EVALUATION OF POLYMERASE CHAIN REACTION AND RESTRICTION ENZYME ANALYSIS FOR ROUTINE IDENTIFICATION OF MYCOBACTERIA: ACCURACY, RAPIDITY, AND COST ANALYSIS

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Abstract. Polymerase chain reaction and restriction enzyme analysis (PCR-REA) of the *hsp65* gene was evaluated for use as a routine identification method for identifying mycobacteria. The accuracy, rapidity, and cost were assessed compared with the conventional biochemical method. Five hundred and forty-one mycobacterial clinical isolates obtained from the Department of Microbiology, Faculty of Medicine at Siriraj Hospital, Mahidol University, were submitted for PCR-REA and biochemical identification. PCR-REA showed high concordant result with 100, 96.2, and 94.1% for identification of *Mycobacterium tuberculosis*, rapid- and slow-growing mycobacteria, respectively. Discordant results were obtained from 24 (4.4%) out of 541 isolates, consisting of 9 rapid growers (6 *M. chelonae*, 2 *M. abscessus*, and 1 *M. fortuitum*) and 15 slow growers (9 *M. scrofulaceum*, 2 *M. gordonae*, 1 *M. avium*, 1 *M. kansasii*, 1 *M. malmoense*, and 1 *M. terrae* complex). PCR-REA demonstrated not only accurate results but was also less expensive (2.1 US$/sample). This method was rapid with a turn-around time of 30 hours compared with 2-4 weeks for the conventional method.

INTRODUCTION

The continued threat of the ancient disease tuberculosis (TB) is still a public health hazard, especially in the developing world. A staggering 1.9 million people around the globe die of tuberculosis each year; another 1.9 billion are latently infected with *Mycobacterium tuberculosis* and are at risk for active disease (Dye *et al*, 1994). Moreover, the increasing number of immunocompromized hosts, mostly due to HIV infection, accelerates infection by opportunistic mycobacterial pathogens, *eg M. avium* complex (MAC) (Falkinham III, 1996).

The treatment of infections with nontuberculous mycobacteria (NTM) is different from that of tuberculosis. Species identification of the causative agent is therefore important for selecting an appropriate therapeutic regimen. The identification of mycobacteria has been conventionally dependent on the biochemical method which requires many weeks until a result is obtained. In addition, the results are often uninterpretable among the closely-related species. This has led to the development of methods which can be used to identify mycobacteria more rapidly and accurately. Many methods have been introduced to fulfill this requirement. Mycolic acid analysis by chromatographic methods has shown good results but still has limitations due to the cost of the instruments and accessories (Chou *et al*, 1996; Leite *et al*, 1998; Kellog *et al*, 2001). Identification using liquid hybridization such as AccuProbe (Gen-Probe) or PCR-based identification with species-specific primers is restricted only to some species (Lumb *et al*, 1993; Park *et al*, 2000). Sequencing of 16S rDNA provides the best identification results for currently known species but some species cannot be differentiated (Springer *et al*, 1996;

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Kirschner *and* Böttger, 1998; Reischl *et al*, 1998; Tønjum *et al*, 1998; Patel *et al*, 2000). In addition, the cost of the sequencing method is still too expensive for routine laboratory use (Cook *et al*, 2003).

Among the promising methods used for mycobacterial identification in a routine laboratory, polymerase chain reaction and restriction enzyme analysis (PCR-REA), otherwise named PRA or PCR-RFLP, is one of the simple and rational methods. Of the published target genes, *viz hsp65* (Plikaytis *et al*, 1992; Telenti *et al*, 1993; Devallios *et al*, 1997; Taylor *et al*, 1997), *rpoV* (Comincini *et al*, 1998), ITS (Sansila *et al*, 1998; Roth *et al*, 2000), *rpoB* (Lee *et al*, 2000), and 16S rDNA (de Baere *et al*, 2002), *hsp65* is a promising target because PCR-REA based on this target has been widely used since 1992 and the technique employs only two restriction enzymes for broad identification of mycobacterial species. Therefore, this study evaluated the possible use of PCR-REA in a routine mycobacteriology laboratory for identification of cultured mycobacteria in terms of its accuracy, rapidity, and cost compared with the biochemical identification.

