

## Capillary Electrophoretic Restriction Fragment Length Polymorphism Patterns for the Mycobacterial *hsp65* Gene

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**PCR-restriction fragment length polymorphism (RFLP) analysis is a nonprobe method for the rapid identification of *Mycobacterium* species. We demonstrate the separation of DNA or restriction fragments digested from the mycobacterial gene encoding the 65-kDa heat shock protein (*hsp65*) by capillary electrophoresis (CE). By using a pair of unlabeled primers, Tb11 and Tb12, and only one restriction enzyme, HaeIII, we investigated a total of 52 reference and clinical strains encompassing 12 *Mycobacterium* species. The electrophoretic separation of high-resolution CE required <20 min and was capable of identifying fragments as small as 12 bp. A good agreement of measurement was observed between the sizes of restriction fragments resolved by CE, and the real sizes were deduced from the sequence analysis. Distinct differentiations were also well demonstrated between some species and subspecies by an extra HaeIII digestion site. With the advantage of the complete RFLP pattern available from CE, it appears to be more convenient to use an electropherogram rather than performing the cumbersome slab gel electrophoresis plus diagnostic algorithm to identify *Mycobacterium* species. Beyond the agarose and polyacrylamide gel electrophoresis, high-resolution CE provides an alternative for rapid identification of *Mycobacterium* species that is feasible for automation and routine use without the need for costly probes.**

The traditional identification method for tuberculosis used to take several weeks because of the slow growth rate of mycobacteria. In addition, antibiotic treatment varies according to the species of mycobacteria. Therefore, it is particularly important to diagnose *Mycobacterium* species as rapidly as possible. For many years, the rapid identification of *Mycobacterium* species, including both probe and nonprobe methods, had been available for clinical use. The probe method depends upon PCR and hybridization with labeled probes. The nonprobe method requires PCR of the 65-kDa heat shock protein (*hsp65*) gene and electrophoretic separation of the digested products to obtain the restriction pattern for each species. However, there are reasons why the rapid method cannot be widely applied. For the probe method such as the GenProbe AccuProbes test (11), its identifiable numbers of species are limited and the cost of probes remains high. For the nonprobe method (2, 15), the processes of slab gel electrophoresis are cumbersome and unable to separate the low-molecular-weight fragments. If capillary electrophoresis (CE) could solve the current problems of the nonprobe method, it could be used more commonly with the advantage of additional identifiable species, feasibility for automation, and lower cost compared to the method of Hernandez et al. (6). To achieve restriction fragment length polymorphism (RFLP) detection by automatic fluorescent fragment analysis, Hernandez et al. combined PCR-RFLP analysis of the *hsp65* and 16S rRNA genes by using

two labeled primer sets and four restriction enzymes. Although the unique patterns were obtained for 19 species analyzed, the method could not be applied to identify species where end fragments were not distinctive. It required a very expensive instrumentation, separate runs, multiple enzymes, and primers with labels.

The first application using PCR-RFLP analysis to the differentiation of *Mycobacterium* species was developed by Plikaytis et al. (9) in 1992. Since then, similar approaches for rapid identification of mycobacteria to the species level based on evaluation of the gene encoding for the 65-kDa heat shock protein had been studied by Telenti et al. (15) and others (1, 5, 13, 14). In all of these studies, the identification of *Mycobacterium* species required the use of two restriction enzymes (BstEII and HaeIII) and the separation of restriction fragments by 3% agarose gel electrophoresis. However, restriction fragments shorter than 60 bp were not available in the diagnostic algorithm differentiating *Mycobacterium* species. In 2001, Brunello et al. (2) replaced 3% agarose with 10% polyacrylamide in the electrophoresis of PCR-RFLP analysis. It provided a more precise estimate of restriction fragments and extended the maximal resolution to ca. 40 bp. Recently, Chang's laboratory team has developed a high-resolution CE with laser-induced fluorescence detection to separate DNA fragments in the size range of 11 to 2,176 bp accomplished in <20 min (3, 4, 7, 8, 16, 17). Compared to the probe method, the CE method does not involve any probes or hybridization steps. Unlike the conventional CE methods, DNA migrates against electro-osmotic flow, and thus the migration times for small DNA fragments are longer in the high-resolution CE method. As a result, this method provides a high-resolving power for small DNA fragments. Because a better resolution of low-molecular-weight fragments would make the RFLP pattern more informative, we conducted the present study to apply the new strategy for

