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Use of PCR and Reverse Line Blot Hybridization Macroarray Based on 16S-23S rRNA Gene Internal Transcribed Spacer Sequences for Rapid Identification of 34 *Mycobacterium* Species

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The aim of this study was to develop a PCR and reverse line blot hybridization (PCR-RLB) macroarray assay based on 16S-23S rRNA gene internal transcribed spacer sequences for the identification and differentiation of 34 mycobacterial species or subspecies. The performance of the PCR-RLB assay was assessed and validated by using 78 reference strains belonging to 55 *Mycobacterium* species, 219 clinical isolates which had been identified as mycobacteria by high-performance liquid chromatography or gas chromatography, three skin biopsy specimens from patients with suspected leprosy which had been shown to contain acid-fast bacilli, and isolates of 14 nonmycobacterial species. All mycobacteria were amplified in the PCR and hybridized with a genus-specific probe (probe MYC). The 34 species-specific probes designed in this study hybridized only with the corresponding *Mycobacterium* species. The mycobacterial PCR-RLB assay is an efficient tool for the identification of clinical isolates of mycobacteria; it can reliably identify mixed mycobacterial cultures and *M. leprae* in skin biopsy specimens.

Mycobacterium tuberculosis infection continues to increase worldwide at an estimated annual rate of 3.5% (http://www.who.int/tb/publications/global_report/2005/pdf/Full.pdf). The rapidly spreading human immunodeficiency virus (HIV) epidemic in many parts of the world will further increase the number of HIV-related cases of tuberculosis as well as the number of nontuberculous mycobacterial infections. The rapid and accurate identification of clinically significant mycobacterial species is necessary for optimal medical and public health interventions (3, 21).

The results of traditional methods for the identification of closely related *Mycobacterium* species by growth characteristics (pigment, growth rate, colonial morphology) and biochemical tests are often difficult to interpret, even for some very common species. The tests are cumbersome and time-consuming, require expertise, lack sensitivity and reproducibility (21), and often use nonstandardized reagents. Rapid biochemical methods, such as high-performance liquid chromatography (HPLC) of mycolic acids and gas chromatography (GC) of fatty acids, are available only in specialized laboratories (2).

The rapid identification of mycobacteria to the species level is recommended in clinical laboratories to ensure accurate diagnosis and effective therapy. This has stimulated the development in recent years (1, 4–9, 11–20, 22, 25, 29, 30) of a number of rapid and more accurate identification tools based on molecular technology.

Several DNA sequences or genes have been targeted for the differentiation of *Mycobacterium* species, including the 16S rRNA gene (8, 9), the 23S rRNA gene (13, 15), the 16S-23S rRNA gene spacer region (15), the 32-kDa protein gene (22), *dnaA* (14), *dnaJ* (25), *gyrB* (4), *recA* (1), *rpoB* (6, 17), *secA1* (29), and *sod* (30). There are at least two commercially available systems for the identification of *Mycobacterium* spp. based on line probe hybridization, namely, the INNO-LiPA Mycobacterium system (Immunogenetics, Ghent, Belgium), which targets the 16S-23S rRNA gene spacer region and which identifies 16 *Mycobacterium* species, and the GenoType Mycobacterium CM/AS system (Hain Life Science, Nehren, Germany), which targets the 23S rRNA gene and which identifies 13 species (14). None of these methods can differentiate all of the *Mycobacterium* species commonly isolated in the clinical laboratory; more discriminatory methods are still needed for clinical and epidemiological investigations.

In this study, we developed a PCR-reverse line blot hybridization (PCR-RLB) assay based on the 16S-23S rRNA gene internal transcribed spacers (ITSs) for the identification of a larger number of mycobacteria to the species or the subspecies level.

MATERIALS AND METHODS

Mycobacterial reference strains. Reference strains of the following *Mycobacterium* species were included in this study: (i) the members of the *M. tuberculosis* complex *M. africanum* ATCC 25420, *M. bovis* ATCC 19210, *M. bovis* BCG (National Institute for the Control of Pharmaceutical and Biological Products, China), *M. microti* ATCC 19422, and *M. tuberculosis* H37Rv and (ii) the nontuberculous mycobacteria *M. abscessus* ATCC 19977, *M. agri* ATCC 27406, *M. aichiense* ATCC 27280, *M. asiaticum* ATCC 25276, *M. aurum* ATCC 23366, *M. austroafricanum* ATCC 33464, *M. avium* ATCC 25291, *M. chelonae* subsp. *chelonae* ATCC 35752, *M. chitae* ATCC 19627, *M. chubuense* ATCC 27278, *M. diernhoferi* ATCC 19340, *M. duvalii* ATCC 43910, *M. fallax* ATCC 35219, *M. farcinogenes* ATCC 35753, *M. flavescens* ATCC 14474, *M. fortuitum* subsp. *fortuitum* ATCC 6841, *M. gadium* ATCC 27726, *M. gastri* ATCC 15754, *M. gilvum* ATCC 43909, *M. gordonae* ATCC 14470, *M. hemophilum* ATCC 29548, *M. intracellulare* ATCC 13950, *M. kansasii* ATCC 12478, *M. komossense* ATCC