MATERIALS AND METHODS

Bacterial strains and growth condition

Twenty mycobacterial reference strains and 541 clinical isolates were obtained from the Division of Mycology and Mycobacteriology, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, during January 2000 - March 2004, except that 11 isolates of *M. marinum*, isolated in 1998, were obtained from stock cultures. The reference strains are listed in Table 1. Mycobacterial clinical isolates, consisting of 50 strains of *M. tuberculosis* and 491 strains of NTM, were isolated from various clinical samples. These strains were cultured and maintained on the Löwenstein-Jensen (LJ) medium (BBL) and incubated at 37ºC (or room temperature for *M. marinum*) until colonies appeared. Growth rate, colony morphology and pigment production were determined as preliminary identification.

Biochemical identification

All clinical isolates were initially differentiated into *M. tuberculosis* complex (MTC) and NTM based on the results of preliminary identification. Both MTC and NTM were subsequently identified by a biochemical approach (a total of 13 tests) according to standard protocols (Kent and Kubica, 1985).

Polymerase chain reaction and restriction enzyme analysis

Genomic DNA was extracted by a boiling method. In brief, one loopful of mycobacterial cells was scraped from the surface of the LJ medium and suspended in 250 μl of TE (10 mM Tris, pH 8.0 and 1 mM EDTA) buffer. The suspension was boiled at 100ºC for 15 minutes and cell debris was removed by centrifugation at 13,000*g* for 2 minutes. The supernatant was stored at -20ºC until used.

Amplification of *hsp65* was performed as previously described by Telenti *et al* (1993) with some modifications. The PCR mixture (50 μl) contained 1x PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin), 200 μM dNTPs (Amersham Biosciences, USA), 10 pmol each primer [Tb11 (5'-ACCAACG ATGGTGTGTCCAT-3') and Tb12 (5'-CTTGTCG AACCGCATACCCT-3')], 1 unit of *Taq* DNA polymerase (Amersham Biosciences, USA), and 5 μl of DNA template. The PCR condition consisted of initial denaturation at 94ºC for 5 minutes; amplification was performed in the Touchgene Gradient (Techne) for 35 cycles of 1 minute of denaturation at 94ºC, 1 minute of annealing at 60ºC, and 1 minute of primer extension at 72ºC with an additional 10 minutes of elongation after the last cycle.

After amplification, the 439-bp amplified product of *hsp65* was detected by using 2.5% agarose gel electrophoresis (Organic Research). The amplified product was then digested separately with two restriction enzymes, *Bst*EII (Amersham Pharmacia) and *Hae*III (Amersham Pharmacia). The digestion reaction was conducted in a 1.5-ml microcentrifuge tube, consisting of 12.5 μl of amplified product, 1.5 μl of 10x reaction buffer and 2 units of restriction enzyme for a total volume of 15 μl. After incubation at 37ºC overnight, the digested products were separated using 4% agarose gel electrophoresis [~2.5 V/cm] using 25-bp DNA ladder

(Invitrogen) and the digested products of *M. tuberculosis* as a molecular size marker and internal size marker, respectively. The sizes of digested bands were measured, compared to those of the 25-bp DNA ladder and the internal marker, and the species was identified by using the published algorithms (Devallois *et al*, 1997).

Cost analysis

The cost of biochemical identification and PCR-REA was considered only in terms of material costs, including materials and reagents used in each method. Labor and capital costs (building and laboratory instruments) were excluded from the cost assessment because of the different costs among countries. The cost is based on the material price (year 2002) in baht and converted to the US$ (1 US$ = 40 baht).

