Novel Algorithm Identifies Species in a Polymycobacterial Sample by Fluorescence Capillary Electrophoresis-Based Single-Strand Conformation Polymorphism Analysis

Tomotada Iwamoto,^{1*} Toshiaki Sonobe,¹ and Kozaburo Hayashi²

Department of Bacteriology¹ and Department of Parasitic Agents,² Kobe Institute of Health, 4-6 Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan

Received 10 June 2002/Returned for modification 5 August 2002/Accepted 17 September 2002

An algorithm to directly identify multiple mycobacterial species in a sample by using fluorescence capillary electrophoresis (FCE)-based single-strand conformation polymorphism (SSCP) analysis was developed. Part of the 16S-23S ribosomal DNA internal transcribed spacer (ITS) region in 37 reference strains and 73 clinical isolates representing 19 mycobacterial species and *Mycobacterium tuberculosis* complex was PCR amplified with a fluorescence-labeled mycobacterium-specific primer, 6-carboxyfluorescein-labeled primer Sp1f, and 5-hexachlorofluorescein-tagged Sp2r. FCE-SSCP analysis was applied to both undigested PCR products and the corresponding *Hae*III-digested restriction fragments (RF) from each strain. The 23 resultant SSCP patterns distinguished all 19 species and *M. tuberculosis* complex. The technique is applicable for the detection of multiple mycobacterial species in a sample. It was demonstrated by analyzing two model mycobacterial communities consisting of five species with both rapidly and slowly growing species (model A) and four species commonly encountered in clinical practice (model B). The sensitivity study with spiked sputum samples with different amounts of *M. tuberculosis* H37Rv, *M. avium*, and *M. intracellulare* cells indicated that up to 25% of the amount of each mycobacterium could be detected relative to the two other species. Fifty-one sputum specimens analyzed by FCE-RF-SSCP were compared with the Amplicor assay (Roche Diagnostics GmbH). Species identified by both assays were always the same. Moreover, FCE-RF-SSCP could identify *M. abscessus* and *M. kansasii*, which are not targeted by Amplicor. FCE-RF-SSCP of sputum obtained from a patient with mixed *M. avium* and *M. intracellulare* infection gave SSCP patterns corresponding to these two species.

Recent increases in infections caused by *Mycobacterium tuberculosis* and nontuberculous mycobacteria are a serious hazard to public health (2, 6, 11–13, 20). More than 30 mycobacterial species are recognized as pathogenic or potentially pathogenic. Mixed infections with two or more species (1, 18, 29, 31) and nosocomial infections (3, 8, 19, 25, 27) are always troublesome. Therefore, timely identification of causative mycobacteria is imperative for appropriate therapy and epidemiological analyses.

Genotyping the sequence polymorphisms in the genus *Mycobacterium* can identify species quickly and unequivocally. PCR-restriction fragment polymorphism analysis (PRA) of the *hsp65* gene (4, 21, 22, 28), 16S ribosomal DNA (rDNA) (5, 14, 15, 30), 16S-23S rDNA internal transcribed spacer (ITS) (23), *dnaJ* (26), *rpoB* (17), and *gyrB* (16) has been intensively studied. However, these methods are not applicable for a polymycobacterial sample because too many restriction fragments from multiple species of mycobacteria in it blur the analysis.

We developed a fluorescence capillary electrophoresis (FCE)-based restriction fragment–single-strand conformation polymorphism (FCE-RF-SSCP) technique which can simultaneously identify multiple mycobacterial species in a sample. We first established an algorithm for species identification by applying this technique to 110 defined mycobacterial strains.

The technique was further validated on “spiked” specimens, clinical sputum specimens, and sputum obtained from a patient with mixed *M. avium* and *M. intracellulare* infection.

(This work was presented in part at the 102nd Annual Meeting of the American Society for Microbiology, Salt Lake City, Utah, 20 May 2002.)

MATERIALS AND METHODS

Mycobacteria and DNA preparation. Genomic DNAs used for the establishment of an algorithm were isolated from 37 reference strains and 73 clinical isolates representing 19 mycobacterial species and *M. tuberculosis* complex with Isoplant (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions (Table 1). Clinical isolates were identified by conventional biochemical tests, Accu-Probe (Gen-Probe, Inc., San Diego, Calif.), DDH Mycobacteria ‘Kyokuto’ (Kyokuto Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), or partial 16S rDNA sequencing.