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TABLE 1. *Mycobacterium* strains studied for the RFLP pattern of the *hsp65* gene by CE

Organisms	No. of strains	Source of strains <sup>a</sup>
<i>M. avium</i>	1	ATCC 15441
<i>M. fortuitum</i>	1	ATCC 15320
<i>M. gordonae</i>	1	ATCC 14470
<i>M. intracellulare</i>	1	ATCC 13950
<i>M. phlei</i>	1	ATCC 10707
<i>M. smegmatis</i>	1	ATCC 10708
<i>M. tuberculosis</i>	1	ATCC 10709
<i>M. asiaticum</i>	1	S27
<i>M. avium</i>	3	S49, S51, S54
<i>M. chelonae</i> subsp. <i>chelonae</i>	6	S9, S10, S16, S18, S43, S58
<i>M. chelonae</i> subsp. <i>abscessus</i>	6	S7, S17, S37, S52, S59, M11
<i>M. fortuitum</i>	5	S13, S14, S15, S44, S47
<i>M. gastri</i>	2	S22, S24
<i>M. gordonae</i>	3	S38, S40, S55
<i>M. intracellulare</i>	4	S6, S33, S39, S41
<i>M. kansasii</i>	6	S19, S21, S23, S34, S56, S57
<i>M. simiae</i>	1	S26
<i>M. tuberculosis</i>	8	S30, S45, S53, M8, M9, M10, M44, M45

<sup>a</sup> ATCC, American Type Culture Collection. All other strains were clinical isolates from the Mycobacteriology Laboratory of Taipei Municipal Hospital for Chronic Diseases.

optimizing sensitivity, speed, and resolution in capillary electrophoretic separation of mycobacterial *hsp65* gene fragments.

#### MATERIALS AND METHODS

**Mycobacterium strains.** A total of 52 reference and clinical strains encompassing 12 *Mycobacterium* species were used in the present study (Table 1). Seven reference strains were obtained from the American Type Culture Collection (Rockville, Md.). The other 45 clinical isolates were collected at the mycobacteriology laboratory of Taipei Municipal Hospital for Chronic Diseases. Identification at the species level was done by classical biochemical tests and further confirmed by DNA sequence analysis.

**DNA extraction, amplification, and restriction enzyme digestion.** A loop of mycobacteria grown on solid medium (Löwenstein-Jensen medium at 37°C) was suspended in 500  $\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA; pH 8) and inactivated by boiling for 30 min. Template DNA was extracted by using a Puregene DNA purification kit (Gentra Systems) according to the manufacturer's instructions. A pair of unlabeled primers, Tb11 (5'-ACCAACGATGGTG TGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT), was used in a PCR to amplify the *hsp65* gene, which was a 439-bp fragment between positions 398 and 836 as reported previously (12). PCR mixtures contained 10 mM Tris-

HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 0.4  $\mu$ M concentrations of each primer, 200  $\mu$ M concentrations of each of four deoxynucleoside triphosphates, 1.25 U of *Taq* polymerase (Qiagen, Hilden, Germany), and 3  $\mu$ l of the DNA template in a final volume of 50  $\mu$ l. Thermocycling of reaction mixtures was performed in a model 2400 Thermocycler (Perkin-Elmer-Applied Biosystems) programmed for 35 cycles of 60 s at 94°C, 60 s at 60°C, and 60 s at 72°C, followed by a 10-min incubation at 72°C. Amplification products were then digested with the restriction endonuclease HaeIII (New England Biolabs). Thus, 10  $\mu$ l of the amplified reaction solution was added to a mixture containing 1  $\mu$ l of enzyme (5 U), 5  $\mu$ l of restriction buffer (10 $\times$ ), and 34  $\mu$ l of sterile distilled water. The mixtures were incubated for 60 min at 37°C for HaeIII digestion.