RESULTS

Identification of *Mycobacterium* reference strains

All reference strains revealed a correlated result with PCR-REA. The REA pattern after digestion with *Bst*EII and *Hae*III was identical to previous reports, with the exception of *M. neolactis* that was absent in those studies (Devallois *et al*, 1997; Comincini *et al*, 1998; Sansila *et al*, 1998). The PCR-REA pattern of *M. neolactis* showed digested fragments of 120 and 325 bp and 60 and 155 bp after digestion with *Bst*EII and *Hae*III, respectively.

Identification of *Mycobacterium tuberculosis* complex (MTC)

Among 50 MTC clinical isolates, no intraspecies variation was observed using PCR-REA (*Bst*EII: 245/120/80 and *Hae*III: 160/140/70). Comparison of PCR-REA and the biochemical method showed 100% concordant result as summarized in Table 2.

Identification of nontuberculous mycobacteria

Rapidly growing mycobacteria. Two hundred and thirty-six isolates of rapidly growing mycobacteria including *M. abscessus*, *M. chelonae*, and *M.*

Table 1
List of *Mycobacterium* reference strains used in this study.

Mycobacterium species	Strain	Source
M. avium	ATCC 25291	Dr C Abe
M. bovis BCG	ATCC 35735	Dr P Palittapongarnpim
M. duvalii	MNC 442	TB division, Thailand
M. flavescens	ATCC 23035	TB division, Thailand
M. fortuitum	ATCC 6841	TB division, Thailand
M. gordonae	ATCC 144701	Dr C Abe
M.intracellulare	ATCC 13950	Dr C Abe
M. kansasii	ATCC 12478	Dr C Abe
M. marinum	ATCC 927	Dr C Abe
M. microti	KK 1401	Dr C Abe
M. neolactis	S 152	TB division, Thailand
M. nonchromogenicum	ATCC 19530	Dr C Abe
M. phlei	ATCC 23042	TB division, Thailand
M. scrofulaceum	ATCC 19981	Dr C Abe
M. smegmatis[a]	mc^2155	Snapper *et al*, 1990.
M. szulgai	JATA 3201	Dr C Abe
M. terrae	ATCC 15775	TB division, Thailand
M. tuberculosis	ATCC 27294	TB division, Thailand
M. tuberculosis	H37Rv Japan	TB division, Thailand
M. xenopi	ATCC 19250	Dr C Abe

ATCC, American Type Culture Collection, Rocksville, Maryland
JATA, Japan Anti-Tuberculosis Association, Kiyose-shi, Tokyo
[a]*M. smegmatis* was kindly provided by Prof Dr EC Böttger.

Table 2
Summary of identification results of the biochemical method, PCR-REA, and 16S rDNA sequencing.

Biochemical identification	PCR-REA	16S rDNA sequencing[a]
M. tuberculosis (50)	*M. tuberculosis* complex (50)	-
Slowly growing mycobacteria		
Photochromogens		
M. kansasii (37)	*M. kansasii* I, IV (36)	-
	M. aurum / neoaurum (1)	*M. neoaurum*
M. marinum (13)	*M. marinum* (13)	-
Scotochromigens		
M. gordonae (13)	*M. gordonae* III, IV, VI (11)	-
	M. simiae II (2)	-
M. scrofulaceum (9)	*M.asiaticum* (1)	*Mycobacterium* IWGMT 90237
	M. gordonae III (2)	-
	M. gordonae III (1)	*M. gordonae*
	M. gordonae IV (1)	*M. gordonae*
	M. intracellulare (1)	*M. intracellulare*
	M. simiae I (1)	*Mycobacterium* IWGMT 90236
	Unpublished pattern	*Mycobacterium* MCRO 33
	M. simiae I (1)	-
M. szulgai (1)	*M. szulgai* (1)	-
Nonchromogens		
M. avium complex (180)	*M. avium* (153)	-
	M. intracellulare[b] (26)	-
	M. simiae I (1)	-
M. malmoense (1)	*M. kansasii* I (1)	*M. kansasii / gastri*
M. terrae complex (1)	*M. kansasii* I (1)	*M. kansasii*
Rapidly growing mycobacteria		
M. chelonae / abscessus (45)	*M. abscessus* I, II (43)	-
	M. chelonae I (1)	-
	M. fortuitum (1)	*M. fortuitum*
M. abscessus (97)	*M. abscessus* (95)	-
	M. fortuitum (2)	-
M. chelonae (5)	*M. abscessus* I, II (5)	-
M. fortuitum (89)	*M. fortuitum* (88)	-
	M. abscessus II (1)	-