Clinical sputum specimens. Fifty-one sputum specimens from patients with suspected *M. tuberculosis* or nontuberculous mycobacterial infections were obtained from Kobe City General Hospital. After decontamination with *N*-acetyl-L-cysteine-NaOH, DNA was extracted from these specimens with a respiratory specimen preparation kit (Roche Diagnostics GmbH). The sputum specimens were also subjected to acid-fast staining and cultured. One frozen sputum sample obtained from a patient with mixed *M. avium* and *M. intracellulare* infection was retrospectively analyzed by FCE-RF-SSCP.

DNA amplification and restriction enzyme digestion. Genus *Mycobacterium*-specific primers Sp1f and Sp2r, designed by Roth et al. (23), were used to amplify part of the 16S-23S rRNA gene spacer. A reaction mixture (50 μ l) containing 20 pmol of 6-carboxyfluorescein (FAM)-labeled Sp1f and 5-hexachlorofluorescein (HEX)-tagged Sp2r primers, 1.5 mM MgCl₂, 3.5 μ l of dimethyl sulfoxide, and 1 to 20 μ l of DNA solution was amplified with the Expand high-fidelity PCR system (Roche Diagnostics GmbH) following the thermal profile method of Roth et al. (23). Fivefold-diluted PCR product (9 μ l) was digested at 37°C for 3 h

* Corresponding author. Mailing address: Department of Bacteriology, Kobe Institute of Health, 4-6 Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan. Phone: 81 78 302 6251. Fax: 81 78 302 0894. E-mail: kx2t-iwmt@asahi-net.or.jp.

TABLE 1. Strains used to establish an algorithm for species identification

Species	Strain ^a	Serovar	Sequevar	No. of tests run
<i>M. avium</i>	ATCC 15769	1		1
	ATCC 25291	2	Mav A	5
	ATCC 35718	3		1
	ATCC 35767	4	Mav A	1
	ATCC 35768	5		1
	ATCC 35773	6		1
	ATCC 35771	8		1
	ATCC 35774	9	Mav D	3
	ATCC 35765	10	Mav B	3
	ATCC 35766	11	Mav A	1
	Clinical isolates (14)			1
<i>M. intracellulare</i>	ATCC 35762	12	Min A	5
	ATCC 35769	13		1
	ATCC 35761	14	Min A	1
	ATCC 35848	15	Min A	1
	ATCC 13950	16	Min A	1
	ATCC 35763	17	Min A	1
	ATCC 35770	18	MAC D	5
	ATCC 35772	19	Sequevar V	5
	ATCC 35764	20	Min A	1
	Clinical isolates (18)			1
<i>M. tuberculosis</i> complex	H37Rv			4
	BCG Tokyo			3
	Clinical isolates (12)			1
<i>M. kansasii</i>	ATCC 12478		MKa A	6
	Clinical isolates (7)			1
<i>M. marinum</i>	ATCC 927			5
	Clinical isolate (1)			5
<i>M. scrofulaceum</i>	ATCC 19981		Mscro A	4
<i>M. simiae</i>	ATCC 25275		Sequevar I	3
<i>M. goodii</i>	ATCC 14470		Mgo A	4
	Clinical isolates (9)			1
<i>M. szulgai</i>	ATCC 35799			4
<i>M. lentiflavum</i>	Clinical isolate (1)			4
<i>M. xenopi</i>	ATCC 19250		Mxe A	3
<i>M. nonchromogenicum</i>	ATCC 19530			3
<i>M. gastri</i>	ATCC 15754			3
<i>M. terrae</i>	Clinical isolate (1)			3
<i>M. chelonae</i>	ATCC 19237			3
	Clinical isolate (1)			3
<i>M. abscessus</i>	ATCC 19977			6
	Clinical isolates (4)			2
<i>M. smegmatis</i>	ATCC 14468			3
<i>M. phlei</i>	ATCC 11758		Mphle A	3
<i>M. diernhoferi</i>	ATCC 19340			3
<i>M. fortuitum</i>	ATCC 6841		Mfo A	3
	ATCC 19542			3
	Clinical isolates (5)			1

^a The number of clinical isolates tested is shown in parentheses.