**CE.** Digested fragments were subjected to CE by using poly(ethylene oxide) solution in the presence of electroosmotic flow (3, 4, 7, 8, 16, 17). Briefly, a high-voltage power supply (Gamma High Voltage Research, Inc., Ormond Beach, Fla.) was used to drive electrophoresis. The entire detection system was enclosed in a black box with a high-voltage interlock. The high-voltage end of the separation system was put in a laboratory-made Plexiglas box for safety. A 4.0-mW He-Ne laser with 543.6-nm output from Uniphase (Mantence, Calif.) was used for excitation. The light was collected with a  $\times$ 10 objective lens (numerical aperture = 0.25). One RG 610 cutoff filter was used to block scattered light before the emitted light reached the phototube (Hamamatsu R928). The amplified currents were transferred directly through a 10-k $\Omega$  resistor to a 24-bit A/D interface at 10 Hz (Borwin; JMBS Developments, Le Fontanil, France) and stored in a personal computer. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, Ariz.) with an internal diameter of 75  $\mu$ m were used for DNA separations without any further coating process. The capillary length was 40 cm, and the length to the detector was 30 cm. Tris-borate buffer was positioned at both anode and cathode sides. Ethidium bromide was added for the intercalation within double-stranded DNA fragments as the source of fluorescence. Using a syringe, the fresh polymer was injected into the capillary at the anode side. The samples were introduced at the cathode side by hydrodynamic injection with a height of 30 cm for 10 s. Electrophoresis was performed at a voltage of 20 kV for 20 min at ambient temperature.

**Analysis of amplified *hsp65* gene sequence.** According to the manufacturer's instructions, the sequences of amplified *hsp65* gene products were obtained by using a DNA sequencing kit (ABI Prism 377 sequence detection system; Applied Biosystems). For each sample, the sequence of amplified *hsp65* gene was further analyzed by using the Genedoc and Sequencer 4.1.4 programs to yield the real sizes of fragments deduced from the position of HaeIII digestion. The results from the sequence and fragment analysis were used as a reference of comparison for the fragment sizes estimated by CE.

#### RESULTS

With strictly controlled conditions of CE, the size of DNA restriction fragment was estimated by the established calibration with a 10-bp double-stranded DNA ladder (1  $\mu$ g/ $\mu$ l; Invitrogen/Life Technologies, Carlsbad, Calif.) as the molecular size standard. Since our home-made CE had only a single

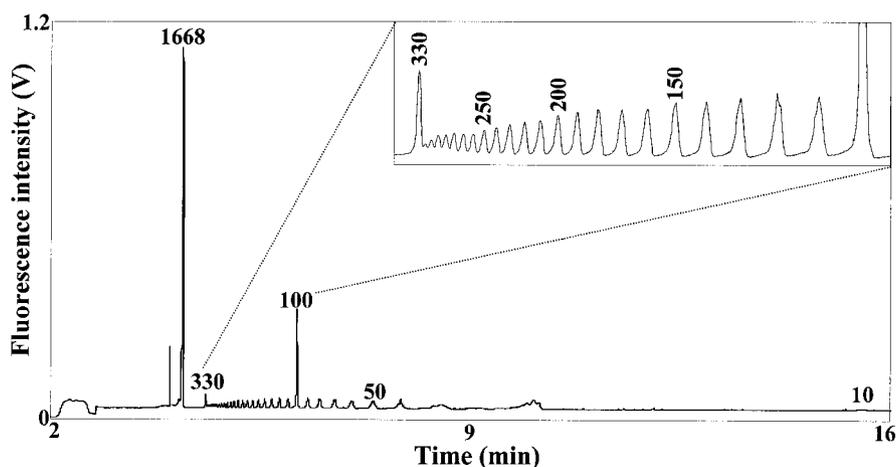


FIG. 1. CE of 10-bp DNA ladder showing 33 10-bp repeats plus a fragment of 1,668 bp.

TABLE 2. Fragment sizes of *hsp65* genes from 12 *Mycobacterium* species after HaeIII digestion and CE compared to those deduced by sequence analysis

