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Mycobacteriology

The *hsp65* gene patterns of less common *Mycobacterium* and *Nocardia* spp. by polymerase chain reaction-restriction fragment length polymorphism analysis with capillary electrophoresis

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Abstract

To rapidly identify *Mycobacterium* and *Nocardia* spp. without costly probes, we had implemented capillary electrophoresis (CE) in polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis to analyze their 65-kDa heat shock protein (*hsp65*) gene. The PCR–RFLP analysis with CE (PRACE) involved only one restriction enzyme, *Hae*III, and a single electrophoretic separation less than 10 min. Full-range (10–200 bp) RFLP patterns of 12 less common *Mycobacterium* and 7 *Nocardia* spp. were investigated. A good agreement was observed between the sizes of restriction fragments resolved by CE and the real sizes deduced from sequence analysis. Including *hsp65* gene patterns of 12 *Mycobacterium* spp. published earlier, differentiation was distinct among 24 *Mycobacterium* and 7 *Nocardia* spp. Some closely related species exhibiting similar biochemical characteristics could be well discriminated by an extra *Hae*III digestion site. Thus, PRACE offers a nonprobe alternative for rapid identification of various cultured *Mycobacterium* and *Nocardia* to the species level.

Keywords: Restriction fragment length polymorphism; PCR–RFLP analysis; Capillary electrophoresis; Mycobacteria other than tuberculosis; Nocardia

1. Introduction

So far, more than 70 *Mycobacterium* spp. and at least 12 members of the genus *Nocardia* have been identified. Opportunistic infections due to mycobacteria other than tuberculosis (MOTT) and *Nocardia* spp. have been on the rise, predominantly affecting immunocompromised patients. The well-documented species in MOTT infections include *Mycobacterium avium* complex, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, *Mycobacterium simiae*, *Mycobacterium szulgai*, *Mycobacterium ulcerans*, *Mycobacterium xenopi*, and *Mycobacterium fortuitum* (Falkinham, 1996). Occasionally, infections are attributed to those species that are often encountered as a contaminant in clinical samples, such as *Mycobacterium gastri*, *Mycobacterium gordonae*, and *Mycobacterium terrae* (Wayne and Sramek, 1992). Differentiation of pathogenic and nonpathogenic MOTT from *Mycobacterium tuberculosis* complex (MTC) has become a relevant diagnostic issue, considering that many MOTT are resistant to the antibiotics used for tuberculosis treatment. Although rapid identification of *Mycobacterium* spp. is essential for the early diagnosis, the current use of rapid genotypic methods is limited by cost and variety of probes available for MOTT species. Meanwhile, differentiation between *Mycobacterium* and *Nocardia* spp. also warrants our greater attention because of their similar staining,

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Table 1
Mycobacterium and *Nocardia* strains studied for the *hsp65* gene patterns by the PCR–RFLP analysis with CE

Organisms	No. of strains	Source of strains[a]
M. avium Chester	1	ATCC 700898
M. celatum	1	CCF 56
M. lacticola[b]	1	CCF 18
M. marinum	4	TMC 927, CCF 31, CCF 32, CCF 33
M. mucogenicum	3	CCF 22, CCF 23, CCF 24
M. neoaurum[b]	1	CCF 19
M. peregrinum	1	CCF 57
M. scrofulaceum	2	ATCC 19981, CCF 39
M. simiae	1	CCF 34
M. terrae	1	CCF 40
Mycobacterium triplex	1	CCF 35
M. xenopi	3	CCF 36, CCF 37, CCF 38
N. abscessus	1	CCF 64
Nocardia brasiliensis	1	CCF 60
Nocardia cyriacigeorgica	1	CCF 61
N. farcinica	1	CCF 59
N. nova complex	1	CCF 58
N. pseudobrasiliensis	1	CCF 63
N. transvalensis complex	1	CCF 62

TMC = Trudeau Mycobacterial Culture Collection.

[a] The sources of other strains are the culture collection of clinical isolates from the Section of Clinical Microbiology, Cleveland Clinic, Cleveland, OH.

[b] Two mycobacterial strains (CCF18 and CCF19) were biochemically identified as *M. lacticola*/*neoaurum*. They were further differentiated as *M. lacticola* (CCF18) and *M. neoaurum* (CCF19) by DNA sequence analysis.

morphology, colonial, and cultural characteristics (McNeil and Brown, 1994; Olson et al., 1998; Patterson et al., 1992; Short et al., 2005; Staneck et al., 1981). Rapid diagnosis of *Mycobacterium* and *Nocardia* infection may allow earlier initiation of effective therapy, thus, improving patient outcome. Therefore, it would be beneficial to develop an accurate, rapid, automatic, and cost-effective method capable of simultaneously distinguishing all species of *Mycobacterium* and *Nocardia*.