The number of isolates are indicated in brackets; - represents that 16S rDNA sequencing was not done
[a]The 16S rDNA sequencing was performed as described by Kirschner and Böttger (1998)
[b]Three isolates showed novel pattern but 16S rDNA sequencing confirmed as *M. intracellulare*

fortuitum, identified by the biochemical method, were submitted for identification using PCR-REA. Although the *M. chelonae* clade has been differentiated into *M. chelonae* and *M. abscessus* by a NaCl tolerance test and utilization of sodium citrate, 45 of 147 isolates used in this study were not separated by those two tests. All of them were biochemically identified as *M. chelonae/ abscessus* whereas the others were separated either into *M. abscessus* or *M. chelonae*. PCR-REA identified all 147 isolates as *M. abscessus* (143 isolates), *M. chelonae* (1 isolate), and *M. fortuitum* (3 isolates). Likewise, 1 of 89 isolates of *M. fortuitum* was discordantly identified as *M. abscessus* by PCR-REA (Table 2).

Slowly growing mycobacteria. Two hundred and fifty-five clinical isolates of slowly growing mycobacteria including photochromogenic,

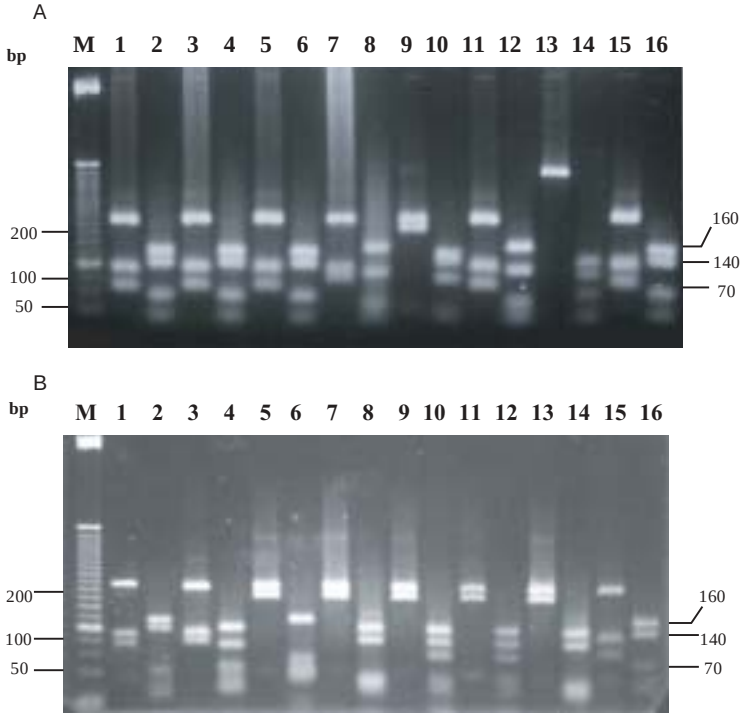

Fig 1–PCR-REA patterns of reference strains and clinical isolates after digestion with *Bst*EII (lanes 1,3,5,7,9,11,13,15) and *Hae*III (lanes 2,4,6,8,10,12,14,16).

A) Reference strains. M: 25-bp DNA ladder; lanes 1-2: *M. tuberculosis* H37Rv; lanes 3-4: *M. tuberculosis* ATCC 27294; lanes 5-6: *M. bovis* BCG ATCC 35735; lanes 7-8: *M. gordonae*; lanes 9-10: *M. scrofulaceum* ATCC 19981; lanes 11-12: *M. gordonae* ATCC 14470; lanes 13-14: *M. szulgai* JATA 3201; lanes 15-16: an internal marker *M. tuberculosis*.