FIG. 1. FCE-RF-SSCP analysis of the internal standard, *Mycobacterium avium* ATCC 25291 undigested ITS fragment and *Hae*III-digested fragment. 6-carboxy-X-rhodamine-labeled internal standard, FAM-labeled fragments, and HEX-labeled fragments are shown as red peaks, blue peaks, and green peaks, respectively. Arbitrary electrophoretic mobility values for internal standard were assigned as follows: peak 1, 3859; peak 2, 3960; peak 3, 4096; peak 4, 4125; peak 5, 4157; peak 6, 4219; peak 7, 4404; peak 8, 4426; peak 9, 4462; peak 10, 4493; peak 11, 4817; peak 12, 4880; peak 13, 4919; peak 14, 5150; peak 15, 5188; peak 16, 5454; peak 17, 5485; peak 18, 5538; peak 19, 5563; peak 20, 5675; peak 21, 5702; peak 22, 5716; and peak 23, 5739.

in a buffer containing 5 U of *Hae*III enzyme (Takara Shuzo Co., Ltd., Shiga, Japan).

Artificially constructed mycobacterial community. Two model mycobacterial communities were constructed. Model A represents a mixture of rapid- and slow-growing species consisting of *M. abscessus* ATCC 19977, *M. avium* ATCC 25291, *M. chelonae* ATCC 19237, *M. intracellulare* ATCC 35772, and *M. xenopi* ATCC 19250. Model B is a mixture of *M. avium* ATCC 25291, *M. intracellulare*

ATCC 13950, *M. tuberculosis* H37Rv, and *M. kansasii* ATCC 12478, which is responsible for more than 95% of the cases of mycobacteriosis in Japan (24). Five nanograms of purified genomic DNAs from each species was suspended together in the PCR mixture and used for FCE-RF-SSCP analysis after the PCR.

Spiked specimen preparation. *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950, and *M. tuberculosis* H37Rv were individually cultured in Middlebrook

TABLE 2. Algorithm for the identification of *Mycobacterium* species by FCE-RF-SSCP

Electrophoretic mobility values (SD)				Species and SSCP genotype	Reference sequevar	No. of strains (reference/clinical)
ITS 6-FAM	ITS HEX	<i>Hae</i> III 6-FAM	<i>Hae</i> III HEX			
4323.0 (2.0)	4435.3 (1.5)	3643.0 (1.3)	4100.1 (1.5)	<i>M. xenopi</i>	Mxe A	1/0
4359.7 (1.5)	4484.0 (2.0)	3683.6 (1.5)	3732.7 (2.0)	<i>M. szulgai</i>		1/0
4362.8 (1.3)	4530.8 (1.4)	3688.0 (1.0)	3794.3 (2.1)	<i>M. lentiflavum</i>		0/1
4383.7 (1.4)	4465.0 (3.4)	No digestion		<i>M. gordonae</i>	Mgo A	1/9
4396.8 (3.3)	4490.8 (2.5)	3414.6 (2.8)	3918.4 (2.2)	<i>M. tuberculosis</i> complex		2/12
4422.1 (2.9)	4479.5 (1.8)	4021.3 (1.5)	3849.1 (0.3)	<i>M. scrofulaceum</i>	Mscro A	1/0
4423.4 (3.2)	4479.5 (2.9)	3688.5 (4.8)	3885.5 (2.4)	<i>M. avium</i>	Mav A, Mav B, Mav D	10/14
4429.8 (2.4)	4521.6 (2.2)	No digestion		<i>M. kansasii</i>	MKa A	1/7
4435.2 (2.4)	4563.2 (1.4)	No digestion		<i>M. marinum</i>		1/1
4436.5 (2.2)	4557.5 (1.5)	3691.3 (1.1)	3798.3 (1.1)	<i>M. simiae</i>	Sequevar I	1/0
4441.6 (1.4)	4538.2 (1.8)	3960.9 (1.5)	3890.8 (1.0)	<i>M. intracellulare</i> III	MAC D	1/0
4444.0 (1.9)	4515.0 (1.9)	3686.3 (4.3)	3890.4 (2.0)	<i>M. intracellulare</i> I	Min A	7/14
4448.5 (1.6)	4519.5 (1.0)	3696.8 (1.1)	4164.9 (0.8)	<i>M. gastri</i>		1/0
4462.5 (2.3)	4521.3 (2.8)	4024.5 (2.9)	3835.2 (2.6)	<i>M. intracellulare</i> II	Sequevar V	1/4
4592.1 (1.8)	4667.0 (2.1)	4161.3 (1.3)	3968.9 (2.1)	<i>M. fortuitum</i> II		1/0
4614.9 (2.5)	4673.1 (3.4)	No digestion		<i>M. chelonae</i>		1/1
4633.8 (2.5)	4735.1 (3.3)	4168.9 (3.5)	3875.1 (2.9)	<i>M. smegmatis</i>		1/0
4635.8 (3.4)	4743.2 (2.1)	No digestion		<i>M. abscessus</i>		1/4
4649.5 (1.9)	4749.5 (1.3)	4256.5 (1.1)	3977.0 (4.5)	<i>M. fortuitum</i> I	Mfo A	1/5
4731.5 (1.3)	4867.3 (2.3)	4323.0 (1.4)	4013.4 (1.2)	<i>M. phlei</i>	Mphle A	1/0
4736.9 (2.0)	4762.4 (0.5)	3680.9 (1.2)	3997.3 (1.3)	<i>M. diernhoferi</i>		1/0
4715.4 (2.5) ^b	4846.4 (1.9)	3809.0 (2.1)	3971.1 (1.0)	<i>M. terrae</i>		0/1
4736.7 (1.9)						
4784.7 (2.4)	4827.2 (2.1) ^a	4228.4 (1.4)	3920.0 (2.3)	<i>M. nonchronogenicum</i>		1/0
	4876.0 (1.8)					