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TABLE 1. Identification of 34 *Mycobacterium* species, by PCR-RLB

Species	Total no. of isolates	Identification method[a] (no. of isolates)	PCR result	RLB result with probe MYC[b]	Species-specific probe used for RLB[c] (no. of isolates)	ITS sequencing result (no. of isolates)
M. abscessus	12	GC (8)	+	+	ABS (12)	ND[d]
		HPLC (4)				
M. chelonae complex	28	GC (28)				ND
M. abscessus			+	+	ABS (26)	ND
M. chelonae a/b			+	+	CHE1 (2)	ND
M. chelonae	4	HPLC (4)	+	+	CHE1 (3), CHE and FOR1 (1)	ND
M. chelonae a/b						Uninterpretable[e] (1)
M. chelonae c					None found	ND
MAC	3	GC (3)	+	+	INT (3)	ND
M. avium	23	HPLC (23)	+	+	AVI (11), INT (10), AVI and KAN1 (2)	*M. intracellulare* (1), uninterpretable (2)
M. fortuitum complex	29	GC (22)	+	+	FOR1 (12); FOR2 and FOR3 (1); FOR1, FOR2, and FOR3 (9)	Uninterpretable (2)
		HPLC (7)	+	+	FOR1 (4); FOR2 and FOR3 (1); FOR1, FOR2, and FOR3 (2)	Uninterpretable (3)
M. gordonae	28	GC (20)	+	+	GOR (16); GOR and ABS (2); GOR and FOR1 (1); GOR and FOR1, FOR2, and FOR3 (1)	Uninterpretable (4)
		HPLC (8)	+	+	GOR (8)	ND
M. intracellulare	2	HPLC (2)	+	+	AVI (1), INT (1)	*M. avium* (1), *M. intracellulare* (1)
M. kansasii	3	GC (1)	+	+	KAN1 (1)	ND
		HPLC (2)	+	+	KAN1 (1), KAN2 (1)	ND
M. lentiflavum	2	HPLC (2)	+	+	LEN (2)	*M. lentiflavum* (2)
M. leprae	3[f]	ND	+	+	LEP (2), MTBC and INT (1)	*M. leprae* (2), uninterpretable (1)
M. malmoense	1	HPLC (1)	+	+	MAL (1)	ND
M. nonchromogenicum	11	GC (11)	+	+	− (10),[g] FOR1 (1)	*M. fortuitum* a (1)
M. phlei a	12	GC (12)	+	+	PHL1 (12)	ND
M. phlei b					None found	
M. scrofulaceum	15	GC (8)	+	+	SCR (7), SCR and INT (1)	Uninterpretable (1)
		HPLC (7)	+	+	SCR (7)	ND
M. smegmatis	2	HPLC (2)	+	+	SME (2)	ND
M. szulgai	1	GC (1)	+	+	SZU (1)	ND
M. triplex	2	HPLC (2)	+	+	TRI (2)	*M. triplex* (2)
M. tuberculosis complex	39	GC (20)	+	+	MTBC (39)	ND
		HPLC (19)				
M. ulcerans/*M. marinum*	1	HPLC (1)	+	+	ULC/MAR (1)	ND
M. xenopi	1	HPLC (1)	+	+	XEN (1)	ND
Total	222[h]					

[a] GC was performed with isolates obtained from Shenzhen Chronic Disease Hospital and Guangzhou Thoracic Hospital, China; HPLC was performed with isolates from the Mycobacterium Reference Laboratory, Centre for Infectious Diseases and Microbiology, ICPMR, Westmead, Australia.
[b] *MYC*, *Mycobacterium* genus-specific probe; +, positive hybridization.
[c] For the specificities of the probes on RLB, see Table 2.
[d] ND, not done; sequencing was not performed if the HPLC or GC and PCR-RLB assay results were the same.
[e] All cultures in which mixed species were present, as identified by the PCR-RLB assay, produced uninterpretable sequencing results.
[f] Three clinical biopsy specimens with acid-fast bacilli on microscopy presumed to be *M. leprae*.
[g] No probes specific for *M. nonchromogenicum* were included in the RLB.
[h] The total includes 219 clinical isolates and three skin biopsy specimens.