B) Clinical isolates. M: 25-bp DNA ladder; lanes 1-2: *M. intracellulare*; lanes 3-4: a novel pattern *M. intracellulare*; lanes 5-6: *M. abscessus* I; lanes 7-8: *M. avium*; lanes 9-10: *M. kansasii* I; lanes 11-12: *M. kansasii* I; lanes 13-14: *M. avium*; lanes 15-16: an internal marker *M. tuberculosis*.

scotochromogenic, and nonchromogenic groups were submitted for identification using the biochemical method and PCR-REA. For the photochromogenic group, 50 clinical isolates were identified using the biochemical method as *M. kansasii* (37 isolates) and *M. marinum* (13 isolates). PCR-REA results demonstrated that 97% (36 of 37) of *M. kansasii* isolates were concordantly identified whereas one isolate was identified as *M. aurum / neoaurum*. All 13 isolates of *M. marinum* were 100% concordantly identified by the two methods.

In the case of the scotochromogenic group, biochemical identification specified 23 clinical isolates as *M. gordonae* (13 isolates), *M. scrofulaceum* (9 isolates), and *M. szulgai* (1 isolate). Using PCR-REA, 11 isolates of *M. gordonae* were concordantly identified as *M. gordonae* while two isolates were identified as *M. simiae*. All nine isolates of *M. scrofulaceum* were discordantly identified as *M. gordonae* (4 isolates), *M. simiae* (2 isolates), *M. asiaticum* (1 isolate), and *M. intracellulare* (1 isolate). The remaining single isolate showed a unique pattern that could not be identified using the published algorithms (*Bst*EII: 245/120/80 and *Hae*III: 140/100). However, one isolate of *M. szulgai* was correctly identified (Table 2).

For the nonchromogenic group, the conventional method identified 180 clinical isolates as *M. avium* complex (MAC) whereas PCR-REA identified 153 isolates as *M. avium* and 23 isolates as *M. intracellulare.* Three isolates of MAC displayed a novel PCR-REA pattern (*Bst*EII: 245/120/100 and *Hae*III: 140/90/60, see Fig 1) which was absent in the published algorithm; however, these isolates were confirmed by 16S rDNA sequencing as *M. intracellulare* (data not shown). A similar result was obtained after submitting to the PRA database (http://app.chuv.ch/prasite); this unknown pattern was identified as *M. intracellulare* type IV. The remaining isolate was identified as *M. simiae*. Two nonchromogenic mycobacteria, which were biochemically identified as *M. malmoense* and *M. terrae* complex, were discordantly identified as *M. kansasii* by PCR-REA (Table 2).

Table 3
Cost of biochemical identification.

Biochemical test	Mycobacteria					Cost (US$/test)	Duration of test
	A	B	C	D	E		
Arylsulfatase	-	√	√	√	√	0.36 for 3 days 0.73 for 14 days	3 and 14 days
Carbon sources assimilation	-	-	-	-	√	0.32	4 weeks
Heat-stable catalase	√	√	√	√	√	0.03	20 minutes
Growth on MacConkey	-	-	-	-	√	0.07	11 days
Iron uptake	-	-	-	-	√	0.1	4 weeks
NaCl tolerance	-	-	-	-	√	0.1	4 weeks
Niacin accumulation	√	-	-	-	-	1.3	90 minutes
Nitrate reduction	√	√	√	√	√	0.02	2 hours
PNB tolerance	√	-	-	-	-	0.1	4 weeks
Pyrazinamidase	-	√	√	√	-	0.2	4 and 7 days
Tellurite reduction	-	-	-	√	√	0.22	10 days
Tween hydrolysis	-	√	√	√	-	0.02	10 days
Urease	-	√	√	√	√	0.02	7 days

A = TB complex; B = Photochromogen; C = Scotochromogen; D = Nonchromogen; E = Rapid grower

Table 4
Cost of PCR-REA identification.