The genotypic approach including probe and nonprobe methods have been used to rapidly identify *Mycobacterium* spp. for over 10 years. The probe method depends upon genetic amplification and hybridization with labeled probes. The nonprobe method requires the enzymatic digestion of amplified genetic products and electrophoretic separation to obtain the restriction fragment length polymorphism (RFLP) pattern. An algorithm based on the nonprobe method called either polymerase chain reaction (PCR)-restriction enzyme pattern analysis or PCR–RFLP analysis (PRA) has been developed for differentiating mycobacterial species (Brunello et al., 2001; Ringuet et al., 1999; Taylor et al., 1997; Telenti et al., 1993; Wilson et al., 1998). However, there are reasons why both methods cannot be applied to most clinical specimens. For the probe method, such as the Gen-Probe AccuProbes test (Reisner et al., 1994), its identifiable numbers of species are limited and the cost of probes remains high. For the nonprobe method (Brunello et al., 2001; Taylor et al., 1997), the processes of slab gel electrophoresis are cumbersome and unable to separate the low molecular weight fragments. In recent years, Chang's laboratory team has developed a high-resolution capillary electrophoresis (CE) with laser-induced fluorescence detection to separate DNA fragments in the size range of 11 to 2176 bp in less than 10 min (Chen and Chang, 1999a, 1999b; Hsieh et al., 2000; Huang et al., 2001; Tseng and Chang, 2001; Tseng et al., 2001). Our application of CE in PRA could potentially solve some shortcomings of both methods by offering high-resolution simultaneous screening of all RFLP available species and feasibility of automation. We had improved PRA by implementing CE to analyze the mycobacterial 65-kDa heat shock protein (*hsp65*) gene. In the previous study (Ho et al., 2004), species-specific *hsp65* gene patterns were established from 12 common *Mycobacterium* spp. As compared with the report of separate runs using 4 enzymes and fluorescence-labeled primers (Hernandez et al., 1999), the CE-based analysis could be achieved at a lower cost using a much smaller loading amount (internal diameter, 75 μm), only one restriction enzyme, and a pair of unlabeled primers. The aim of our current study is to obtain and analyze additional *hsp65* gene patterns of 12 less common *Mycobacterium* and 7 *Nocardia* spp. collected for the database for identification purpose.

2. Materials and methods

2.1. Study strains

We examined 20 *Mycobacterium* strains encompassing 12 species and 7 *Nocardia* strains from different species (Table 1). Except for one *M. scrofulaceum* strain from the American Type Culture Collection (ATCC), Rockville, MD, and one *M. marinum* strain from the Trudeau Mycobacterial Culture Collection, Denver, CO, the source of strains was the culture collection of clinical isolates from the Clinical Microbiology Section, Cleveland Clinic, Cleveland, OH. Identification at the species level was done by classic biochemical tests and further confirmed by DNA sequence analysis with pyrosequencing (Tuohy et al., 2005).

2.2. DNA preparation, PCR amplification, and RFLP analysis

Mycobacterial DNA preparation, PCR amplification of *hsp65* gene, and RFLP analysis by CE were performed, according to the methods previously described (Ho et al., 2004).

3. Results

A total of 27 strains encompassing 12 *Mycobacterium* and 7 *Nocardia* spp. were subjected to the PRA with CE (PRACE). The electrophoregrams of *hsp65* gene fragments after *Hae*III digestion were obtained for all strains, and 19

representative ones from each species were shown (Figs. 1 and 2). The fragment sizes of representative strains estimated by CE and deduced by sequence analysis were also compared (Table 2). Close peaks differing by only 1 or 2 bp can be resolved, such as 23- and 22-bp peaks from *Mycobacterium mucogenicum* (Fig. 1E), 19- and 17-bp peaks from *M. simiae* (Fig. 1I), *Nocardia nova* complex (Fig. 2E), as well as *Nocardia transvalensis* complex (Fig. 2G). However, some of them may be overlapped, such as 22- and 21-bp peaks from *M. marinum* (Fig. 1D),

