Detection and Genotyping of *Mycobacterium* Species from Clinical Isolates and Specimens by Oligonucleotide Array

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Identification of pathogenic *Mycobacterium* species is important for a successful diagnosis of mycobacteriosis. The purpose of this study was to develop an oligonucleotide array which could detect and differentiate mycobacteria to the species level by using the internal transcribed spacer (ITS) sequence. Using a genus-specific probe and 20 species-specific probes including two *M. avium-intracellulare* complex (MAC)-specific probes, we have developed an ITS-based oligonucleotide array for the rapid and reliable detection and discrimination of *M. tuberculosis*, MAC, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. kansasii*, *M. goodii*, *M. scrofulaceum*, *M. szulgai*, *M. vaccae*, *M. xenopi*, *M. terrae*, *M. flavesens*, *M. smegmatis*, *M. malmoense*, *M. simiae*, *M. marinum*, *M. ulcerans*, *M. gastri*, and *M. leprae*. All mycobacteria were hybridized with a genus-specific probe (PAN-03) for detection of the genus *Mycobacterium*. Mycobacterial species were expected to show a unique hybridization pattern with species-specific probes, except for *M. marinum* and *M. ulcerans*, which were not differentiated by ITS-based probe. Among the species-specific probes, two kinds of species-specific probes were designed for MAC in which there were many subspecies. The performance of the oligonucleotide array assay was demonstrated by using 46 reference strains, 149 clinical isolates, and 155 clinical specimens. The complete procedure (DNA extraction, PCR, DNA hybridization, and scanning) was carried out in 4.5 h. Our results indicated that the oligonucleotide array is useful for the identification and discrimination of mycobacteria from clinical isolates and specimens in an ordinary clinical laboratory.

The increase in mycobacterial infections is a matter of serious public health concern. The increasing global burden of mycobacteria is linked to improper antibiotic therapy and immunocompromised patients, especially those with AIDS (3, 6). One of the prevention strategies is the accurate and rapid detection and discrimination of mycobacteria. Discrimination of nontuberculous mycobacteria (NTM) from *Mycobacterium tuberculosis* is needed for patient management, considering that many NTM are resistant to the antibiotics used for the treatment of tuberculosis. The identification of mycobacterial isolates to the species level has been accomplished by the analysis of the phenotypic and biochemical characteristics of the organisms after culture (25). These approaches can be limited in their ability to detect specific mycobacteria because of time-consuming processes and poor discrimination (11).

In order to overcome these disadvantages, the development of molecular assays has accelerated diagnosis (19). Molecular assays require highly technical tools and target genes. Technical improvements in PCR, sequencing, and oligonucleotide array have increased the sensitivity, specificity, and speed of assays (4, 8, 11, 13, 24). Among these improvements, the oligonucleotide array has recently become a powerful tool for microbial genotyping, drug resistance associated with mutations, and single-nucleotide polymorphisms. This technology also permits the simultaneous monitoring and analysis of a

large number of target genes depending on sequence diversity (5). Several target genes, such as 16S rRNA, *rpoB*, *hsp65*, internal transcribed spacer (ITS), and *gyrB*, have been used as targets for the genotyping of microorganisms (10, 17, 19, 21, 23). The important feature of target genes is that these genes are present in all bacteria and contain both conserved and polymorphic regions (11, 18). This sequence diversity of target genes provides a possibility in the speedy and accurate design of molecular assays.

We have previously studied the PCR assay by using the ITS region for identifying mycobacteria to the species level (16). Based on the previous PCR assay, we tried to develop molecular testing that could identify and discriminate medically important mycobacteria in a single reaction. The purpose of this study was to develop an oligonucleotide array based on the ITS sequence for the genotyping of medically important mycobacteria containing *M. tuberculosis* and 19 NTM. We evaluated the oligonucleotide array with reference strains, clinical isolates, and clinical specimens.