33014, *M. malmoense* ATCC 29571, *M. marinum* ATCC 927, *M. neoaurum* ATCC 25795, *M. nonchromogenicum* ATCC 19530, *M. obuense* ATCC 27023, *M. parafortuitum* ATCC 19686, *M. phlei* ATCC 11758, *M. porcinum* ATCC 33776, *M. pulveris* ATCC 35154, *M. rhodesiae* ATCC 27024, *M. scrofulaceum* ATCC 19981, *M. senegalense* ATCC 35796, *M. shimoidei* ATCC 27962, *M. simiae* ATCC 25275, *smegmatis* ATCC 19420, *M. szulgai* ATCC 35799, *M. terrae* ATCC 15755, *M. thermoresistibile* ATCC 19527, *M. tokaiense* ATCC 27282, *M. triviale* ATCC 23292, *M. ulcerans* ATCC 19423, *M. vaccae* ATCC 29678, and *M. xenopi* ATCC 19250).

Nonmycobacterial reference strains. Reference strains of the following nonmycobacterial species were included: *Corynebacterium paurometabolum* ATCC 8368, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 25304, *Legionella pneumophila* subsp. *pneumophila* ATCC 33153, *Mycoplasma pneumoniae* ATCC 29342, *Nocardia asteroides* ATCC 19247, *Nocardia brasiliensis* ATCC 19296, *Nocardia farcinica* ATCC 3308, *Nocardia transvalensis* ATCC 6856, *Rhodococcus equi* ATCC 6939, *Rhodococcus erythropolis* ATCC 25544, *Rhodococcus rhodochrous* ATCC 13808, *Rhodococcus sputi* ATCC 29627, *Rhodococcus wratislaviensis* ATCC 51786, and *Streptococcus pneumoniae* SSISP pn2L.

Clinical isolates. A total of 219 mycobacterial isolates were obtained from the following sources: Center for Infectious Disease and Microbiology (CIDM), Institute of Clinical Pathology and Medical Research, Westmead, Australia (n = 85); the Institute of Tuberculosis, Shenzhen Chronic Disease Hospital, China (n = 64); and the Clinical Laboratory, Guangzhou Thoracic Hospital, China (n = 70) (Table 1). They included isolates of the *M. tuberculosis* complex and the following nontuberculous *Mycobacterium* species: *M. abscessus*, *M. avium*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. lentiflavum*, *M. malmoense*, *M. nonchromogenicum*, *M. phlei*, *M. scrofulaceum*, *M. smegmatis*, *M. szulgai*, *M. triplex*, *M. ulcerans*, and *M. xenopi*. All isolates were from different

TABLE 2. Specificities and sequences of probes used for *Mycobacterium* PCR-RLB assay