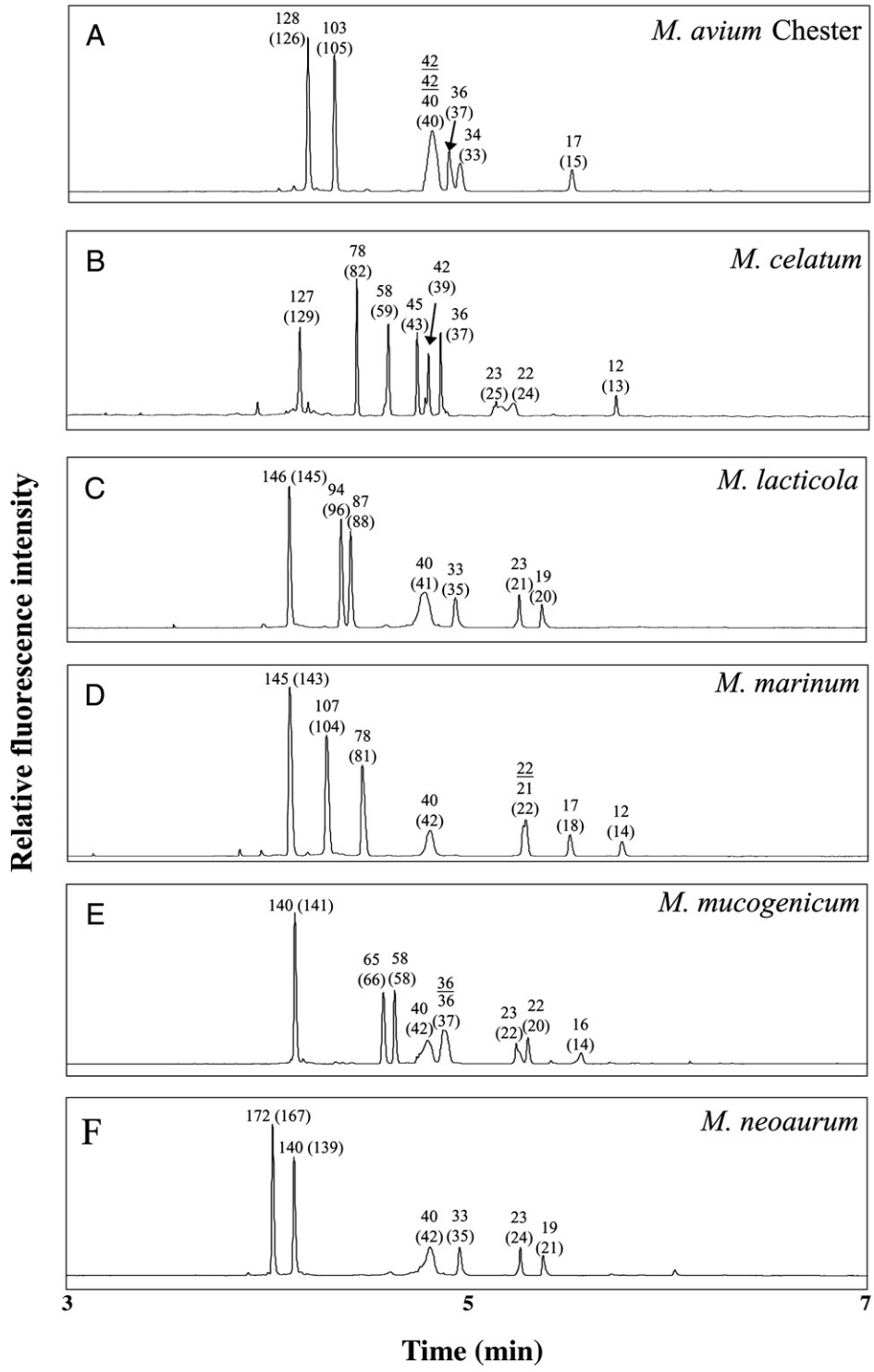

Fig. 1. Electrophoregrams of 12 mycobacterial *hsp65* genes with *Hae*III digestion. The numbers of base pairs without parentheses represent the real sizes of fragments derived from sequence analysis, whereas those within parentheses were estimated by CE.

42- and 40-bp peaks from *M. avium* Chester (Fig. 1A), as well as *Nocardia pseudobrasiliensis* (Fig. 2F). There are tiny peaks (<10 bp) not detected by CE (Table 2), including 6-bp peaks from *M. mucogenicum* and *Mycobacterium neoaurum* as well as 9-bp peaks from *M. neoaurum* and all 7 *Nocardia* strains. Although our CE could only detect the presence of peaks as small as 12 bp seen in *Mycobacterium celatum* (Fig. 1B), *M. marinum* (Fig. 1D), and *M. scrofulaceum* (Fig. 1H), it provides enough polymorphic patterns necessary for the differentiation of available species. Such