^a Fragments yielded two peaks.

FIG. 2. FCE-RF-SSCP analysis of 16S-23S rDNA ITS fragments amplified from artificially constructed mycobacterial communities. Electrophoretic mobility values are indicated at each peak on the electropherograms for FAM-labeled fragments (in blue) and HEX-labeled fragments (in green).

7H9 broth until the turbidity equaled a McFarland no. 1 standard and was then adjusted to the same cell density with an appropriate dilution. Each of these liquid cultures (10 μ l) was mixed with 100 μ l of acid-fast bacillus-negative sputum sample that was diluted with an equivalent amount of phosphate-buffered saline. The amount of *M. tuberculosis* H37Rv was serially diluted twofold, while the other strains were kept constant to produce five levels: no dilution, twofold, fourfold, eightfold, and 16-fold dilutions. One hundred microliters of each mixture was used for DNA extraction after NALC-NaOH treatment.

SSCP electrophoresis. The PCR products were analyzed for SSCP on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) by Gillman's method (7) with modifications: 0.5 μ l of 0.3 N NaOH was added to the SSCP analysis master mix, the SSCP analysis mixture was then denatured by heating at 95°C for 5 min and left at room temperature, and the retention times of the fluorescently labeled fragments were determined against that of the 6-carboxy-X-rhodamine-labeled internal standard (Fig. 1). In addition, the conditions for electrophoresis were set as follows: 5-s injection time, 15.0-kV injection

voltage, 13-kV electrophoresis voltage, 210-s syringe pump time, 30°C constant temperature of the capillary at a room temperature of 23 to 24°C, and a 23-min collection time. The PCR products and corresponding *Hae*III restriction fragments were run in two separate sample tubes after appropriate dilution with sterilized water (in most cases, diluted 1:10).

RESULTS

FCE-RF-SSCP analysis of 19 mycobacterial species and *M. tuberculosis* complex showed 23 SSCP patterns that could be used as the algorithm to identify the species and subtypes of these mycobacteria (Table 2). *M. tuberculosis* H37Rv and *M. bovis* BCG Tokyo could not be discriminated because the 16S-23S rDNA ITS sequences are identical among *M. tuberculosis*

TABLE 3. Identification of mycobacterial species from the SSCP patterns shown in Fig. 2^a