MATERIALS AND METHODS

Bacterial strains. The type strains of 31 mycobacterial species, *M. abscessus* (ATCC 19977), *M. acapulensis* (ATCC 14473), *M. agri* (ATCC 27406), *M. asiaticum* (ATCC 25276), *M. avium* (ATCC 25291), *M. chelonae* (ATCC 35752), *M. flavesens* (ATCC 14474), *M. fortuitum* (ATCC 6841), *M. goodii* (ATCC 19710), *M. gastri* (ATCC 15754), *M. gilvum* (ATCC 43909), *M. goodii* (ATCC 14470), *M. intracellulare* (ATCC 13950), *M. kansasii* (ATCC 12478), *M. lentiflavum* (ATCC 51985), *M. malmoense* (ATCC 29571), *M. marinum* (ATCC 927), *M. phlei* (ATCC 354), *M. peregrinum* (ATCC 14467), *M. porcinum* (ATCC 33776), *M. scrofulaceum* (ATCC 19981), *M. shimoidi* (ATCC 27962), *M. simiae* (ATCC 25275), *M. smegmatis* (ATCC 21701), *M. szulgai* (ATCC 35799), *M. terrae* (ATCC 15755), *M. triviale* (ATCC 23292), *M. tuberculosis* (ATCC 27294), *M. ulcerans*

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(ATCC 19423), *M. vaccae* (ATCC 15483) and *M. xenopi* (ATCC 19250), and 15 other human pathogenic bacteria, *Aeromonas salmonicida* (ATCC 33658), *Corynebacterium diphtheriae* (ATCC 11913), *Enterobacter aerogenes* (ATCC 13048), *Enterococcus faecalis* (ATCC 19433), *Mycoplasma pneumoniae* (ATCC 15293), *Klebsiella pneumoniae* (ATCC 15380), *Neisseria gonorrhoeae* (ATCC 19424), *Neisseria meningitidis* (ATCC 13077), *Pseudomonas aeruginosa* (ATCC 10145), *Salmonella enteritidis* (ATCC 4931), *Shigella flexneri* (ATCC 9199), *Shigella sonnei* (ATCC 25931), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), and *Streptococcus agalactiae* (ATCC 13813), were purchased from the American Type Culture Collection (ATCC). Also, 99 NTM clinical isolates were provided by the Korean National Tuberculosis Association and 50 *M. tuberculosis* isolates and clinical specimens were provided by Pusan National University Hospital, Kosin University Gospel Hospital, and National Masan Tuberculosis Hospital. The clinical isolates and specimens were identified by conventional methods (acid-fast bacillus [AFB] staining, biochemical test, or culture). The mycobacteria and other bacteria were grown on Ogawa media for several weeks and on blood agar, respectively. The clinical isolate of *M. leprae* was provided by the Institute of Hansen's Disease, College of Medicine, Catholic University of Korea. Footpad granulomata from *M. leprae*-infected nude mice were also dissected, soaked in 1% iodine solution, and finely chopped with number 10 and 15 disposable scalpels. This sample was then homogenized in 2 ml of Dulbecco's phosphate-buffered saline with 25 to 30 glass beads in a homogenizer (Mickle Laboratory Engineering Co., Surrey, United Kingdom) (14). We performed two approaches used to identify the clinical samples. The clinical isolates were identified by saline biochemical test prior to performing diagnosis. The clinical specimens were performed blind, and the results were compared at a later date.

Preparation of genomic DNA and PCR. Clinical specimens, taken from sputum, had to be liquefied, decontaminated, and concentrated using the NaOH method. Then, the DNA was extracted using an InstaGene matrix kit (Bio-Rad Laboratories Inc., Hercules, Calif.) according to the manufacturer's protocol. PCR was performed as previously described with thermal cycler system PCT-100 (MJ Research, Waltham, Mass.) (16). The biotin-labeled primers ITS-F (forward, 5'-TGGATCCGACGAAGTCGTAACAAGG-3') and PAN-04R (reverse, 5'-ATGCTCBAABCACTATCCA-3') were used for PCR amplification.