Species (strain)	Fragment sizes (bp) as determined by:		Fig. 3 panel
	Sequence analysis <sup>a</sup>	CE	
<i>M. kansasii</i> (S34)	128, 103, <b>78</b> , 40, 34, <b>23</b> , 19, 17	131, 108, 78, 42, 36, 24, 22, 20	A
<i>M. gastri</i> (S24)	128, 103, <b>101</b> , 40, 34, 19, 17	128, 106, 100, 44, 31, 21, 18	B
<i>M. avium</i> (S3)	128, <b>103</b> , 59, <b>42</b> , 40, 36, 34	125, 104, 55, 40, 39, 36, 34	C
<i>M. intracellulare</i> (ATCC 13950)	<b>145</b> , 128, 59, 40, 36, 34	147, 127, 59, 40, 36, 34	D
<i>M. smegmatis</i> (ATCC 10708)	146, 123, 58, 40, <b>36</b> , 23, <b>16</b>	147, 123, 57, 40, 37, 24, 17	E
<i>M. phlei</i> (ATCC 10707)	146, 123, 58, <b>52</b> , 40, 23	147, 122, 55, 49, 38, 23	F
<i>M. chelonae</i> subsp. <i>abscessus</i> (S17)	<b>146</b> , 69, 58, <b>52</b> , 48, 40, 23, 6*	151, 69, 58, 52, 48, 40, 24	G
<i>M. chelonae</i> subsp. <i>chelonae</i> (S9)	<b>198</b> , 69, 58, 48, 40, 23, 6*	198, 69, 56, 46, 39, 24	H
<i>M. tuberculosis</i> (ATCC 10709)	152, 128, 69, 42, 22, 17, 12	151, 127, 68, 43, 24, 19, 14	I
<i>M. goodii</i> (S28)	162, 112, 59, 40, 36, 33	162, 113, 57, 41, 37, 35	J
<i>M. fortuitum</i> (S47)	146, 123, 58, 52, 40, 23	146, 121, 54, 48, 37, 22	K
<i>M. asiaticum</i> (S27)	112, 107, 40, 36, 36, 34, 33, 23, 21	114, 109, 40, 35 <sup>b</sup> , 33, 32, 24, 23	L
<i>M. simiae</i> (S26)	145, 128, 40, 36, 35, 23, 19, 17	145, 126, 39, 35, 33, 23, 20, 18	

<sup>a</sup> Prior to HaeIII digestion, the sequences of amplified *hsp65* gene products were obtained, and the locations of restriction sites were further analyzed by using the Genedoc and Sequencer 4.1.4 programs. The boldface numbers represent the fragments different between Fig. 3A and B. Similar comparisons were also observed in the figure pairs Fig. 3C and D, E and F, and G and H. \*, not detected in CE.

<sup>b</sup> Containing two peaks (at 36 and 36 bp).

channel, the 10-bp DNA standard was run after every 10 specimens as a reference. As one example of the runs demonstrated (Fig. 1), 33 10-bp repeats plus a fragment of 1,668 bp in the 10-bp DNA ladder were nicely separated by CE. The relationship between the sizes of 10-bp DNA ladder fragments and their corresponding electrophoretic migration times was analyzed (Fig. 2). The sizes of DNA ladder fragments ranging between 200 and 100 bp, 100 and 20 bp, and 30 and 10 bp were inversely proportional to electrophoretic migration times with linear regression ( $R^2$ ) values of 0.9998, 0.9994, and 0.9939, respectively.

A total of 52 strains encompassing 12 *Mycobacterium* species were subjected to the PCR-RFLP analysis with CE. The electropherograms of *hsp65* gene fragments after HaeIII digestion were obtained for all *Mycobacterium* strains. The fragment sizes estimated by CE and deduced by sequence analysis of the representative *Mycobacterium* species (including two subspecies of *Mycobacterium chelonae*) are shown in Table 2 and presented in Fig. 3. Except for the smallest fragment of 6 bp from *M. chelonae*, the sizes of all fragments for every strain ranged from 198 to 12 bp, which is within the calibration range of 200 to 10 bp. The electrophoretic separation of these fragments mainly occurred between 4 and 16 min after the load of specimens. Each peak stood for a fragment of double-stranded DNA intercalated with ethidium bromide that was detected and recorded by laser-induced fluorescence during electrophoresis. Thus, the heights of peaks or relative fluorescence intensities were proportional to the DNA fragment sizes. This proportional relationship was consistent throughout each of the electropherograms. For a similar reason, the peak of estimated 35 bp appeared to be taller than the neighboring peaks observed in *M. asiaticum* (Fig. 3L); its size was doubled due to the presence of two real 36-bp fragments. Also, here within a distance of 8 bp (40 to 32 bp), CE was able to detect four different peaks. Such an excellent resolution became more prominent for the low-molecular-weight range. A nice separation of peaks in the high-molecular-weight range was also noted, such as between the 103- and 101-bp fragments seen in *M. gastri* (Fig. 3B). In no case were peaks in the range of primers

or primer-dimers observed. Such a powerful resolution offered a full-ranged profile of restriction fragments that was not available with slab gel electrophoresis. Although our CE could only detect the presence of peaks as small as 12 bp seen in *M. tuberculosis* (Fig. 3I), it provided enough polymorphic patterns necessary for the differentiation of species and subspecies among the available strains.