Probe	Strain	Melting temp (°C)	GenBank accession no.	Sequence
MYC	*Mycobacterium* genus	68.59	L15623	301-GTGGTGGGGTGTGGTGTTTG-320
ABS	*M. abcessus*	65.37	AJ314869	32-GGG AAC ATA AAG TAG GCA TCT GTA GTG-58
ASI	*M. asiaticum*	72.03	AF191087	35-TGC AGG CCG TGT GGA GTT CTC-55
AVI	*M. avium*	69.12	AJ314863	126-AAC ACT CGG TCC GTC CGT GT-145
CHE1	*M. chelonae* a/b[a]	62.81	AJ314873	33-GGA ACA TAA AGC GAG TTT CTG TAG-56
CHE2	*M. chelonae* c	67.75	AJ291584	55-AGT GGA TGC ATG CTT GGT GAA-75
DIE	*M. diernhoferi*	66.16	AF186463	221-GTT CGG ACG GTG TCT GTT GTT-241
FOR1	*M. fortuitum* a/c	66.11	AJ291587/89	89-ACA AAC TTT TTT GAC TGC CAG ACA C-113
FOR2	*M. fortuitum* b	72.55	AJ291588	112-TTT GCG GTG ATG GGA CTG CC-131
FOR3	*M. fortuitum* e	68.51	AJ291591	107-GAT TTT GCG GTG ATG GGA CC-126
FLA	*M. flavescens*	65.20	AJ291586	121-GGA ACA CTG CTT TGA GGA ATC AT-143
GAS	*M. gastri*	71.46	X97633	79-GTG CGC AAC AGC AAG CAA GC-98
GIL	*M. gilvum*	71.85	AJ314876	138-CGG TTT TCC GGG GTT GTG GT-157
GOR	*M. gordonae*	67.27	AJ315574	43-CGT GAG GGG TCA TCG TCT GTA-63
HAE	*M. hemophilum*	68.86	AY579400	130-CAC TCA GGC TTG TCC CAT GTT G-151
INT	*M. intracellulare*	69.21	AJ306711	133-GGT CGA TCC GTG TGG AGT CC-152
KAN1	*M. kansasii* a/b	66.72	L42263	213-GGG TGC GCA ACT GTA AAT GAA T-234
KAN2	*M. kansasii* c	67.74	L42264	150-AAA AGT GCC CCA ATT GGT GG-169
LEN	*M. lentiflavum*	69.65	AF318174	266-CCG ACT TTG GTC GAC GTG GT-285
LEP	*M. leprae*	65.48	X56657	172-TTG TCC CTC ATC TTT GGT GGT-192
MAL	*M. malmoense*	73.38	Y14184	126-AAC ACT CGG CCA GTC CGC GT-145
PHL1	*M. phlei* a	70.80	AJ291596	128-AAC ACC GTG TCG AGG ACT GCC-148
PHL2	*M. phlei* b	66.89	AJ291597	107-AAA CGT TTG TCG TTC GGT GG-126
SCR	*M. scrofulaceum*	67.40	AJ314884	129-ACT CGG CTC GTT CTG AGT GGT-149
SEN	*M. senegalense*	66.28	Y10385	55-GTG AGG AGT CTG TGC GCT GTA G-72
SHI	*M. shimoidei*	66.34	X99219	84-TGC ACA ACA ACA AGC GAG AAG-104
SIM	*M. simiae*	65.16	Y14186	138-ACT TCG GTT GAA GTG GTG TCC-158
SME	*M. smegmatis*	68.82	AJ291599	91-AAC GTT GAG ATG CGG TGT GGT-110
SZU	*M. szulai*	69.36	X99220	131-AGG CTT GGC CAG AGC TGT TGT-1513
TER	*M. terrae*	65.90	AJ314868	93-GTG CAG AGG AAT TAC GAA CAA CAA-116
TRI	*M. triplex*	69.21	AF214587	84-CAA CAG CAG ACA ATC GCC AGA C-105
MTBC	*M. tuberculosis* complex	68.04	AJ315568	79-TGC ATG ACA ACA AAG TTG GCC-99
ULC/MAR	*M. ulcerans*/*M. marinum*	61.52	X99217	124-AAC ATC TCT GTT GGT TTC GG-143
VAC	*M. vaccae*	72.60	AJ291600	134–AAT GCC GGC GAG GGA AAT CA-153
XEN	*M. xenopi*	69.39	AJ314866	66-TGT TGG GCA GCA GGC AGT AAC-86

[a] Identification of subtypes a and b of *M. chelonae*, *M. fortuitum*, *M. kansasii*, and *M. phlei* was based on sequence variations in their ITS regions.

patients. Skin biopsy specimens from three patients with presumed leprosy which had been shown to contain acid-fast bacilli by microscopy were included in the study.

Mycobacteria were grown on Löwenstein-Jensen (L-J) slants with weekly inspection until visible growth was detected or in 7H12 broth (MGIT liquid medium; Becton Dickinson) until a positive growth index was signaled by the automated system. Colonies were scraped from the L-J slants and suspended in 1 ml Tris-HCl buffer (10 mM; pH 7.5). The bacteria were killed by heating them to 100°C for 30 min and were then stored at 4°C. The clinical isolates used had been identified to the species level by standard biochemical procedures, and their identities were confirmed by HPLC (CIDM isolates) or GC (isolates from both Chinese laboratories) (2).

Probe and primer design. Primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Sp2 (5′-GAT GCT CGC AAC CAC TAT *Y*CA-3′), which target the 16S-23S ITS region of the *M. tuberculosis* spacer sequence (GenBank accession number L15623), were modified from a previous publication (19); 35 species-specific probes based on the published 16S-23S ITS sequences of various *Mycobacterium* species were designed (Table 2). All probes and primers were checked for specificity against all sequences in GenBank by using QueryBD, WebANGIS GCG, SeqSearch, and Browse code in the Australian National Genomic Information Services (ANGIS) programs (http://www1.angis.org.au/WebANGIS/WebFM). The probes were designed to have similar melting temperatures above 62°C, and their lengths varied from 20 bp to 27 bp. All oligonucleotide probes and primers were synthesized with a 5′-terminal amino group and biotin labels (Sigma-Aldrich, St. Louis, Mo.), respectively.