Model	Undigested 6-FAM		Undigested HEX		HaeIII 6-FAM		HaeIII HEX		Identification
	Mobility	Possible species	Mobility	Possible species	Mobility	Possible species	Mobility	Possible species	Mixture of:
A	4326.4	<i>M. xenopi</i>	4433.4	<i>M. xenopi</i>	3643.7	<i>M. xenopi</i>	3836.1	<i>M. intracellulare</i> II	<i>M. xenopi</i>
	4426.2	<i>M. scrofulaceum</i>	4478.7	<i>M. szulgai</i>	3690.2	<i>M. lentiflavum</i>	3884.8	<i>M. avium</i>	<i>M. avium</i>
		<i>M. avium</i>		<i>M. scrofulaceum</i>		<i>M. avium</i>			<i>M. intracellulare</i> II
		<i>M. kansasii</i>		<i>M. avium</i>		<i>M. simiae</i>			<i>M. chelonae</i>
						<i>M. intracellulare</i> I			<i>M. abscessus</i>
	4460.9	<i>M. intracellulare</i> II	4521.7	<i>M. kansasii</i>	4021.5	<i>M. scrofulaceum</i>	4101.2	<i>M. xenopi</i>	
B				<i>M. gastri</i>		<i>M. intracellulare</i> II			
	4614.1	<i>M. chelonae</i>	4671.9	<i>M. fortuitum</i> II	4612.5	<i>M. chelonae</i>	4670.2	<i>M. chelonae</i>	
	4637.1	<i>M. smegmatis</i>	4745.6	<i>M. abscessus</i>	4636.4	<i>M. abscessus</i>	4744.5	<i>M. abscessus</i>	
		<i>M. abscessus</i>		<i>M. fortuitum</i> I					
	4395.1	<i>M. tuberculosis</i> complex	4479.5	<i>M. szulgai</i>	3415.2	<i>M. tuberculosis</i> complex	3886.1	<i>M. avium</i>	<i>M. tuberculosis</i>
				<i>M. scrofulaceum</i>				<i>M. intracellulare</i> III	complex
	4421.0	<i>M. scrofulaceum</i>	4490.9	<i>M. avium</i>	3690.0	<i>M. lentiflavum</i>	3918.1	<i>M. intracellulare</i> I	<i>M. avium</i>
		<i>M. avium</i>		<i>M. tuberculosis</i> complex		<i>M. avium</i>		<i>M. tuberculosis</i> complex	<i>M. kansasii</i>
						<i>M. simiae</i>		<i>M. nonchronogenicum</i>	<i>M. intracellulare</i> I
	4432.0	<i>M. kansasii</i>	4516.5	<i>M. gastri</i>	4433.6	<i>M. kansasii</i>	4522.1	<i>M. kansasii</i>	
		<i>M. marinum</i>		<i>M. intracellulare</i> I		<i>M. marinum</i>			
	4442.9	<i>M. intracellulare</i> III	4523.9	<i>M. intracellulare</i> II					
		<i>M. intracellulare</i> I		<i>M. kansasii</i>					
				<i>M. gastri</i>					
				<i>M. intracellulare</i> II					

^a See Table 2, footnote a.

complex isolates (9, 16). *M. terrae* and *M. nonchronogenicum* had multiple peaks (Table 2). The method is highly reproducible, with reasonable intraspecies stability of SSCP (Table 2). Based on the standard deviation of SSCP peak placement, we applied a tolerance level of ± 5 data points in the algorithm for species identification.

The established polymorphisms among SSCP patterns for the 19 species and *M. tuberculosis* complex suggest that species can be distinguished even when multiple mycobacterial species are found in a single sample. To test this hypothesis, two model mycobacterial communities representing a mixture of rapid- and slow-growing species (model A) and the most ubiquitous mycobacterial species found in clinical practice (model B) were constructed. The FCE-RF-SSCP analysis of the model communities yielded characteristic SSCP patterns (Fig. 2) from which all of the mixed strains were correctly identified (Table 3).

The species belonging to each peak in Fig. 2 were identified as follows (Table 3). In the initial step, we listed all of the possible species under the peaks with the algorithm, then the species that appeared on all four peaks, undigested 6-FAM, undigested HEX, HaeIII 6-FAM, and HaeIII HEX, were judged as the existing species in a sample. The blue peak (3690.0 data points) and green peak (3886.1 data points) in the HaeIII fragments of model B are considered overlapping peaks originating from *M. avium* and *M. intracellulare* I. Besides these two model communities, our study of various combinations of three to five species demonstrated that this technique would work clinically (data not shown).

When sputum samples spiked with *M. avium*, *M. intracellulare*, and different amounts of *M. tuberculosis* H37Rv cells were analyzed, the peaks of single-stranded DNA from *M. tuberculosis* H37Rv were discernible in fourfold- but not in eightfold-diluted samples (Fig. 3). No unexpected peaks or interference

from the spiked samples were observed. These results indicate that the detection limit for each mycobacterium is up to 25% of the amount relative to the other two species.