The mean sizes and standard deviations of all fragments

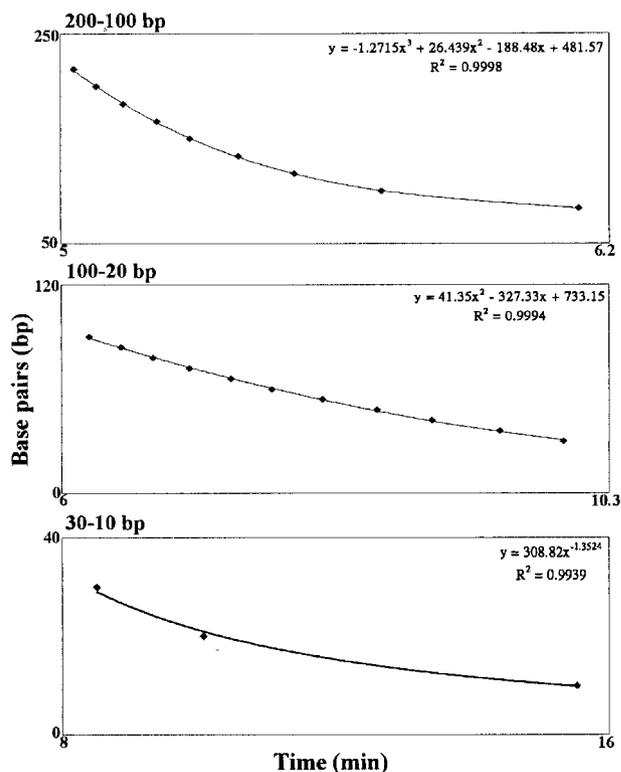


FIG. 2. Relationship between the sizes of DNA ladders (10 to 200 bp) and the corresponding electrophoretic migration time.