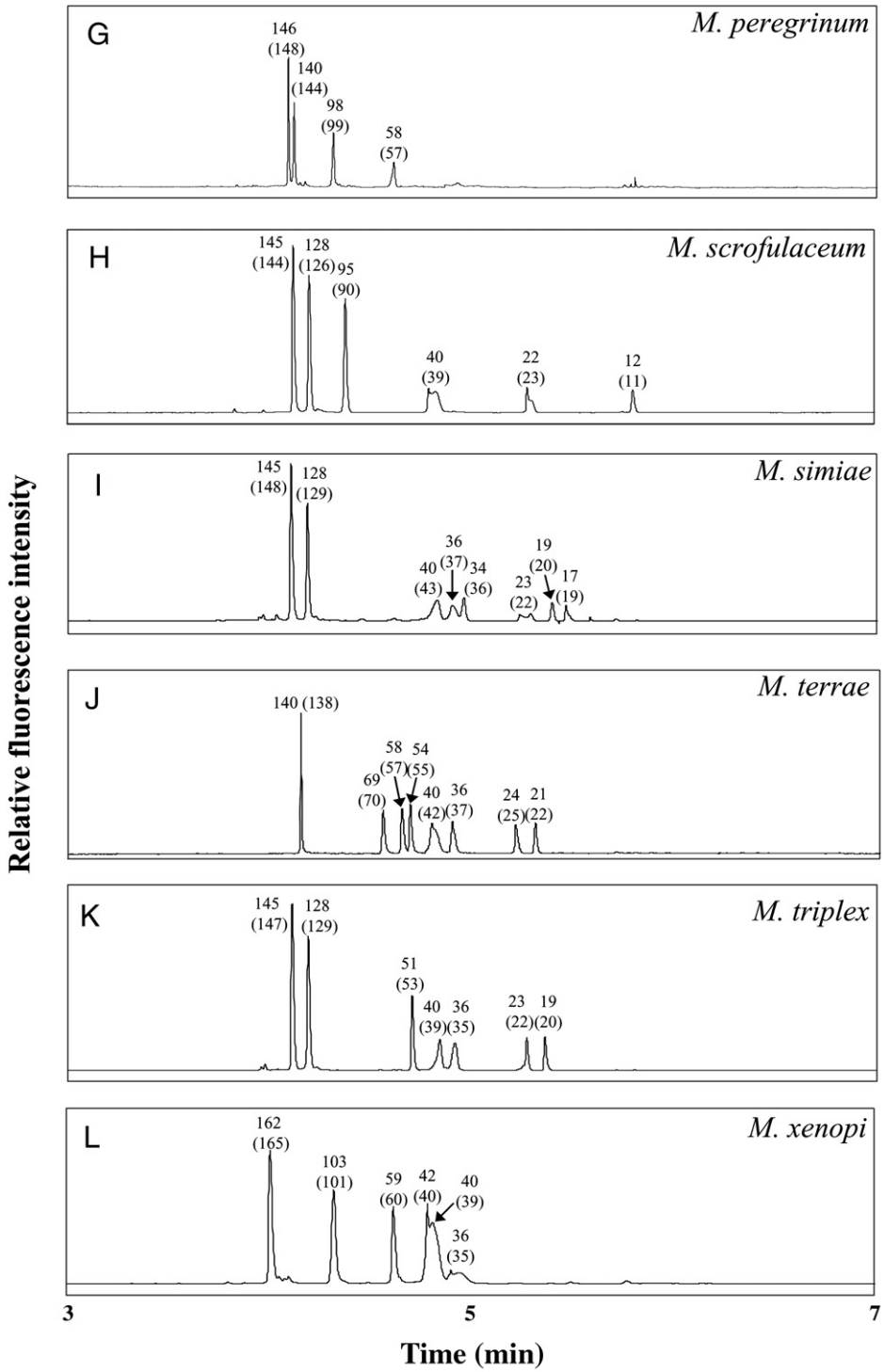

Fig. 1. continued.