Fifty-one sputum specimens from patients with suspected *M. tuberculosis* or nontuberculous mycobacterial infections were tested by FCE-RF-SSCP and the Amplicor assay (Table 4). These specimens were also subjected to acid-fast staining and cultured. Species identified by FCE-RF-SSCP, Amplicor, and the culture method were always the same. In addition to the three species targeted by Amplicor, FCE-RF-SSCP could identify *M. abscessus* and *M. kansasii*. Two specimens were negative by smear and culture tests but showed positive results only in the Amplicor (specimens 32 and 33); this may be due to the higher sensitivity of Amplicor than FCE-RF-SSCP or may represent false-positive results of the Amplicor test. The 51 sputum specimens were not amplified nonspecifically. The results also support the specificity of the primer set used in this study and warrant the application of our technique to the direct identification of mycobacterial species in a sputum specimen.

FCE-RF-SSCP of sputum obtained from a patient with mixed *M. avium* and *M. intracellulare* infection gave SSCP patterns corresponding to these two species (4421.3 data points of blue and 4480.0 of green for *M. avium*, and 4442.0 blue and 4515.9 green for *M. intracellulare* I). The blue peak (3687.0) and green peak (3886.2) in HaeIII are the overlapping peaks originating from these species (Fig. 4). This result demonstrates the effectiveness of FCE-RF-SSCP analysis in the direct identification of mixed infections from patient specimens.

DISCUSSION

We developed a simple algorithm using FCE-RF-SSCP with genus *Mycobacterium*-specific primers (23) to simultaneously

FIG. 3. FCE-RF-SSCP analysis of PCR amplification products for sputum samples spiked with *M. avium* ATCC 25291, *M. intracellulare* ATCC 13950, and various amounts of *M. tuberculosis* H37Rv. The amount of *M. tuberculosis* H37Rv was serially twofold diluted, while that of the other strains was kept constant: no dilution (A), -fold (B), 4-fold (C), 8-fold (D), or 16-fold (E) dilution. Electrophoretic mobility values at each peak and their corresponding strains are indicated on the electropherograms: av, *M. avium* ATCC 25291; int, *M. intracellulare* ATCC 13950; TB, *M. tuberculosis* H37Rv.

TABLE 4. Comparison of FCE-RF-SSCP with Amplicor assay for direct identification of mycobacteria in sputum specimens

Specimen no.	Smear result ^a	Species identification of culture isolates ^b	Species identification	
			Amplicor	FCE-RF-SSCP
1	P	<i>M. abscessus</i>	Not detected	<i>M. abscessus</i>
2	P	<i>M. abscessus</i>	Not detected	<i>M. abscessus</i>
3	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
4	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
5	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
6	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
7	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
8	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
9	P	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
10	P	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
11	P	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
12	P	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
13	P	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
14	P	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> II
15	P	<i>M. kansasii</i>	Not detected	<i>M. kansasii</i>
16	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
17	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
18	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
19	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
20	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
21	P	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
22	P	Culture negative	<i>M. avium</i>	<i>M. avium</i>
23	P	Culture negative	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
24	N	<i>M. abscessus</i>	Not detected	<i>M. abscessus</i>
25	N	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
26	N	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
27	N	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
28	N	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i> I
29	N	<i>M. kansasii</i>	Not detected	<i>M. kansasii</i>
30	N	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
31	N	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
32	N	Culture negative	<i>M. avium</i>	No amplification
33	N	Culture negative	<i>M. avium</i>	No amplification
34	N	Culture negative	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex
35–51	N	Culture negative	Not detected	No amplification

^a P, positive for acid-fast bacilli; N, negative.
^b Identification was determined by conventional biochemical tests, Accu Probe, DDH Mycobacteria ‘Kyokyo,’ or partial 16S rDNA sequencing.

distinguish multiple mycobacterial species in a polymicrobial sample. This is the first report to expand the PRA method to this application. Compared with ordinary PRA methods, this technique is unique because it analyzes only terminal restriction fragments and undigested fragments. In this method, the troublesome gel-to-gel inconsistencies seen in agarose-based

methods are avoided, as FCE gives high resolution and reproducible analyses by with an internal standard in each sample. Recently, Gillman et al. (7) used FCE for the identification of mycobacterial species by using four pairs of fluorescent primers of the 16S rRNA gene and subsequently analyzed the SSCPs. Without using a restriction enzyme, they obtained spe-

FIG. 4. FCE-RF-SSCP analysis of sputum from a patient with mixed mycobacterial *M. avium* and *M. intracellulare* infection. Electrophoretic mobility values at each peak are indicated on the electropherograms.

cies-specific SSCP patterns for 30 of the common mycobacteria. However, since the PCR primers used in their method are not genus *Mycobacterium* specific, prior cultivation is required for the analysis.