Designs of genotype-specific probes and quality control (QC) probes. (i) **Genotype-specific probes.** The ITS sequences of mycobacteria were obtained from GenBank. According to the multiple alignment analysis data obtained by using CLUSTALW, genus- and species-specific probes were designed from the conserved and polymorphic regions of the ITS sequences of mycobacteria, respectively. The uniqueness of the sequences of the probes designed from the mycobacterial ITS region was analyzed with the BLAST search. The probes were designed to meet the following parameters. The oligonucleotides were between 15 and 22 nucleotides long, and the position of the potential mismatch in similar sequences was close to the center of the probe. The 5' end of each probe was modified by adding poly(T) and an aminolink group to enable covalent immobilizing on the aldehyde-coated glass surface.

(ii) **QC probe.** We prepared QC probes to check the spot uniformity of each array. The 6-carboxytetramethylrhodamine (TAMRA)-labeled QC probe [20-mer poly(T)] was also modified by an aminolink group at the 5' end. We spotted mixed probes of genotype-specific and QC probes. Before hybridization, we could confirm the proper spotting of genotype-specific probes by scanning at a 532-nm wavelength.

Fabrication of oligonucleotide arrays. Probes were printed in glass slides as shown in Fig. 1W. The oligonucleotide arrays were spotted on silylated glass slides (Cell Associated, Inc., Pearland, Texas) by using a PixSys nQUAD 4500 Microarrayer (Cartesian Technologies, Inc., Irvine, CA). The spotting solution contained a mixture of genotype-specific and QC probes (9:1 ratio) in solution. The spotted slides were dried for 3 h at 50°C and treated for 5 min with a freshly prepared 0.25% NaBH₄ solution. The slides were washed once for 5 min with 0.2% sodium dodecyl sulfate and distilled water to remove unbound probes.

Hybridization, dye conjugation, and scanning. PCR aliquots (2 µl) from each sample and Cy5-labeled streptavidin (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) were applied to the oligonucleotide array area and covered with coverslips to prevent evaporation of the sample in the 50-ml Corning tube during hybridization. Hybridization was performed at 40°C for 30 min. Slides were washed once with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature and once with 0.2× SSC and dried by centrifugation to completely remove any remaining solution. The oligonucleotide arrays were scanned with a GenePix 4000A array scanner (Axon Instruments, Inc., Union City, CA). The fluorescent image of the genotype-specific probes was obtained at 635 nm (Cy5), and that of the QC probes was obtained at 532 nm (TAMRA). The fluorescent signals from each spot were measured by using

CombiView software (GeneIn, Inc., Busan, Korea) for the analysis of the diagnostic oligonucleotide array.

Sequence analysis of mycobacterial ITS. For the sequencing of the ITS of the mycobacteria, ITS-F (forward, 5'-TGGATCCGACGAAGTCGTAACAAGG-3') and ITS-R (reverse, 5'-TGGATCCTGCCAAGGCATCCACCAT-3') primers were designed to amplify the ITS region to approximately 350 to 450 bp in length. The amplicons were purified using a QIAquick PCR purification kit (QIAGEN, Inc., Valencia, Calif.) according to the manufacturer's protocol. The sequence of PCR products was determined using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Inc., Foster City, Calif.) in the BaseStation-1 DNA fragment analyzer (MJ Research).

RESULTS

Genus- and species-specific probes of mycobacteria. The ITS contained both conserved and polymorphic regions. There were four conserved regions at the ITS that could be used to design mycobacterial genus-specific probes (PAN-01, PAN-02, PAN-03, and PAN-04) (Table 1). Initially, the PAN-01 probe was ruled out considering its low melting temperature. The PAN-02 probe was a degenerate genus-specific probe because there were polymorphic sequences at the ITS of *M. abscessus*, *M. bovis*, *M. chelonae*, and *M. xenopi*, among others (data not shown). The PAN-03 probe had conserved sequences among mycobacteria and showed a more significantly unique hybridization pattern than that of the PAN-02 probe. The PAN-04 probe was used as a biotin-labeled reverse primer to amplify the genus *Mycobacterium* for hybridization of the oligonucleotide array.