DNA extraction and PCR amplification. The extraction of DNA was carried out with a commercially available kit (InstaGene Matrix; Bio-Rad Laboratories), according to the manufacturer's specifications. Amplification of an ITS segment was performed with primers Sp1 and Sp2 in a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM (each) deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dUTP), 25 pmol of each primer, 0.75 U of *Taq* DNA polymerase (all reagents were from QIAGEN, Pty. Ltd., Australia), and 5 μl of DNA. The thermal profile involved initial denaturation for 5 min at 96°C and 35 cycles with the following steps: 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. The final extension was for 10 min at 72°C. The PCR products were analyzed with SRBR Safe DNA gel stain (Molecular Probes Europe BV, The Netherlands).

Nucleotide sequencing. The purified PCR products were directly sequenced with forward primers Sp1 and Sp2 by using an Applied Biosystems model 373A DNA sequencer (Perkin-Elmer Applied Biosystems) and a BigDye Terminator cycle sequencing kit. Sequences were identified by using the FastA program group accessed through WebANGIS.

RLB assay. The RLB assay was performed by using a system which has previously been described in detail (28, 31). Briefly, the slots in a Miniblotter 45 apparatus (Immunetics) were filled with 150 μl of optimized concentrations of probe solution. Each PCR product was denatured and immediately chilled on ice. Hybridization was performed at 60°C for 40 min. The washed membrane was incubated in peroxidase-labeled streptavidin conjugate (Roche, Mannheim, Germany) at 42°C for 40 min. The washed membrane was then incubated in chemiluminescence blotting substrate (ECL Direct system; Roche) for 1 min, covered with Hyperfilm X-ray film (Amersham) for detection of chemiluminescence, and exposed to the X-ray film for 7 min.

Sensitivities of species-specific PCR and ITS PCR-RLB assay. *M. tuberculosis* H37Rv and *M. abscessus* stock suspensions were prepared from cultures after 2 and 3 weeks of growth, respectively, on Löwenstein-Jensen slants and adjusted to a 0.5 McFarland standard. DNA was extracted as described above. The detection limits of the individual species-specific PCR and the PCR-RLB assay were