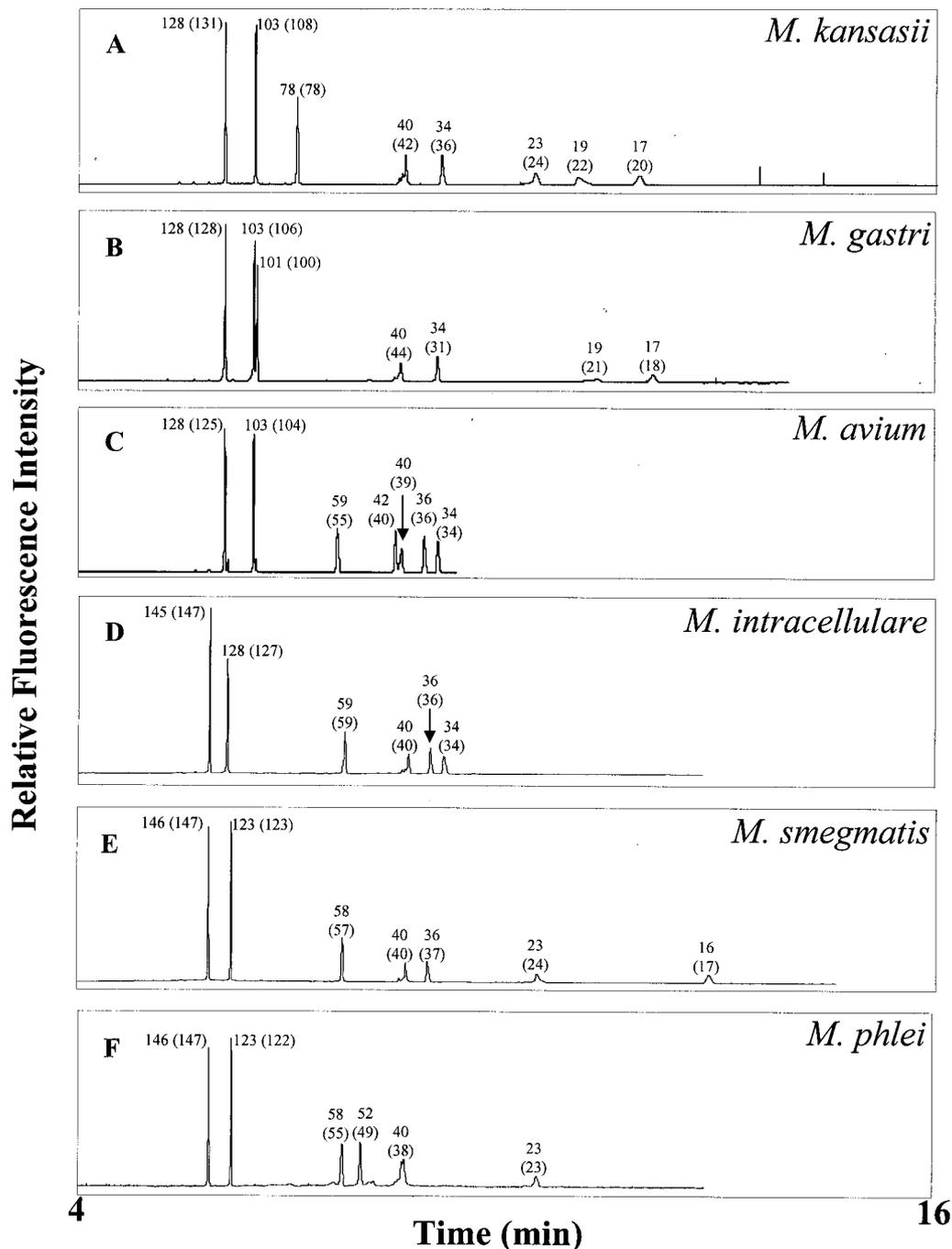


FIG. 3. Electropherograms of mycobacterial *hsp65* genes with HaeIII digestion. The numbers of base pairs without parentheses represent the real sizes of fragments derived from sequence analysis, whereas those within parentheses were estimated by CE.

estimated by CE were analyzed for seven species, with one showing two subspecies, each containing four to nine strains (Table 3). For the estimated mean sizes in comparison with the corresponding real sizes of 53 fragments, 44 (83%) fragments differed by <4 bp and 9 (17%) fragments differed by 4 to 5 bp. In terms of the standard deviation, it yielded a reproducible pattern with the variations of 51 (96%) fragments being less than 4 bp; those of two (4%) fragments were equal to 5 bp. In a practical sense, the image analysis for differentiating the

RFLP patterns could be programmed to accept the variations in fragment sizes within these limits.

Interestingly, among the species and subspecies described in the present study, four pairs were found to be clearly distinguished by only one different HaeIII digestion site. For example, both species *M. kansasii* and *M. gastri* (Fig. 3A and B) had fragments 128, 103, 40, 34, 19, and 17 bp in common. The fragments that differed were 78 and 23 bp in *M. kansasii* and 101 bp in *M. gastri*, as indicated by the boldface numbers in