The presence of several polymorphic regions within the ITS enabled us to design individual species-specific probes. We designed a species-specific probe to detect *M. marinum* and *M. ulcerans* at the same time, because the similarity of ITS between *M. marinum* and *M. ulcerans* was 100% (data not shown).

We selected the optimal genus-specific probe and species-specific probes through hybridization with the clinical isolate of *M. leprae* as well as with reference strains of 31 mycobacterial species and of 15 other pathogenic bacteria. We produced amplicons with biotin-labeled primers in order to hybridize the oligonucleotide array. The amplicon size ranged from 270 to 400 bp from species to species. The results of independent experiments on mycobacterial species and other pathogenic bacteria for genotyping are presented in Fig. 1. The genus-specific probe (PAN-03) showed a unique hybridization pattern with all mycobacteria (Fig. 1A through T) and a mismatch with 15 other bacteria containing *C. diphtheriae*, which is taxonomically close to mycobacteria, and *M. pneumoniae*, which is the most common bacterium causing respiratory tract infections (Fig. 1U). The genus *Mycobacterium* can be identified with a single genus-specific probe (PAN-03). The most optimal species-specific probes, among 2 to 13 individual probes for discriminating mycobacteria, are shown in Table 1. Each mycobacterial species was expected to show a unique hybridization pattern with the species-specific probes (Fig. 1). For example, *M. tuberculosis* was expected to hybridize with the PAN-03 and *M. tuberculosis* complex (MTB)-specific probes (Fig. 1A), and other mycobacteria were hybridized with the PAN-03 probe but not with the MTB probe. The PAN-03 probe and each species-specific probe (MTB, MAC, FOR, CHE, ABC, KAN, GOR, SCO, SZU, VAC, XEN, TER, FLA, SME, MAL, SIM, M-U, GAS, and LEP) were hybridized specifically with *M. tuberculosis*, *M. avium-intracellulare* complex,

FIG. 1. Hybridization results with mycobacterial species and other bacteria by oligonucleotide array. (A through T) Each species of mycobacterium was expected to show a unique pattern of reactivity to each set of probes. (U) Hybridization result is shown for other pathogenic bacteria. (V) Image by QC probe before hybridization is shown. (W) Scheme of probes is laid out in oligonucleotide array. The probes were patterned in duplicate. Probe names are as explained in Results. PAN, PAN-03 probe.

M. fortuitum, *M. chelonae*, *M. abscessus*, *M. kansasii*, *M. goodii*, *M. scrofulaceum*, *M. szulgai*, *M. vaccae*, *M. xenopi*, *M. terrae*, *M. flavescens*, *M. smegmatis*, *M. malmoense*, *M. simiae*, *M. marinum* and *M. ulcerans*, *M. gastri*, and *M. leprae*, respectively. But only the PAN-03 probe was hybridized with *M. acapulensis*, *M. agri*, *M. asiaticum*, *M. gallinarum*, *M. gilvum*, *M. lentiflavum*, *M. phlei*, *M. peregrinum*, *M. procinum*, *M. shimoidei*, and *M. triviale*, which were not species-specific probes contained in the oligonucleotide array (Fig. 1T). The oligonucleotide array allowed for the differentiation of *M. chelonae*

from *M. abscessus* and *M. kansasii* from *M. gastri* but could not differentiate *M. marinum* from *M. ulcerans*. The mycobacterial species except MAC were each identified with a respective single probe. For MAC, in which there are many subspecies, a combination probe of MAC-01 (degenerate species-specific probe) and MAC-05 was designed. The species-specific probes could discriminate mycobacteria to the species level under uniform hybridization conditions.