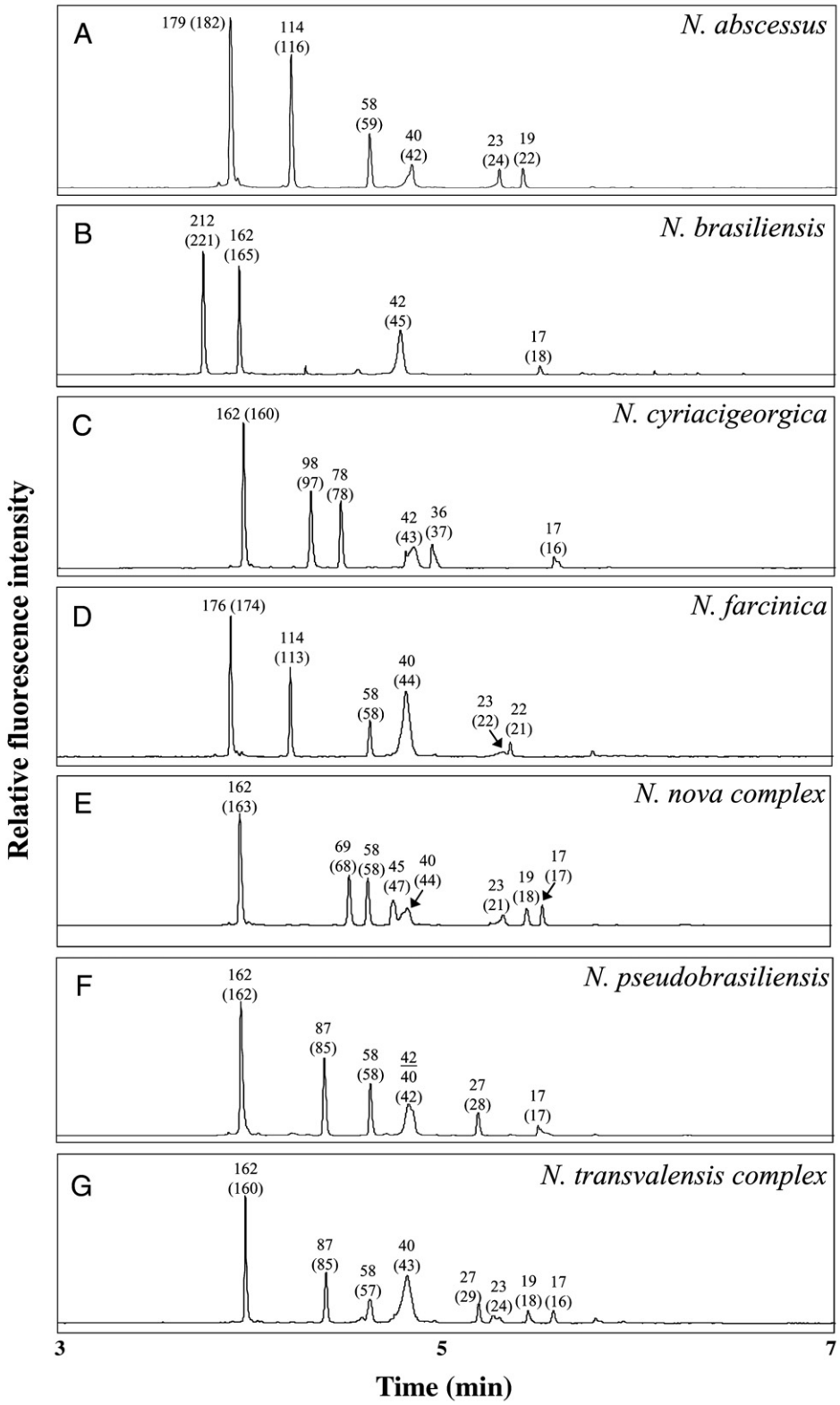

Fig. 2. Electrophoregrams of 7 nocardial *hsp65* genes with *Hae*III digestion. The numbers of base pairs without parentheses represent the real sizes of fragments derived from sequence analysis, whereas those within parentheses were estimated by CE.

Table 2
Fragment sizes (bp) of *hsp65* genes from 12 *Mycobacterium* and 7 *Nocardia* spp. resolved by PRACE in comparison with those deduced from sequence analysis

Species (strain)	Fragment sizes (bp) as determined by:		Figure
	Sequence analysis[a]	PRACE	
M. avium Chester (ATCC 700898)	128, 103, 42, 42, 40, 36, 34, 17	126, 105, 40[b], 37, 33, 15	Fig. 1A
M. celatum (CCF 56)	127, 78, 58, 45, 42, 36, 23, 22, 12	129, 82, 59, 43, 39, 37, 25, 24, 13	Fig. 1B
M. lacticola (CCF 18)	**146**, **94**, **87**, 40, 33, 23, 19	145, 96, 88, 41, 35, 21, 20	Fig. 1C
M. marinum (TMC 927)	145, 107, 78, 40, 22, 21, 17, 12	143, 104, 81, 42, 22[b], 18, 14	Fig. 1D
M. mucogenicum (CCF 22)	140, 65, 58, 40, 36, 36, 23, 22, 16, 6*	141, 66, 58, 42, 37[b], 22, 20, 14	Fig. 1E
M. neoaurum (CCF 19)	**172**, **140**, 40, 33, 23, 19, **9***, **6***	167, 139, 42, 35, 24, 21	Fig. 1F
M. peregrinum (CCF 57)	146, 140, 98, 58	148, 144, 99, 57	Fig. 1G
M. scrofulaceum (ATCC 19981)	145, 128, 95, 40, 22, 12	144, 126, 90, 39, 23, 11	Fig. 1H
M. simiae (CCF 34)	145, 128, 40, 36, 34, 23, 19, 17	148, 129, 43, 37, 36, 22, 20, 19	Fig. 1I
M. terrae (CCF 40)	140, 69, 58, 54, 40, 36, 24, 21	138, 70, 57, 55, 42, 37, 25, 22	Fig. 1J
M. triplex (CCF 35)	145, 128, 51, 40, 36, 23, 19	147, 129, 53, 39, 35, 22, 20	Fig. 1K
M. xenopi (CCF 36)	162, 103, 59, 42, 40, 36	165, 101, 60, 40, 39, 35	Fig. 1L
N. abscessus (CCF 64)	179, 114, 58, 40, 23, 19, 9*	182, 116, 59, 42, 24, 22	Fig. 2A
N. brasiliensis (CCF 60)	212, 162, 42, 17, 9*	221, 165, 45, 18	Fig. 2B
N. cyriacigeorgica (CCF 61)	162, 98, 78, 42, 36, 17, 9*	160, 97, 78, 43, 37, 16	Fig. 2C
N. farcinica (CCF 59)	176, 114, 58, 40, 23, 22, 9*	174, 113, 58, 44, 22, 21	Fig. 2D
N. nova complex (CCF 58)	162, 69, 58, 45, 40, 23, 19, 17, 9*	163, 68, 58, 47, 44, 21, 18, 17	Fig. 2E
N. pseudobrasiliensis (CCF 63)	162, 87, 58, **42**, 40, 27, 17, 9*	162, 85, 58, 42[b], 28, 17	Fig. 2F
N. transvalensis complex (CCF 62)	162, 87, 58, 40, 27, **23**, **19**, 17, 9*	160, 85, 57, 43, 29, 24, 18, 16	Fig. 2G