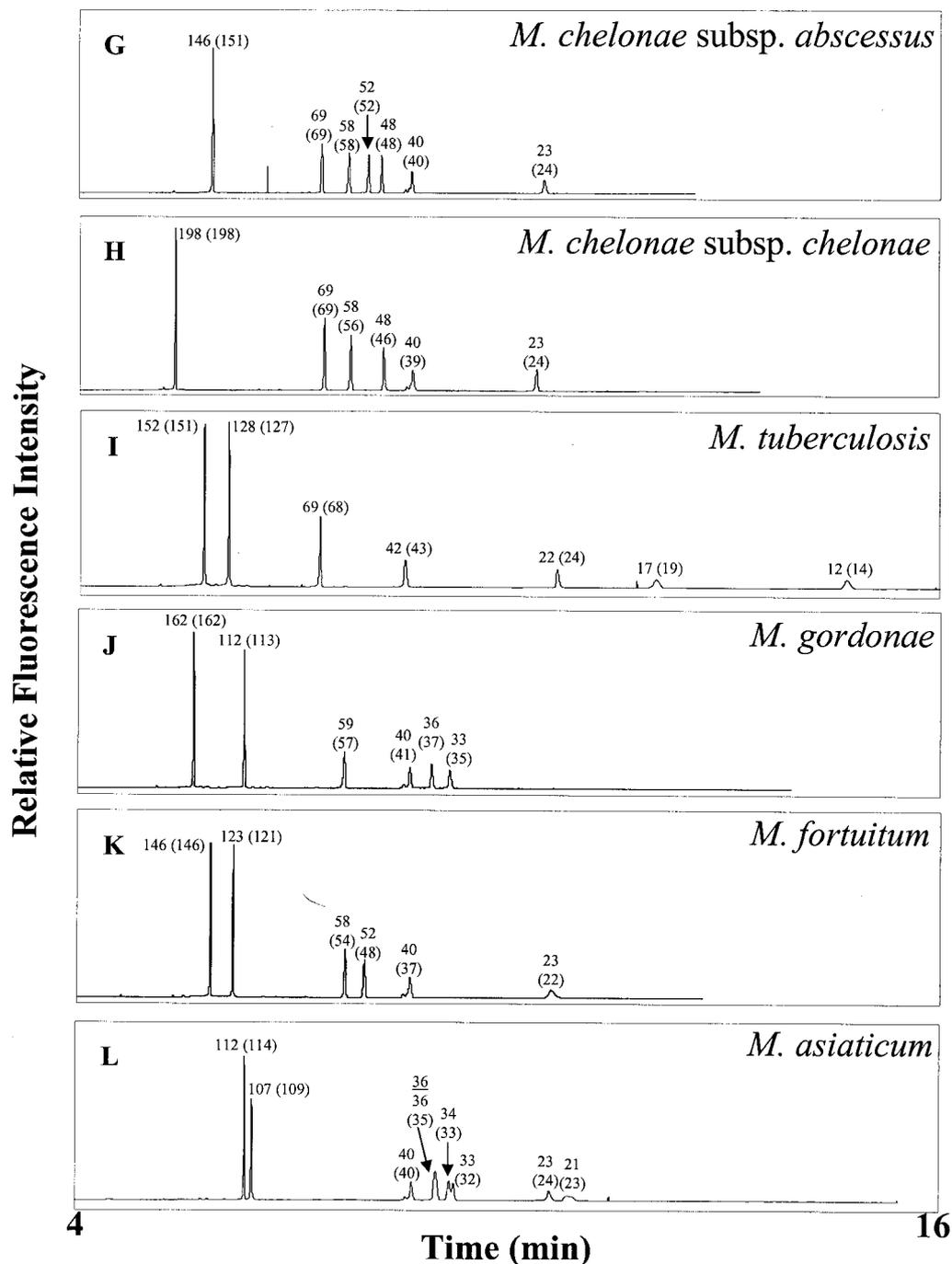


FIG. 3—Continued.

Table 2. Such a difference was considered to be the result of an additional HaeIII digestion site within the 101-bp fragment of *M. kansasii* compared to *M. gastri*. Similar observations were also noted for the comparisons between species *M. avium* and *M. intracellulare* (Fig. 3C and D) and species *M. smegmatis* and *M. phlei* (Fig. 3E and F), as well as subspecies *abscessus* and *chelonae* (Fig. 3G and H) of *M. chelonae*. These observations were confirmed by the position of HaeIII digestion in fragment and sequence analysis. Thus, some species and subspecies with similar *hsp65* gene sequences could be well differentiated by

the electrophoregram due to the presence of an extra HaeIII digestion site.

**DISCUSSION**

In the present study, the CE-based method provided more precise estimates of fragment sizes compared to those obtained by 10% polyacrylamide gel electrophoresis (PAGE), as well as the sharp resolution for fragments of <40 bp that are not available by slab gel electrophoresis. As shown in the capillary

TABLE 3. Fragment lengths of mycobacterial *hsp65* genes detected by CE compared to those deduced from sequence analysis