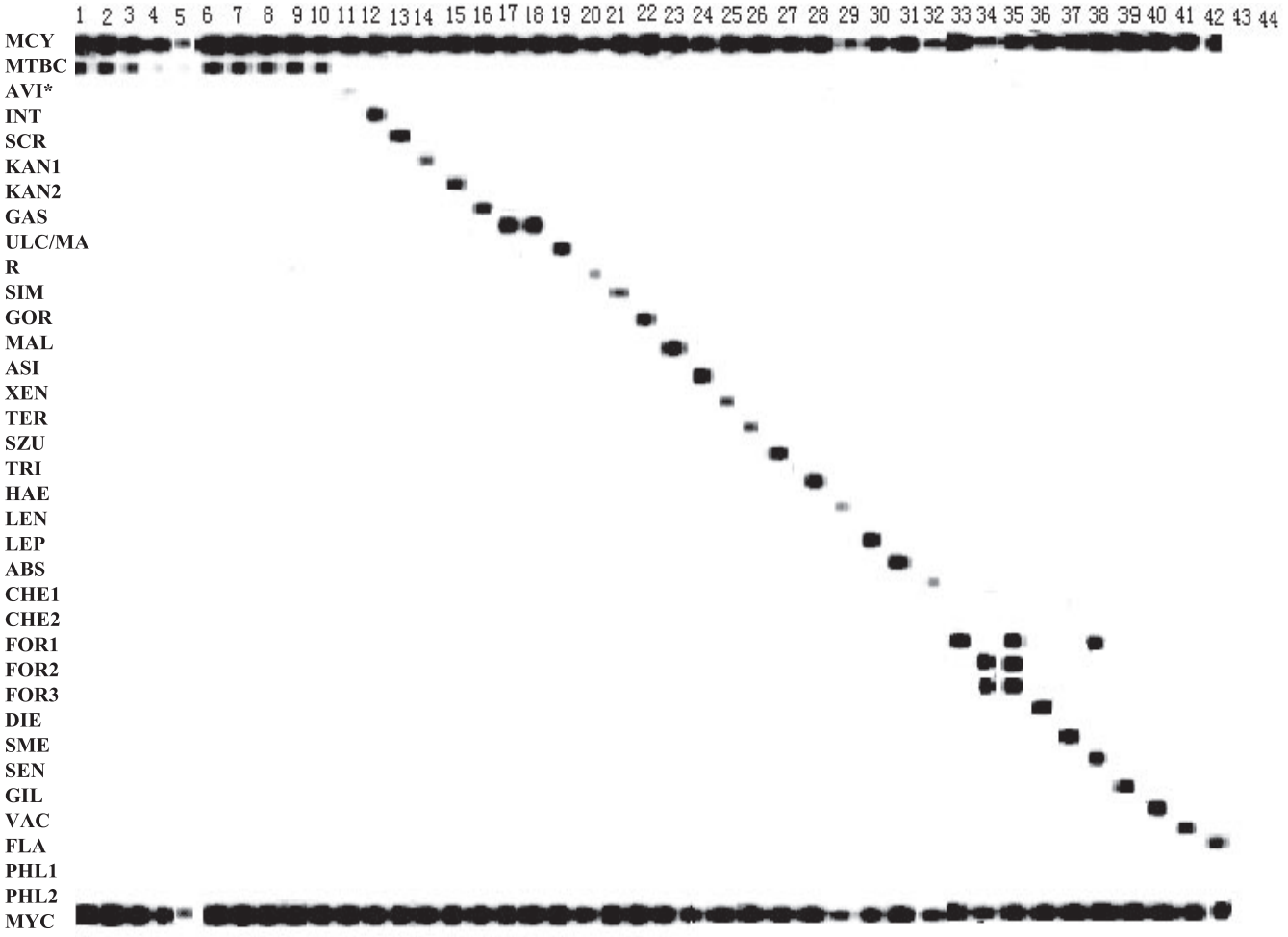

FIG. 1. Hybridization results with *Mycobacterium* species and other bacteria by RLB assay. Lanes 1 to 5, dilutions of *M. tuberculosis* H37Rv (500 pg/μl, 50 pg/μl, 5 pg/μl, 500 fg/μl, and 50 fg/μl, respectively); lane 6, *M. tuberculosis* H37Rv; lane 7, *M. bovis*; lane 8, *M. microti*; lane 9, *M. africanum*; lane 10, BCG; lane 11, *M. avium*; lane 12, *M. intracellulare*; lane 13, *M. scrofulaceum*; lane 14, *M. kansasii* a/b; lane 15, *M. kansasii* c; lane 16, *M. gastri*; lane 17, *M. ulcerans*; lane 18, *M. marinum*; lane 19, *M. simiae*; lane 20, *M. gordonae*; lane 21, *M. triplex*; lane 22, *M. malmoense*; lane 23, *M. asiaticum*; lane 24, *M. shimoidei*; lane 25, *M. xenopi*; lane 26, *M. terrae*; lane 27, *M. szulai*; lane 28, *M. lentiflavum*; lane 29, *M. hemophilum*; lane 30, *M. lepre*; lane 31, *M. abcessus*; lane 32, *M. chelonae* a/b; lane 33, *M. fortuitum* a/c; lane 34, *M. fortuitum* b; lane 35, *M. fortuitum* e; lane 36, *M. diernhoferi*; lane 37, *M. smegmatis*; lane 38, *M. senegalense*; lane 39, *M. gilvum*; lane 40, *M. vacca*; lane 41, *M. flavescens*; lane 42, *M. phlei* a; lane 43, *R. equi*; lane 44, *N. farcinica*. ∗, a weakly hybridizing band (lane 11, *M. avium*) was due to a low input DNA concentration.

determined by amplification and hybridization of the DNA of each species serially diluted from 5 ng/μl to 500 fg/μl with molecular biology-grade water and tested in parallel.

RESULTS

Sensitivities of PCR-RLB assay. The detection limits of the species-specific PCRs for both *M. tuberculosis* H37Rv and *M. abscessus* DNA were 5 pg/μl (data not shown). The PCR-RLB assay was 10 times more sensitive than the species-specific PCR assays for both *M. tuberculosis* H37Rv (Fig. 1) and *M. abscessus* DNA (data not shown); i.e., it was able to detect 500 fg/μl. The MYC (genus-specific) probe was more sensitive (50 fg/μl) than the MTBC (species-specific) probe for the detection of *M. tuberculosis* H37Rv (Fig. 1) but was less sensitive (500 fg/μl) than the ABS probe (50 fg/μl) for the detection of *M. abscessus* (data not shown).

Specificity of species-specific mycobacterial PCR-RLB assay. PCR amplification with primers Sp1 and Sp2 of DNA from all 78 mycobacterium reference strains produced single bands ranging in length from 200 to 320 bp. The amplicons from the slowly growing *Mycobacterium* species were smaller (200 to 250 bp) than those from the rapidly growing species (250 to 320 bp). *M. xenopi* produced the smallest product (~200 bp), and *M. gilvum* produced the largest product (~320 bp).

