosis* isolates. According to the signal intensity of probes 17 and 18, this isolate was considered to have mutations in codon 531 (CTG→CCG) of the *rpoB* gene.

TABLE 3. Results of susceptibility testing and oligonucleotide chip testing of 243 isolates of *M. tuberculosis*

Drug susceptibility	No. of isolates with characteristic					
	Rifampin		Isoniazid			
			Mutation			Wild type
	rpoB mutation	Wild type	*katG*	*inhA*	Both	
Susceptible	2	126	0	3	0	121
Resistant	107	8	29	43	13	34

Multiplex PCR. The specific amplification of each target gene was performed by single PCRs using specific PCR primer sets, which produces amplicons of 157 bp for the *rpoB* gene, 149 bp for the *katG* gene, and 160 bp for the *inhA* gene. The identity of each amplicon was further confirmed by sequencing. When multiplex PCR was applied to amplify all three amplicons in one reaction, the PCR product chip hybridization reaction proved that all three targets were successfully amplified, even though the PCR product was shown as an indiscriminate band. The detection limit of the multiplex PCR-based oligonucleotide chip was 10 pg/μl of the template DNA (data not shown).

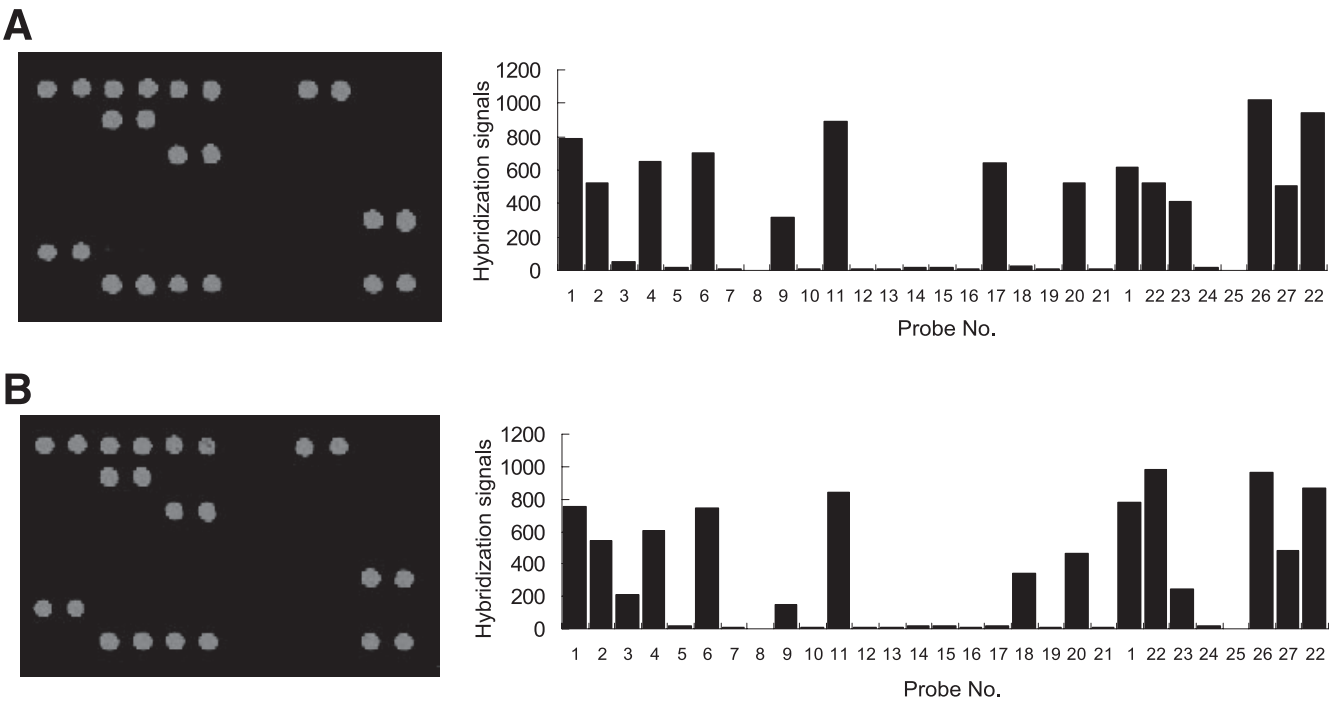


FIG. 2. Hybridization pattern of clinical isolates with oligonucleotide chip. (A) Wild-type *M. tuberculosis*. (B) RIF-resistant and INH-susceptible *M. tuberculosis*. Probe numbers: 1 to 21, probes for detection of resistance to RIF; 22 to 27, probes for detection of resistance to INH.