Oligonucleotide array assay with clinical isolates and specimens of mycobacteria. We tested 149 clinical isolates and 155

TABLE 1. Genus- and species-specific probes used for the genotyping of mycobacteria

Target species and probe name	Position	Sequence ^a
Mycobacteria		
PAN-01	1–11	5'-ARGGAGCACCA-3'
PAN-02	105–124	5'-CACACTRTTGGGYYYYGAGG-3'
PAN-03	163–186	5'-TGGTGGGGTGTGGTGTTTGA-3'
PAN-04	190–209	5'-TGGATAGTCGVTGTVGAGCAT-3'
<i>M. tuberculosis</i> MTB	76–97	5'-T ₁₅ -GGGTGCATGACAACAAAGTTGG-3'
MAC		
MAC-01	118–137	5'-T ₁₅ -CCCTGAGACAACACTCGGTC-3'
MAC-05	139–158	5'-T ₁₅ -ACAACACTCGGTCSRTCCGT-3'
<i>M. fortuitum</i> FOR	158–177	5'-T ₁₅ -TGGCATCCGGTTGCGGGTGT-3'
<i>M. chelonae</i> CHE	30–49	5'-T ₁₅ -TTGGGAACATAAAGCGAGTTGT-3'
<i>M. abscessus</i> ABC	28–49	5'-T ₁₅ -ACTAGGGAACATAAAGTAGGCA-3'
<i>M. kansasii</i> KAN	18–34	5'-T ₁₅ -GCATCCCAACAGAGTGGG-3'
<i>M. gordonae</i> GOR	127–143	5'-T ₁₅ -CACCTCGGGTGCTGTC-3'
<i>M. scrofulaceum</i> SCO	131–150	5'-T ₁₅ -TCGGCTCGTTCTGAGTGGTG-3'
<i>M. szulgai</i> SZU	131–150	5'-T ₁₅ -AGGCTTGGCCAGAGCTGTTG-3'
<i>M. vaccae</i> VAC	134–151	5'-T ₁₅ -AATGCCGCGAGGGAAAT-3'
<i>M. xenopi</i> XEN	76–95	5'-T ₁₅ -CAGGCAGTAACCGCCGGCAC-3'
<i>M. terrae</i> TER	142–161	5'-T ₁₅ -AGGAGTCCTTGGGGGTTTCT-3'
<i>M. flavescens</i> FLA	125–146	5'-T ₁₅ -CACTGCTTTGAGGAATCATCAG-3'
<i>M. smegmatis</i> SME	117–136	5'-T ₁₅ -CTGTTTCGATGCCAG-3'
<i>M. malmoeense</i> MAL	131–145	5'-T ₁₅ -TCGGCCAGTCCGCGT-3'
<i>M. simiae</i> SIM	136–155	5'-T ₁₅ -CGACTTCGGTTGAAGTGGTG-3'
<i>M. marinum-ulcerans</i> M-U	122–141	5'-T ₁₅ -GCAACATCTCTGTTGGTTTC-3'
<i>M. gastri</i> GAS	147–161	5'-T ₁₅ -CGTCCAAGAGTGTG-3'
<i>M. leprae</i> LEP	146–162	5'-T ₁₅ -TTGTCCCTCATCTTTGG-3'

^a Positions are based on the nucleotide positions of the ITS of *M. tuberculosis* (GenBank accession no. L15623). R, S, V, and Y show mixed bases; R, A + G; S, G + C; V, G + A + C; Y, C + T.

clinical specimens with the oligonucleotide array. We performed two approaches used to identify the clinical samples. The clinical isolates were identified by using typing results known prior to diagnosis. The 155 clinical specimens were performed blind, and the results were compared at a later date. Some discrepant results occurred when the results by conventional identification were compared with those by oligonucleotide array (Table 2). But the results by oligonucleotide array and sequencing were identical in all cases for each species. In cases where there was a mismatch between the phenotypic and genotypic identifications, we repeated the test three times to confirm our oligonucleotide array and sequencing results. Of the nine *M. chelonae* isolates, which were identified by conventional methods, eight were identified as *M. chelonae* and one was identified as *M. abscessus* by oligonucleotide array. Of the nine *M. gordonae* isolates, six were identified as *M. gordonae*, two were identified as *M. tuberculosis*, and one was identified as *M. flavescens* by oligonucleotide array. The PCR and direct sequencing results matched oligonucleotide array results (Table 2). Among the clinical specimens, two were shown to hybridize with the PAN-03 probe and were identified as *Mycobacterium* spp. by oligonucleotide array. By sequencing, they were identified as *M. celatum* and *M. lentiflavum*. The oligonucleotide array allowed for the identification of a number of mycobacterial species in one reaction. It is advantageous for diagnostic purposes to identify a number of bacteria simultaneously in a clinical sample.