There were cross-reactions between the *Mycobacterium* genus-specific primers (Sp1 and Sp2) and DNA extracts from *Nocardia* spp. and *Rhodococcus* spp., which produced two to three copies of the amplicon (Fig. 2). However the genus-specific probe (MYC) hybridized only with *Mycobacterium* spp., and with a few exceptions, all species-specific probes hybridized only with the corresponding species. The exceptions were as follows: the probe for *M. marinum* cross-reacted with

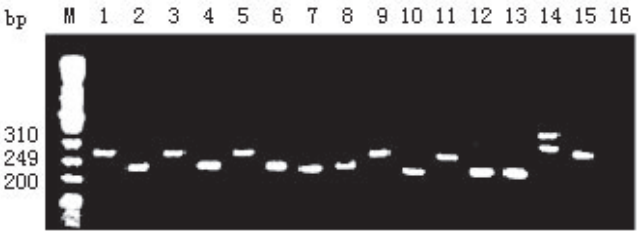

FIG. 2. PCR results for *Mycobacterium* species and other bacteria obtained by using primers Sp1 and Sp2 (based on the 16S-23S rRNA gene ITS). Lanes: M, DNA size standards (Hinf DNA; 24, 40, 66, 82, 118, 140, 151, 200, 249, 311, 413, 417, 427, 500, 553, 713, and 726 bp); 1, *M. smegmatis*; 2, *M. triplex*; 3, *M. fortuitum*; 4, *M. scrofulaceum*; 5, *M. flavescens*; 6, *M. lepre*; 7, *M. tuberculosis*; 8, *M. simiae*; 9, *M. gilvum*; 10, *M. ulcerans*; 11, *M. abcessus*; 12, *M. szulai*; 13, *M. avium*; 14, *N. farcinica*; 15, *R. equi*; 16, *K. pneumoniae*. Note the cross-reactions with *Nocardia* and *Rhodococcus* species, which are resolved by RLB assay.

M. ulcerans because the ITS regions of these two species are identical, one *M. senegalense* reference strain (ATCC 35796) reacted with the FOR1 probe (specific for *M. fortuitum* a/c and *M. senegalense*), and one *M. fortuitum* subsp. *fortuitum* (ATCC 6841) reference strain did not react with the SEN probe (specific for *M. senegalese*) (Fig. 1). The PCR-RLB assay allowed differentiation of *M. chelonae* from *M. abscessus*, *M. gastri* from *M. kansasii*, and *M. avium* from *M. intracellulare*. *M. tuberculosis* complex subspecies could not be distinguished from each other.

PCR-RLB assay with clinical isolates of mycobacteria. A total of 219 clinical isolates were tested "blind" (i.e., without knowledge of the species identity) by the PCR-RLB assay. The PCR-RLB assay was repeated with the few isolates for which there were discrepancies between the results of the PCR-RLB assay and those of GC or HPLC (Table 2). The PCR-RLB assay (including duplicate tests) and sequencing results were identical for all isolates. Three isolates that were identified as members of the *M. avium-M. intracellulare* complex by GC were identified as *M. intracellulare* by the PCR-RLB assay. Of 23 isolates identified as *M. avium* by HPLC, 11 were identified as *M. avium*, 10 were identified as *M. intracellulare*, and 2 were identified as *M. avium* mixed with *M. kansasii* a/b by the PCR-RLB assay. Of 28 isolates identified as *M. chelonae-M. abscessus* complex by GC, 26 were identified as *M. abscessus*, 1 was identified as *M. chelonae* a/b, and 1 was identified as *M. chelonae* c by the PCR-RLB assay. Of four isolates identified as *M. chelonae* by HPLC, three were identified as *M. chelonae* a/b and one was identified as *M. chelonae* c by the PCR-RLB assay. Two clinical isolates that had been reported as resembling *M. fortuitum* by HPLC were shown by the PCR-RLB assay and sequencing to be *M. triplex*, and the identities of two clinical isolates presumptively identified as *M. lentiflavum* by HPLC were confirmed to be *M. lentiflavum* by PCR-RLB and sequencing.

In all, the PCR-RLB assay identified 22 mixed cultures (Table 1). In addition to two with a mixture of *M. avium* and *M. kansasii*, there were three in which two potentially pathogenic species were identified: *M. chelonae* and *M. fortuitum*, *M. tuberculosis* complex and *M. intracelluare*, and *M. scrofulaceum* and *M. intracelluare*. Four cultures initially identified as *M. gordonae* (usually a contaminant) were mixed with another species, and 13 cultures apparently contained mixtures of *M. fortuitum* subtypes.