Kim et al. (17) used PRA of *rpoB* DNA for direct identification of mycobacterial species in sputum samples and bronchoalveolar lavage specimens. Although this method showed improved identification of clinical specimens, application is limited to a single mycobacterial infection because too many fragments from a mixed infection blur the analysis. In our method, peaks from four types of fragments were obtained individually and used to identify each species. Since the number of SSCP peaks originating from each primer for a single species is theoretically one, multiple mycobacterial species in a sample can be identified with the algorithm. Multiple peaks obtained from *M. terrae* and *M. nonchronogenicum* are due to different stable conformations of the same fragment (7, 10) or the existence of multicopies of ITS in these species.

The newly developed technique, FCE-RF-SSCP, was validated on artificially constructed mycobacterial communities, clinical sputum specimens, and a sputum specimen from a patient with mixed *M. avium* and *M. intracellulare* infection. All results supported the ability of the technique to directly identify multiple mycobacterial species present in one specimen. Further investigations would accumulate more SSCP patterns from more *Mycobacterium* species and perform a multicenter study to ensure applicability in a laboratory setting.

ACKNOWLEDGMENTS

We thank M. Okazaki and B. Umeda of Kobe City General Hospital for providing sputum samples and the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, for providing *M. tuberculosis* H37Rv and *M. bovis* BCG Tokyo.