* = not detected in CE.

[a] Before *Hae*III digestion, the sequence of amplified *hsp65* gene products were obtained and the locations of restriction sites were further analyzed by the Genedoc and Sequencer 4.1.4 program. The bold numbers represent the fragments of difference in the pairs of Fig. 1C and F as well as Fig. 2F and G.

[b] CE peaks at 40, 22, 37, and 42 bp contain more than one peak at 42, 42, and 40; 22 and 21; 36 and 36; and 42 and 40 bp, respectively.

a powerful resolution offered a full-range (10–200 bp) RFLP pattern that was not obtainable by the slab gel electrophoresis.

In the PRA of mycobacterial *hsp65* gene using 10% polyacrylamide gel electrophoresis (PAGE), the sizes of restriction fragments generated by *Hae*III digestion have been analyzed (Brunello et al., 2001). In that study, the authors concluded that 23 (88%) of the total of 26 fragments generated by *Hae*III digestion showed a size differing from the real size by less than 5 bp and 3 (12%) differed from 5 to 10 bp. In our study (Table 2), among the total of 76 fragments equal to or larger than 40 bp separated by CE, 73 (96%) differed by less than 5 bp and 3 (4%) differed from 5 to 10 bp. Among the total of 50 fragments smaller than 40 bp separated by CE, 49 (98%) differed by less than 3 bp and 1 (2%) differed by 3 bp. Although the strains used for comparison were different between 2 studies, the estimates by CE were more precise and offered resolution for low molecular weight fragments (<40 bp), which were unavailable by 10% PAGE.

Because short DNA fragments intercalated with less amounts of fluorescent dye molecules, they fluoresce weakly. Similar concerns about the reproducibility of minor fragments with relative SD (RSD) had been studied and emphasized in the previous report (Chen and Chang, 1999a). In the current study, the electrophoretic peak patterns for each sample are also quite reproducible with the RSD (5 runs) of migration times and peak heights less than 2.0% and 2.5%, respectively. In addition, the mean size and SD of all fragments estimated by CE were analyzed for 3 species, each containing 3 to 4 strains (Table 3). For the estimated mean sizes in comparison with the corresponding real sizes of 22 fragments, all (100%) fragments differed by

Table 3
Mean and SD of *hsp65* gene fragment sizes (bp) from the strains of 3 *Mycobacterium* spp. detected by PRACE in comparison with those deduced from the sequence analysis

Species (no. of strains)	*M. marinum* (4)		*M. mucogenicum* (3)		*M. xenopi* (3)	
Method of determination	Sequence analysis[a]	PRACE[b]	Sequence analysis[a]	PRACE[b]	Sequence analysis[a]	PRACE[b]
Fragment sizes (bp)	145	143 ± 4	140	141 ± 3	162	165 ± 4
	107	104 ± 4	65	66 ± 2	103	101 ± 2
	78	81 ± 2	58	58 ± 2	59	60 ± 2
	40	42 ± 2	40	42 ± 2	42	40 ± 2
	22	23 ± 2	36	37 ± 1	40	39 ± 1
	21	22 ± 1	23	22 ± 1	36	35 ± 1
	17	18 ± 1	22	20 ± 2		
	12	14 ± 2	16	14 ± 2		

[a] Values are true sizes of fragments.

[b] Values are means ± SDs.

3 bp or less. In terms of the SD, 19 (86%) fragments yielded a reproducible pattern with the variations of 3 bp or less, whereas those of 3 (14%) fragments were equal to 4 bp. In a practical sense, the image analysis for differentiating the RFLP patterns could be programmed to accept such a variation in fragment sizes within these limits.