Detection limit of oligonucleotide array assay. The detection limit of the oligonucleotide array was determined by amplification and hybridization of serial dilutions of mycobacterial DNA (5-ng/μl to 500-fg/μl dilutions of the initial DNA concentrations of *M. tuberculosis*, *M. avium* and *M. fortuitum*). The

detection limits of the oligonucleotide array were as low as the initial 5-pg/μl DNA concentrations of *M. tuberculosis*, *M. avium*, and *M. fortuitum*. We showed only the results of hybridization with 500, 50, and 5 pg/μl and 500 fg/μl of the initial DNA concentration of *M. tuberculosis* (Fig. 2A). It was several hundred to ten thousand times the signal-to-noise value at 500 to 5 pg/μl of the initial DNA concentration of *M. tuberculosis*. At the initial 500-fg/μl DNA concentration, the signal-to-noise value was too low (<2.0) to detect the target. But visible bands of these amplicons were not observed by gel electrophoresis at a 5-pg/μl concentration (Fig. 2B).

DISCUSSION

Molecular assays have been used increasingly over the past 10 years to improve the sensitivity and speed in the diagnosis of infectious diseases. If molecular assays are to replace culture and biochemical methods in the future, they will need to provide the genotyping of microorganisms (9). Together with data from increasing numbers of whole microbial genomes, thousands of sequences can be selected to probe numerous genes. The oligonucleotide array offers the possibility of the rapid examination of larger amounts of DNA sequences with a single hybridization (18). This technology will be a valuable tool for bacterial genotyping from cultures and clinical specimens, especially slow-growing and hard-to-culture mycobacteria. Among species with overlapping phenotypic patterns, the conventional procedures are unable to distinguish organisms (20). Therefore, efforts for the development of more rapid and accurate diagnostic tools for the identification of specific species of mycobacteria by using sequence-based molecular techniques have been undertaken in recent years. In our previous study,

TABLE 2. Interpretations obtained with clinical isolates and clinical specimens of mycobacteria^a