TABLE 4. *rpoB* gene mutation pattern in *M. tuberculosis* clinical isolates

Drug susceptibility (*n*)	*rpoB* base change	*rpoB* codon no. and amino acid change	No. of isolates
RIF resistant (115)	CTG→CCG	533 Leu→Pro	3
	TCG→TTG	531 Ser→Leu	60
	CAC→CGC	526 His→Arg	6
	CAC→TAC	526 His→Tyr	7
	CAC→GAC	526 His→Asp	7
	CAC→CTC	526 His→Leu	4
	TCG→TTG	522 Ser→Leu	2
	GAC→GTC	516 Asp→Val	10
	GAC→TAC	516 Asp→Tyr	3
	CAA→CTA	513 Gln→Leu	1
	CAA→?	513 Gln→?	3
	CUG→CCG	511 Leu→Pro	1
	Wild type	Wild type	8
RIF susceptible (128)	CTG→CCG	533 Leu→Pro	2
	Wild type	Wild type	126

Comparison of conventional susceptibility test and oligonucleotide chip. (i) Resistance to RIF. The oligonucleotide chip could detect mutation of the RRDR of the *rpoB* gene (codons 511, 513, 516, 522, 526, 531, and 533). Among 115 RIF-resistant isolates, 107 (93.0%) had mutations in the *rpoB* gene that would alter the amino acid sequence of the RRDR (Table 3). Two of 128 RIF-susceptible strains showed mutations at codon 533 (CTG->CCG, Leu→Pro), which were confirmed by sequencing (Table 4). Eight RIF-resistant strains (6.9%) that gave the wild-type hybridization pattern on the oligonucleotide chip were confirmed in the absence of any mutations in the *rpoB* gene by sequencing. The most frequent change was found at Ser531 (n = 60 isolates; 52.2%), followed by His526 (n = 24), Asp516 (n = 13), Leu533 (n = 5), Gln513 (n = 4), Ser522 (n = 2), and Leu511 (n = 1). In terms of detecting drug resistance, the diagnostic sensitivity and specificity were 93.0% and 98.4%, respectively.

(ii) Resistance to INH. Among the 119 INH-resistant isolates, 85 (71.4%) isolates had mutations in the *katG* gene or *inhA* gene (Table 3). Of those, 37 (31.1%) isolates had a mutation in codon 315 (AGC→ACC) resulting in a Ser315Thr amino acid substitution in *katG*, which is the most common mutation found in INH-resistant strains. Forty-three (36.1%) strains had a single point mutation, C→T, at position 15 upstream of the start site of the *inhA* gene. Three of 124 INH-susceptible organisms had a single point mutation at position 15 upstream of the start site of the *inhA* gene, which was confirmed by sequencing too (Table 5). The diagnostic sensitivity and specificity of detection of INH resistance were 71.4% and 97.6%, respectively.

DISCUSSION

Tuberculosis is a global public health problem that will continue to increase in prominence, in part due to the AIDS pandemic and lack of new efficacious drugs. In addition, *M. tuberculosis* strains resistant to one or more of the first-line drugs are increasing in frequency and have caused outbreaks with high mortality (2, 3, 6, 8, 21). These problems emphasize the need for rapid and reliable detection methods to identify resistant organisms. The availability of data bearing on the gene mutations responsible for development of antibiotic resistance will assist in accomplishing these goals. Molecular techniques have begun to revolutionize the improving sensitivity and speed of identification and drug susceptibility testing.

In this study, the detection of *M. tuberculosis* strains resistant to RIF and INH in clinical isolates was evaluated by using an oligonucleotide chip system. The specialized oligonucleotide chip allowed detection of six different *rpoB* mutations in the 81-bp region of the *rpoB* gene from the cultured isolates. In general, *rpoB* mutations were found in 96% of RIF-resistant *M. tuberculosis* strains worldwide (15, 20). However, the frequency of genotypic changes of *rpoB* in relation to RIF resistance was low in other parts of the world: i.e., about 10% of RIF-resistant *M. tuberculosis* strains from China lack mutations in the RRDR (12, 26). In the present study, 93% of RIF-resistant strains showed *rpoB* mutations.