Interestingly enough, 2 pairs of closely related species exhibiting similar biochemical characteristics were found to be clearly distinguished by different *Hae*III digestion sites (Table 2). The first pair, *Mycobacterium lacticola* and *M. neoaurum*, had fragments 40, 33, 23, and 19 bp in common. The fragments in difference were 146, 94, and 87 bp in *M. lacticola*, and 172, 140, 9, and 6 bp in *M. neoaurum*, as indicated by the bold numbers. Such difference was considered as the results of a different *Hae*III digestion site within the precursory 181-bp fragment of both species and an extra *Hae*III digestion site within the precursory 146-bp fragment of *M. neoaurum*. The second pair, *N. pseudobrasiliensis* and *N. transvalensis complex*, had 162, 87, 58, 40, 27, 17, and 9 bp in common. The fragments in difference were 42 bp in *N. pseudobrasiliensis* and 23 and 19 bp in *N. transvalensis complex*, as indicated by the bold numbers. Such a difference was considered as the result of an additional *Hae*III digestion site within the 42-bp fragment of *N. transvalensis* complex as compared with *N. pseudobrasiliensis*. The given observations were confirmed by the position of *Hae*III digestion in fragment and sequence analysis. Thus, closely related species with similar *hsp65* gene sequences could be well differentiated by the electrophoregram because of the presence of a different or extra *Hae*III digestion site.

4. Discussion

The use of PRA has been the recent focus for recognition of individual mycobacterial and nocardial species (Hernandez et al., 1999; Laurent et al., 1999; Steingrube et al., 1997; Taylor et al., 1997; Telenti et al., 1993; Wilson et al., 1998) as well as separation of mycobacteria from nocardiae (Lungu et al., 1994). We selected the *hsp65* gene for our CE-based PRA because its sequence has more variability than the 16S rRNA gene and could be exploited to identify both slowly and rapidly growing mycobacteria (RGM) as well as nocardiae (McNabb et al., 2004; Plikaytis et al., 1992; Ringuet et al., 1999; Steingrube et al., 1995b; Wayne and Sramek, 1992). An algorithm based on this approach has been developed for mycobacterial differentiation up to 54 species (Brunello et al., 2001). Following the previous study (Ho et al., 2004), we applied high-resolution CE in the PRA to establish the species-specific RFLP patterns of nocardiae and more mycobacteria. The advantages of high-resolution CE include the more precise estimates of DNA fragment sizes and the available profile of low molecular weight fragments with detection limit reaching 12 bp. As compared with the real sizes deduced from the restriction sites of sequence analysis, 94% of 40 bp or larger fragments differed by less than 5 bp and 98% of fragments smaller than 40 bp differed by less than 3 bp. A reproducible pattern with SDs of all fragments less than 5 bp was also essential for the RFLP analysis. It appears more convenient to adopt the electrophoregram rather than perform the cumbersome slab gel electrophoresis plus diagnostic algorithm for identification. Including the PRA of 12 *Mycobacterium* spp. published earlier (Ho et al., 2004), the full-range (10–200 bp) RFLP patterns of all 24 *Mycobacterium* and 7 *Nocardia* spp. were readily differentiated from each other. The RFLP pattern of *M. tuberculosis* does not coincide or cross-react with any of MOTT species in which *M. celatum* (Fig. 1B) has been reported to cause false-positive results by the use of probe method (Butler et al., 1994; Somoskovi et al., 2000). Closely related species exhibiting similar biochemical characteristics could also be well discriminated by the variance of one *Hae*III digestion site. In good agreement with the *hsp65* gene sequencing, PRACE generates direct unambiguous RFLP patterns and can distinguish medically relevant species. Based on the results, PRACE offers an alternative for simultaneous screening of MTC, MOTT, and *Nocardia* spp.

M. lacticola (Fig. 1C) and *M. neoaurum* (Fig. 1F) are closely related species with similar *hsp65* gene sequences. The clinical presentation of *M. neoaurum* involves infection of indwelling catheters, primarily in immunosuppressed patients (Davison et al., 1988; George and Schlesinger, 1999). Within the RGM, *M. lacticola* is also an unusual cause of vascular catheter-related bacteremia. Currently, no biochemical data were available on isolates identified as "*M. lacticola*" (Kiska et al., 2004). Sequence analysis of 16S rRNA gene was required to identify both organisms, and they were found to have 99.3% identity. By the PRA of *hsp65* gene, *M. neoaurum* and *M. lacticola* demonstrated almost identical RFLP patterns with a slight difference in the size of the lower molecular weight band of *Hae*III digest. The given genetic similarities are in concord with our results in which both species could be undoubtedly distinguished by PRACE. As recalled from our previous study (Ho et al., 2004), rapidly growing *Mycobacterium abscessus* and *Mycobacterium chelonae* differed by 3 bases in the 16S rRNA sequence, as well as slow-growing *M. kansasii* and *M. gastri* with identical 16S rRNA sequence, could also be conspicuously discriminated by PRACE.