DNA from two of the three biopsy specimens from patients with clinical leprosy hybridized with the LEP probe and were confirmed by sequencing to contain *M. leprae*. The other hybridized with both the MTB and the INT probes, but the sequencing result was uninterpretable, suggesting that both *M. tuberculosis* and *M. intracellulare* were present. In addition to this clinical specimen, the PCR-RLB assay identified mixed species in 14 separate clinical isolates and the sequencing results were uninterpretable.

DISCUSSION

In comparison to conventional biochemical methods, GC and HPLC provide rapid results. However, both require expensive instrumentation, reproducible patterns depend on standardized conditions of growth, and interpretation of the results is not always easy. The PCR-RLB assay does not require standardized growth conditions, and it is more accurate and objective than either GC or HPLC. In our study, two clinical isolates which had presumptively been identified as *M. fortuitum* by HPLC were identified by the PCR-RLB assay as *M. triplex*, and this result was confirmed by sequencing. The PCR-RLB assay identified mixed mycobacterial cultures which had not been recognized by HPLC or GC and which gave uninterpretable sequencing results. Some previously unidentified species were potentially clinically significant. The finding of four mixed cultures initially identified as containing only *M. gordonae* indicates that this contaminant *Mycobacterium* species can mask potential pathogens. It is not clear whether the high proportion of mixed subtypes of *M. fortuitum* indicates that cross-reactions between probes occur or that mixtures of subtypes commonly occur in nature.

In addition, the PCR-RLB assay was able to discriminate between *M. chelonae* and *M. abscessus*, which could not be distinguished by GC, and between *M. avium* and *M. intracellulare*, which could not be distinguished by HPLC (Table 1).

Sequencing of the 16S rRNA gene is often regarded as the "gold standard" for the identification of mycobacteria (5), but it has a demonstrated lower variability with several examples of species difficult to separate from the genus *Mycobacterium* (10, 25). Some *Mycobacterium* species share the same 16S rRNA gene sequence (*M. kansasii* and *M. gastri*, *M. senegalense* and *M. farcinogenes*) or have very similar 16S rRNA gene sequences (e.g., *M. malmoense* and *M. szulgai*) (24).

The 16S-23S rRNA gene ITS region has been identified as a potentially suitable target for probes that can differentiate closely related species. It contains both conserved and highly variable signatures and is rather small. The 16S-23S rRNA gene ITS-based PCR produces a relatively small PCR product (200 to 350 bp) (Fig. 2). Roth et al. reported that PCR based on the 16S-23S rRNA gene ITS and restriction fragment length polymorphism (RFLP) analysis is a promising method with advantages over the previously used *hsp65* gene-based method for the reliable and easy identification of mycobacteria (19, 20). However, it has been impeded by difficulties, such as minor differences in band sizes between some species and the occurrence of new patterns not previously reported (17, 27). Moreover, RFLP analysis cannot reliably detect mixtures of

species within samples. Others have also found that the presence of more than one species in a culture or a clinical sample makes it difficult or impossible to identify the species present by sequencing or RFLP analysis of 16S-23S rRNA gene ITS PCR products (10).

In our study, from the analysis of 78 reference strains, 219 clinical isolates, and three skin biopsy specimens, the specificity of our PCR-RLB assay, based on the 16S-23S rRNA gene ITS region, was close to 100% (except that *M. senegalense* reference strain ATCC 35796 reacted with the FOR1 probe). Several other systems that are similar to our PCR-RLB assay system are available: the INNO-LiPA strip (version 2; Immunogenetics) (15, 23, 26), the GenoType Mycobacterium system (Hain Life Science) (13), and an oligonucleotide array (16) can identify 16, 13, and 19 *Mycobacterium* species, respectively. Our PCR-RLB assay based on the 16S-23S rRNA gene ITS can differentiate 34 mycobacterial species and subspecies by the use of 35 probes and has a high degree of specificity.

Our assay is as simple as other reverse hybridization methods, but it is more sensitive because it uses a nonprecipitating enhanced chemiluminescent substrate and Hyperfilm X-ray film. It successfully identified mycobacteria directly in skin biopsy specimens and has the potential to be used for the direct identification of *Mycobacterium* species from other types of clinical specimens. In addition, it has the advantage that the nylon filters can be stripped and reused at least 30 times and therefore is relatively inexpensive.

In conclusion, the PCR-RLB assay method described here is faster than conventional biochemical tests, is more accurate than HPLC or GC, is less expensive than sequencing, and does not require specialized instrumentation. It is more discriminatory than methods based on 16S rRNA gene-specific PCR or PCR-RFLP analysis of other genes (e.g., *rpoB*) and identifies more species than other PCR-RLB assays based on the 16S-23S rRNA gene ITS. It has the potential to be used to identify *Mycobacterium* species directly in clinical specimens.

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