Source (total no.) and organism ^b (no.) or result	No.	Positive probe(s)	Identification by:	
			Oligonucleotide array	Sequencing ^c
Clinical isolates (149)				
<i>M. abscessus</i> (1)	1	PAN, ABC	<i>M. abscessus</i>	<i>M. abscessus</i>
<i>M. avium</i> (10)	10	PAN, MAC	MAC	<i>M. avium</i>
<i>M. avium</i> complex (10)	10	PAN, MAC	MAC	<i>M. avium</i>
<i>M. chelonae</i> complex (9)	8	PAN, CHE	<i>M. chelonae</i>	<i>M. chelonae</i>
	1	PAN, ABC	<i>M. abscessus</i>	<i>M. abscessus</i>
<i>M. fortuitum</i> (4)	4	PAN, FOR	<i>M. fortuitum</i>	<i>M. fortuitum</i>
<i>M. fortuitum</i> complex (9)	9	PAN, FOR	<i>M. fortuitum</i>	<i>M. fortuitum</i>
<i>M. gordonae</i> (9)	6	PAN, GOR	<i>M. gordonae</i>	<i>M. gordonae</i>
	2	PAN, MTB	<i>M. tuberculosis</i>	<i>M. tuberculosis</i>
	1	PAN, FLA	<i>M. flavescens</i>	<i>M. flavescens</i>
<i>M. intracellulare</i> (14)	14	PAN, MAC	MAC	<i>M. intracellulare</i>
<i>M. kansasii</i> (10)	10	PAN, KAN	<i>M. kansasii</i>	<i>M. kansasii</i>
<i>M. leprae</i> (1)	1	PAN, LEP	<i>M. leprae</i>	<i>M. leprae</i>
<i>M. scrofulaceum</i> (3)	3	PAN, SCO	<i>M. scrofulaceum</i>	<i>M. scrofulaceum</i>
<i>M. szulgai</i> (9)	9	PAN, SZU	<i>M. szulgai</i>	<i>M. szulgai</i>
<i>M. terrae</i> complex (10)	10	PAN, TER	<i>M. terrae</i>	<i>M. terrae</i>
<i>M. tuberculosis</i> (50)	50	PAN, MTB	<i>M. tuberculosis</i>	<i>M. tuberculosis</i>
Clinical specimens (155)				
Positive by AFB staining or culture	8	PAN, ABC	<i>M. abscessus</i>	<i>M. abscessus</i>
	1	PAN, CHE	<i>M. chelonae</i>	<i>M. chelonae</i>
	7	PAN, MAC	MAC	<i>M. avium</i>
	16	PAN, MAC	MAC	<i>M. intracellulare</i>
	8	PAN, FOR	<i>M. fortuitum</i>	<i>M. fortuitum</i>
	5	PAN, GOR	<i>M. gordonae</i>	<i>M. gordonae</i>
	2	PAN, MAL	<i>M. malmoeense</i>	<i>M. malmoeense</i>
	1	PAN, M-U	<i>M. marinum</i> or <i>M. ulcerans</i>	<i>M. marinum</i> or <i>M. ulcerans</i>
	1	PAN, SMG	<i>M. smegmatis</i>	<i>M. smegmatis</i>
	3	PAN, SZU	<i>M. szulgai</i>	<i>M. szulgai</i>
	100	PAN, MTB	<i>M. tuberculosis</i>	<i>M. tuberculosis</i>
	1	PAN, VAC	<i>M. vaccae</i>	<i>M. vaccae</i>
	1	PAN	<i>Mycobacterium</i> spp.	<i>M. lentiflavum</i>
	1	PAN	<i>Mycobacterium</i> spp.	<i>M. celatum</i>

^a We performed two approaches to identify the clinical samples. The clinical isolates were identified by biochemical test prior to performing diagnosis, and the clinical specimens were performed blind and the results were compared at a later date. We tested clinical specimens which were positive results by AFB staining or culture.

^b Species identification of microorganisms was the result of biochemical testing.

^c Identification was performed by amplification of the ITS and direct sequencing. PAN, PAN-03 probe.

we showed that the PCR-based detection method was able to differentiate mycobacterial species (16). The genotypes were identified by differences in the molecular weights of the specific-sized DNA fragments amplified with species-specific primers. This method is very valuable in the detection of major pathogenic mycobacteria. But there was a limitation in convenience since it used eight PCR tubes for the detection of eight kinds of mycobacteria.

In this study, we developed the oligonucleotide array to differentiate 20 medically important pathogenic mycobacteria. There were several hurdles in the development of the oligonucleotide array for genotyping of mycobacteria, such as probe design, the adjusting of the hybridization condition of various kinds of probes, and the conforming of spotting of probes, among others. QC is an important aspect of oligonucleotide array fabrication. Among several important QC systems, the monitoring of spot uniformity is critical to manufacturing the oligonucleotide array. Many studies have reported on the QC systems of array (1, 2, 5, 22, 26). These QC systems allow for the confirmation of spot uniformity after hybridization. But we introduced a QC probe which could confirm spot uniformity before hybridization. In our study, the QC probe was manufactured as follows. A TAMRA-labeled QC probe [20-mer

poly(T)] was modified by an aminolink group in the same way as a genotype-specific probe. The spotting solution contained a mixture of a genotype-specific probe and a QC probe (9:1 ratio) in solution. Before the hybridization experiment, a fluorescent image of QC probes was obtained at 532 nm (TAMRA) (Fig. 1V). That is, we could confirm the spot uniformity of the genotype-specific probes before hybridization. This approach may be a valuable tool for the monitoring of spot uniformity. And it was hard to design species-specific probes because there are interspecies variations in several species. MAC shows high interspecies variations. In order to overcome these problems, we tried to design species-specific probes in species-conserved regions. The interspecies variation regions within target sequences were avoided in designing species-specific probes for hybridization by the oligonucleotide array.