M. mucogenicum (Fig. 1E), formerly *M. chelonae*-like organism (Springer et al., 1995), was occasionally found to cause posttraumatic wound infections and catheter-related sepsis. It has unique 16S rRNA gene sequences in comparison with other RGM such as *M. chelonae*, *M. abscessus*, *M. fortuitum*, *Mycobacterium peregrinum*, and *Mycobacterium smegmatis*. Although the 16S rRNA sequencing is now the reference sequencing method, it does not permit the differentiation of closely related RGM species. The *hsp65* gene sequencing was devised for RGM species because it displayed more polymorphism than the 16S rRNA gene sequencing (Ringuet et al., 1999). The PRA of *hsp65* gene

had also been developed to differentiate clinically important species of RGM, but it required 4 separate runs with different restriction enzymes (Steingrube et al., 1995b). We applied high-resolution CE to *hsp65* PRA so that the RFLP patterns of *M. mucogenicum* and *M. peregrinum* (Fig. 1G) in the current study, along with those of other RGM including *M. chelonae*, *M. abscessus*, *M. fortuitum*, and *M. smegmatis* in the previous study (Ho et al., 2004), could be unmistakably distinguished with each other.

Nocardia spp. are Gram-positive aerobic actinomycetes found worldwide as soil saprophytes. At least 6 species are pathogenic for humans and may enter the body via inhalation or wounds with contaminated dust particles (McNeil and Brown, 1994). They are responsible for several infections including pulmonary, central nervous system, and cutaneous infections. Nocardiosis is diagnosed by isolation and culture identification. However, their colonial characteristics and cellular morphology are variable, and may be misidentified as *Mycobacterium* (Olson et al., 1998; Patterson et al., 1992; Short et al., 2005; Staneck et al., 1981; Steingrube et al., 1995a). Accurate identification of pathogenic aerobic actinomycetes is particularly important when aminoglycoside-resistant species (Wilson et al., 1997) such as *N. transvalensis* complex (Fig. 2G) and cephalosporin-resistant species (Steingrube et al., 1993; Wallace et al., 1990) such as *Nocardia farcinica* (Fig. 2D) are involved or when invasive species (Ruimy et al., 1996; Wallace et al., 1995) such as *N. pseudobrasiliensis* (Fig. 2F) are encountered.

A recent study reassessed the capacity of PCR–restriction enzyme pattern analysis of *hsp65* gene to differentiate *Nocardia* spp. Their results concluded that *hsp65* PRA must no longer be used for *Nocardia* spp. identification because many species had the same restriction pattern (Rodriguez-Nava et al., 2006). We understand that such a conclusion is made without the availability of high-resolution RFLP patterns. The major disadvantages of PRA with slab gel electrophoresis are that sizes of fragments are not accurately estimated and fragments of similar sizes are not well discriminated, which are especially difficult for fragments smaller than 60 bp. Some authors have ignored restriction fragments shorter than 60 bp because they may be primer or primer dimer bands (Telenti et al., 1993). Others take into account fragments up to 50 bp (Steingrube et al., 1995b). Thus, interpretation of fragment sizes is sometimes difficult because of the similarity of restriction patterns. Here, high-resolution CE can overcome the disadvantages of slab gel electrophoresis.

On the basis of *hsp65* gene homologies from RGM and *Nocardia* strains, an RFLP analysis of amplified *hsp65* gene was developed to distinguish these 2 genera (Lungu et al., 1994). Because the number of patterns was large and the fragment sizes were difficult to analyze, the profiles obtained were sometimes hard to interpret. Lungu's difficulty in pattern analysis can be attributed to the lack of full-range (10–200 bp) RFLP patterns as shown in Fig. 2. For example, most species of nocardiae have similar large fragments near 162, 114, and 87 bp, which are easily confused with fragments 176, 98, and 78 bp by agarose gel electrophoresis. However, precise estimate of DNA fragment sizes and additional profile in the low molecular weight range evidently make their patterns entirely different and recognizable. For *N. pseudobrasiliensis* (Fig. 2F) and *N. transvalensis* complex (Fig. 2G), which differ by only 2 small peaks of 23 and 19 bp, the identification will be impossible without the help of better resolution and size calibration. With the advantages of high resolution of PRACE in the present and previous studies, discriminations were discrete among all 24 *Mycobacterium* and 7 *Nocardia* spp.

The full-range RFLP patterns provided by technical improvement, as shown by the results of our study, could help us beyond the limitation of identifiable numbers of species and the expensive cost of probes. Nevertheless, there are other questions that remain to be explored, such as additional testing of more than 1 to 2 strains per species, the establishment of all available species-specific RFLP patterns, the improvement of detection limit, and automation. The most important issue is the ability of PRACE to identify mycobacteria or nocardiae directly from clinical isolates. In conclusion, we believe that PRACE offers an excellent nonprobe alternative for rapid identification of multiple cultured *Mycobacterium* and *Nocardia* to the species level. Although this study just establishes the database for identification purpose, routine application of PRACE for rapidly identifying more microbiologic species without probes might be feasible soon.

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