Species (no. of strains)	Fragment length (bp) as determined by:		Species (no. of strains)	Fragment length (bp) as determined by:	
	Sequence analysis <sup>a</sup>	CE <sup>b</sup>		Sequence analysis <sup>a</sup>	CE <sup>b</sup>
<i>M. tuberculosis</i> (9)	152	151 ± 2	<i>M. chelonae</i> subsp. <i>chelonae</i> (6)	198	200 ± 3
	128	126 ± 2		69	65 ± 1
	69	67 ± 2		58	54 ± 2
	42	41 ± 2		48	44 ± 2
	22	23 ± 1		40	36 ± 2
	17	19 ± 1		23	23 ± 1
<i>M. kansasii</i> (6)	128	127 ± 5	<i>M. chelonae</i> subsp. <i>abscessus</i> (6)	146	150 ± 2
	103	108 ± 2		69	68 ± 1
	78	74 ± 5		58	57 ± 1
	40	42 ± 3		52	50 ± 1
	34	37 ± 2		48	46 ± 1
	23	24 ± 2		40	38 ± 2
<i>M. gordonae</i> (4)	162	162 ± 1	<i>M. fortuitum</i> (6)	146	144 ± 3
	112	110 ± 2		123	121 ± 2
	59	56 ± 1		58	54 ± 1
	40	40 ± 2		52	49 ± 1
	36	35 ± 2		40	39 ± 1
	33	33 ± 2		23	22 ± 1
<i>M. avium</i> (4)	128	126 ± 3	<i>M. intracellulare</i> (5)	145	146 ± 2
	103	104 ± 2		128	126 ± 2
	59	55 ± 2		59	58 ± 2
	42	40 ± 2		40	38 ± 2
	40	39 ± 1		36	34 ± 2
	36	35 ± 1		34	31 ± 2
34	33 ± 1				

<sup>a</sup> Values are means.<sup>b</sup> Values are means ± standard deviations.

electropherograms, the resolution between the peaks of double-stranded DNA was excellent, especially for fragments smaller than 60 bp. Its high resolution made the analysis of total fragment patterns with the cheaper ethidium bromide possible. The PCR-RFLP with CE was able to offer a complete RFLP pattern (fragment sizes, >10 bp) with good overall agreement compared to the real sizes deduced from the restriction sites of sequence analysis. With the detection limit of CE reaching as little as 12 bp, it already made the RFLP pattern polymorphic enough for the differentiation of species and subspecies. A reproducible pattern with standard deviations that were ≤3 bp for 96% of fragments was also essential for differentiation.

The sizes of fragments generated by HaeIII digestion have been analyzed by 10% PAGE (2). In that study, the authors concluded that 23 (88%) of 26 fragments generated by HaeIII digestion showed a size differing from the real size by <5 bp and three (12%) differed by 5 to 10 bp. In our study (Table 2), among the total of 51 fragments that were ≥40 bp separated by CE, 49 (96%) differed by <5 bp and 2 (4%) differed by 5 bp. Although the strains used for comparison were different between the two studies, the estimates appeared to be more precise by CE, and the resolution for fragments of <40 bp was not available by 10% PAGE. With CE, these low-molecular-weight fragments were distinctly resolved; 100% of the total 38 fragments differed by 3 bp or less.

Distinctive differentiations are well demonstrated between

some species and subspecies by the presence of an extra HaeIII digestion site. Our CE method is suitable for the identification of mycobacteria other than *M. tuberculosis* but will not differentiate between members of the *M. tuberculosis* complex. Thus, the major advantages of the CE method appear to be in the time saved compared to slab gel analysis, the ability to differentiate between some clinically important species by the presence of an additional HaeIII digestion site, and the possibility of automation. In terms of cost, the CE-based analysis could be achieved by using only one restriction enzyme, a pair of unlabeled primers, and a much smaller loading amount (internal diameter, 75 μm). Compared to the study by Hernandez et al. (6), our method does not require any capillary coating process or the use of expensive fluorescence-labeled primers. These advantages make this method able to meet the aim of lower cost for the rapid identification of *Mycobacterium* species. Instrumental automation is also possible for CE with automatic sample loading, an electrophoretic reader, high-throughput capacity (10), and image database software to differentiate the RFLP patterns. With the advantage of complete RFLP pattern available from CE, it appears more convenient to adopt the electropherogram rather than to perform the cumbersome slab gel electrophoresis plus diagnostic algorithm to identify *Mycobacterium* species. However, there are more issues that remain to be investigated, such as the establishment of complete electropherograms specific for all available species, the detection limit (number of bacilli per microliter) for various known cul-

tured mycobacteria and, most importantly, its ability to identify *Mycobacterium* species directly from clinical isolates before the development of multichannel CE with automatic image analysis.

In conclusion, we believe that an alternative for slab gel electrophoresis-based algorithm of identifying *Mycobacterium* species is available through the high-resolution CE. The complete RFLP pattern provided by technical improvement, as shown by the results of our study, could certainly help us beyond the limitation of identifiable numbers of species and the expensive cost of the probe method. Although this is just the first step for applying the model of PCR-RFLP analysis with CE, automation of rapidly identifying more microbiologic species without probes might be feasible in the near future.

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