REFERENCES

- Branger, B., A. Gouby, A. Oules, J. P. Balucchi, G. Mourad, J. Fourcade, C. Mion, F. Duntz, and M. Ramuz. 1985. *Mycobacterium haemophilum* and *Mycobacterium xenopi* associated infection in a renal transplant patient. Clin. Nephrol. 23:46-49.
- Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma, Jr. 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl. Environ. Microbiol. 65:2492-2496.
- Dawson, D. J., J. F. Armstrong, and Z. M. Blacklock. 1982. Mycobacterial cross-contamination of bronchoscopy specimens. Am. Rev. Respir. Dis. 126: 1095-1097.
- Devallois, A., K. S. Goh, and N. Rastogi. 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. J. Clin. Microbiol. 35:2969-2973.
- Domenech, P., M. C. Menendez, and M. J. Garcia. 1994. Restriction fragment length polymorphisms of 16S rRNA genes in the differentiation of fast-growing mycobacterial species. FEMS Microbiol. Lett. 116:19-24.
- Falkinham, J. O., III. 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. 9:177-215.
- Gillman, L. M., J. Gunton, C. Y. Turenne, J. Wolfe, and A. M. Kabani. 2001. Identification of *Mycobacterium* species by multiple-fluorescence PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. J. Clin. Microbiol. 39:3085-3091.
- Gubler, J. G., H. M. Salfinger, and A. von Graevenitz. 1992. Pseudoepidemic of nontuberculous mycobacteria due to a contaminated bronchoscope cleaning machine. Chest 101:1245-1249.
- Harmsen, D., J. Rothgänger, C. Singer, J. Albert, and M. Frosch. 1999. Intuitive hypertext-based molecular identification of micro-organisms. Lancet 353:291.
- Hayashi, K. 1991. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl. 1:34-38.
- Horsburgh, C. R., Jr. 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. 324:1332-1338.
- Horsburgh, C. R., Jr. 1992. Epidemiology of mycobacterial diseases in AIDS. Res. Microbiol. 143:372-377.
- Horsburgh, C. R., Jr., and R. Selik. 1989. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am. Rev. Respir. Dis. 139:4-7.
- Hughes, M. S., R. A. Skuce, L.-A. Beck, and S. D. Neill. 1993. Identification of mycobacteria from animals by restriction enzyme analysis and direct DNA cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences. J. Clin. Microbiol. 31:3216-3222.
- Kanauija, G. V., V. M. Katoch, C. T. Shivannavar, V. D. Sharma, and M. A. Patil. 1991. Rapid characterization of *Mycobacterium fortuitum-chelonae* complex by restriction fragment length polymorphism of ribosomal RNA genes. FEMS Microbiol. Lett. 61:205-208.
- Kasai, H., T. Ezaki, and S. Harayama. 2000. Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. J. Clin. Microbiol. 38:301-308.
- Kim, B. J., K. H. Lee, B. N. Park, S. J. Kim, G. H. Bai, S. J. Kim, and Y. H. Kook. 2001. Differentiation of mycobacterial species by PCR-restriction analysis of DNA (342 base pairs) of the RNA polymerase gene (*rpoB*). J. Clin. Microbiol. 39:2102-2109.
- Lévy-Frèbault, V., B. Pangon, A. Buré, C. Katlama, C. Marche, and H. L. David. 1987. *Mycobacterium simiae* and *Mycobacterium avium-M. intracellulare* mixed infection in acquired immune deficiency syndrome. J. Clin. Microbiol. 25:154-157.
- Mbithi, J. N., V. S. Springthorpe, S. A. Sattar, and M. Pacquette. 1993. Bactericidal, virucidal, and mycobactericidal activities of reused alkaline glutaraldehyde in an endoscopy unit. J. Clin. Microbiol. 31:2988-2995.
- Peters, M., D. Schürmann, A. C. Mayr, R. Hetzer, H. D. Pohle, and B. Ruf. 1989. Immunosuppression and mycobacteria other than *Mycobacterium tuberculosis*: results from patients with and without HIV infection. Epidemiol. Infect. 103:293-300.
- Plikaytis, B. B., B. D. Plikaytis, M. A. Yakurus, W. R. Butler, C. L. Woodley, V. A. Silcox, and T. M. Shinnick. 1992. Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. J. Clin. Microbiol. 30:1815-1822.
- Ringuet, H., C. Akoua-Koffi, S. Honore, A. Varnerot, V. Vincent, P. Berche, J. L. Gaillard, and C. Pierre-Audigier. 1999. *hsp65* sequencing for identification of rapidly growing mycobacteria. J. Clin. Microbiol. 37:852-857.
- Roth, A., U. Reischl, A. Streubel, L. Naumann, R. M. Kroppenstedt, M. Habicht, M. Fischer, and H. Mauch. 2000. Novel diagnostic algorithm for identification of mycobacteria with genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. J. Clin. Microbiol. 38:1094-1104.
- Sakatani, M. 1999. Nontuberculous mycobacteriosis; the present status of epidemiology and clinical studies. Kekkaku 74:377-384.
- Spach, D. H., F. E. Silverstein, and W. E. Stamm. 1993. Transmission of infection by gastrointestinal endoscopy and bronchoscopy. Ann. Intern. Med. 118:117-128.
- Takewaki, S., K. Okuzumi, I. Manabe, M. Tanimura, K. Miyamura, K. Nakahara, Y. Yazaki, A. Ohkubo, and R. Nagai. 1994. Nucleotide sequence comparison of the mycobacterial *dnaI* gene and PCR-restriction fragment length polymorphism analysis for identification of mycobacterial species. Int. J. Syst. Bacteriol. 44:159-166.
- Takigawa, K., J. Fujita, K. Negayama, S. Terada, Y. Yahaji, K. Kawanishi, and J. Takahara. 1995. Eradication of contaminating *Mycobacterium chelonae* from bronchoscopes and an automated bronchoscope disinfection machine. Respir. Med. 89:423-427.
- Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31:175-178.
- Torres, R. A., J. Nord, R. Feldman, V. LaBombardi, and M. Barr. 1991. Disseminated mixed *Mycobacterium simiae-Mycobacterium avium* complex infection in acquired immunodeficiency syndrome. J. Infect. Dis. 164:432-433.
- Vanechoutte, M., H. De Beenhouwer, G. Claeys, G. Verschraegen, A. De Rouck, N. Pape, A. Elaichouni, and F. Portaels. 1993. Identification of *Mycobacterium* species by with amplified ribosomal DNA restriction analysis. J. Clin. Microbiol. 31:2061-2065.
- Weiszfeiler, J. G., and V. Karasveva. 1981. Mixed mycobacterial infections. Rev. Infect. Dis. 3:1081-1083.