The limits of the phenotype-based identification method have led to the development of methods based on the microbial genotype or DNA sequences which minimize problems with typeability and reproducibility and, in some cases, enable the establishment of large databases of characterized organisms (7, 15). Tortoli et al. (20) reported that *M. gordonae* is misidentified as *M. szulgai* by conventional identification, in-

FIG. 2. Sensitivity of oligonucleotide array for the detection of mycobacteria. We performed experiments of detection limit three times. Among three separate experiments, we used the representative single-experiment result. (A) Image and signal intensities were obtained through hybridization with the amplified DNA of *M. tuberculosis*. The PAN (PAN-03) and MTB probes were the number 1 and number 30 probes and the number 2 probe, respectively, at the result of signal intensity. The detection limit was determined by amplification and hybridization of serial dilutions of DNA (500-pg/ μ l to 500-fg/ μ l dilutions of the *M. tuberculosis* DNA initial concentrations) and based on triplicate samples. Signal intensity (S.I) of hybridization was analyzed by using an intelligent analysis program (CombiView). (B) Before hybridization with oligonucleotide array, ITS of *M. tuberculosis* (5-ng/ μ l to 500-fg/ μ l dilutions of the DNA initial concentrations) was amplified with biotin-labeled primers and separated in a 2% agarose gel. Lanes M, 100-bp DNA ladder size markers.

cluding biochemical investigation. Among strains with overlapping phenotypic patterns, the conventional procedures are unable to distinguish organisms belonging to a certain taxon from the other species. There was a similar result by conventional identification in our study. As shown in Table 2, nine *M. gordonae* isolates which were identified by conventional methods were identified as six *M. gordonae*, two *M. tuberculosis*, and one *M. flavescens* by the oligonucleotide array. Since *M. tuberculosis* is considered clinically significant, whereas *M. gordonae* is not, to distinguish among them is essential. ITS-based oligonucleotide array could discriminate between *M. gordonae* and *M. szulgai*, *M. gordonae* and *M. tuberculosis*, and *M. chelonae* and *M. abscessus*, which are hard to differentiate by a conventional method. Two clinical specimens were shown to hybridize with only the PAN probe. They were interpreted as an unusual or unknown mycobacterium by the oligonucleotide array. After the sequencing of ITS, we could identify them as *M. celatum* and *M. lentiflavum* (Table 2). Therefore, even if mycobacteria are not species-specific probes contained in this oligonucleotide array, we can say that it is a mycobacterial species by hybridization with the PAN probe, so unusual or unknown strains of mycobacteria may be detected from clinical specimens. Consequently, some studies have tried to develop an oligonucleotide array containing a huge number of probes for the detection of many kinds of microorganisms. Photolithography chips displaying over 250,000 oligonucleotides can now be produced to perform sequence analysis on amplified small-subunit rRNA genes (23). A high-density microarray has also been described in the identification of 54 different mycobacterial species by using 82 unique 16S rRNA sequences and all known mutations associated with rifampin and isoniazid resistance in *M. tuberculosis* (12). On the other hand, a relatively small number of probes were included in our oligonucleotide array because of cost-effectiveness. But a genus-specific probe (PAN-03) makes it possible to detect the presence of unusual or unknown mycobacteria. This is a reasonable approach to routine diagnosis with low cost, we believe.

In conclusion, we developed the oligonucleotide array based on the ITS sequence for the identification and discrimination of medically important mycobacterial species in a single hybridization. The advantages of this oligonucleotide array are the identification of 20 mycobacterial species, except for MAC, with a single probe in a single hybridization; the discrimination between closely related species, such as *M. chelonae* and *M. abscessus*; and detection of the presence of unusual or unknown mycobacteria in a rapid, easy-to-perform manner and at a low cost. Our results indicate that oligonucleotide array is very useful for the rapid identification and accurate discrimination of mycobacteria from clinical isolates and specimens in an ordinary clinical laboratory.

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