Unlike RIF resistance, INH resistance is apparently controlled by a more complex genetic system that involves several genes. INH resistance is most frequently associated with a specific mutation in codon 315 of the *katG* gene. This mutation results in production of an altered enzyme that is less able to convert INH to its biologically active form. *katG* (Ser315Thr) is a competent catalase-peroxidase that exhibits a reduced ability to metabolize isoniazid compared with the wild-type *katG* enzyme. The frequency of the amino acid Ser315Thr alteration in *katG*, which is the most common mutation found in INH-resistant strains (5), ranged from 50 to 60% worldwide. Previous studies have shown that mutations in the upstream region of the *inhA* locus resulted in increased levels of *inhA* expres-

TABLE 5. *katG* and *inhA* gene mutation patterns in *M. tuberculosis* clinical isolates

Drug susceptibility (*n*)	*katG* codon 315 mutation	*katG* amino acid change	*inhA* point mutation	No. of isolates
INH resistant (119)	AGC→ACC	Ser→Thr	Wild type	24
	AGC→AAC	Ser→Asn	Wild type	5
	Wild type	Wild type	C1023→T1023	43
	AGC→ACC	Ser→Thr	C1023→T1023	13
	Wild type	Wild type	Wild type	34
INH susceptible (124)	AGC→ACC	Ser→Thr	Wild type	0
	AGC→AAC	Ser→Asn	Wild type	0
	Wild type	Wild type	C1023→T1023	3
	Wild type	Wild type	Wild type	121

sion, thereby elevating the drug target levels and producing INH resistance by a titration mechanism. In our study, we observed the sole mutation of this upstream region was more frequent than the *katG* mutation. Although this mutation has previously been reported by several groups (11), the frequency in our result is relatively high. The probable explanation might be a clonal expansion of the resistant strain, which was not confirmed by medical record review or molecular strain typing. The present understanding of this mutation is that it probably confers resistance by a drug titration effect.

Technically, oligonucleotide chip-based mutation detection is not difficult, and it may be widely applicable in microbiology laboratories in the near future. Sougakoff et al. (23) and Yue et al. (26) have already described using oligonucleotide chips to detect mutations to *rpoB* in *M. tuberculosis* wild-type and drug-resistant strains. Others have published studies about *pncA* mutations (25) and *rpoB*, *katG*, and *rpsL* mutations (24). Putting all accounts together, the application of such rapid molecular methods to the detection of drug resistance will have an important impact on the future control of drug-resistant tuberculosis, especially multidrug-resistant tuberculosis. In our study, the oligonucleotide probes were designed to have various annealing temperatures from 39 to 64°C. At first, we tried to design probes with similar annealing temperature to hybridize both the wild type and mutants. However, the probes were not specific to the respective targets. After redesign of probes with variable lengths containing wild-type or specific mutant sequences, we selected the most specific and appropriate probes by repetitive hybridization experiments. Our results showed that the oligonucleotide chip reaction can discriminate between the wild type and mutants despite the wide ranges of annealing temperatures of probes. Under this circumstance, nonspecific hybridization may occur as shown in Fig. 2. However, our purpose is to determine whether the strain is wild type or mutant in a given gene, by comparing the relative intensities of signals generated from probes representing the same gene. Therefore, signals from other probes do not affect the interpretation of the specific gene mutation.

Results of conventional antimicrobial susceptibility testing using L-J medium did not agree with the oligonucleotide chip in some isolates. Two of five strains with a mutation located at codon 533 (CTG→CCG, Leu→Pro) of the *rpoB* gene were susceptible to RIF by the conventional method in the present study. Also, 3 of 46 strains with a single point mutation at *inhA* were susceptible to INH. One previous study demonstrated that strains preserving these type of mutations showed low-level resistance to each specific drug by the BACTEC method (7), which suggests that the oligonucleotide chip of the present study is a more precise detection system than the conventional antimicrobial susceptibility test.

Given that mutations detected in the oligonucleotide chip were closely related in drug resistance examined by the conventional method and that the detection limit is very low, the hybridization with the specialized oligonucleotide chip could be a promising approach to rapid detection of drug resistance of tubercle bacilli not only from the cultured isolates, as shown in the current study, but also directly in clinical specimens. Furthermore, the detection of the same specific mutation in an epidemiologically related group may contribute to early detection of an outbreak developing from the same source of infection, as suggested above. The results presented here show the potential use of oligonucleotide chips for the detection of clinically important genetic variations in *M. tuberculosis*.

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