Identification process	Cost / test (US$)
DNA isolation	0.15
Amplification and amplified product detection	0.7
Restriction enzyme digestion and products detection	1.25
Total	2.10

In summary, the concordant percentage of identification results of PCR-REA compared to the biochemical method was 96.2% (227/236) and 94.1% (240/255) for rapidly and slowly growing mycobacteria, respectively (Table 3). The discordant result was obtained in 24 isolates, including 9 rapid growers and 15 slow growers. To confirm the identification results, 10 of 24 isolates were submitted for 16S rDNA sequencing. The sequences were then searched for homology in the GenBank database using nucleotide-nucleotide BLAST (blastn) for species identification (Altschul *et al*, 1990). The results demonstrated that most of the isolates tested (7 of 10) showed concordant results with those obtained from PCR-REA whereas the remaining three isolates were identified as *Mycobacterium* IWGMT 90236, *Mycobacterium* IWGMT 90237, and *Mycobacterium* MCRO 33 (Table 2).

Determination of cost and turn-around time

The rapidity and cost of mycobacterial identification, which are important issues for selecting the method to be used in a routine laboratory especially in developing countries, were determined and compared between PCR-REA and the biochemical method. The cost of PCR-REA, approximately US$ 2.1/sample, was not significantly higher than that of the biochemical method (Tables 3-5). PCR-REA had a turn-around time of 2-3 days, beginning from receipt of the bacterial culture to obtaining the result, compared to 2-4 weeks for the biochemical identification.

DISCUSSION

During the past several decades mycobacterial identification has depended on the biochemical method which is time-consuming. In addition, phenotypic variability and phenotypic homogeneity that cause undifferential characteristics, and the bias of traditional identification

Table 5
Comparison of turn-around time and cost for identification of mycobacteria between the biochemical method and PCR-REA.

Mycobacteria	Turn-around time		Total cost (US$ / sample)	
	Biochemical	PCR-REA	Biochemical	PCR-REA
MTB complex	3-4 weeks	30 hours	1.45	2.10
Photochromogens	2 weeks	30 hours	1.38	2.10
Scotochromogens	2 weeks	30 hours	1.38	2.10
Nonchromogens	2 weeks	30 hours	1.60	2.10
Rapid growers	3-4 weeks	30 hours	1.97	2.10

schemes towards established taxa, are critical factors affecting accurate identification. PCR-REA, a molecular method, has been developed and used for identification of mycobacteria. Although several genes or sequences have been selected as targets for PCR-REA, most of them use more than two restriction enzymes for speciation of mycobacterial species. Only *hsp65*- and *rpoB*-based PCR-REA need only two restriction enzymes and are able to differentiate up to 40 species of mycobacteria. This makes the method an attractive routine one for identification of mycobacteria, especially in high TB-burdened countries or in laboratories where expensive instruments are limited.

From the results, the concordant percentage of *M. tuberculosis*, rapidly growing, and slowly growing mycobacteria identification using PCR-REA compared to biochemical identification was 100, 96.2 and 94.1%, respectively. Emphasizing NTM identification, 24 isolates (4.4%) from 491 clinical isolates showed discordant results. Most of the discordantly identified *Mycobacterium* species were members of the scotochromogenic group (11 out of 24), *ie*, having phenotypic heterogeneity, rendering them difficult to interpret by the biochemical identification. Nine isolates of *M. scrofulaceum* were discordantly identified; most of them were misidentified as *M. gordonae* (4 isolates) and *M. simiae* (2 isolates). In this study the biochemical tests were repeated and the results matched with *M. scrofulaceum*, suggesting that there are sequence variations in *hsp65* among members of this group and that PCR-REA should be carefully used in interpreting the results or the third method should be used to verify the species identification. Misidentification between *M. scrofulaceum* and *M. simiae*, a member of the photochromogenic group, was present, if these *M. simiae* strains showed negative for niacin accumulation test as previously reported (Rynkiewicz *et al*, 1998). Similarly, that study also demonstrated that some strains of *M. simiae* did not produce pigment upon exposure to light that could lead to misclassification and misidentification of *M. avium* and *M. simiae* as found in this study.

Misidentification between photochromogenic and nonchromogenic groups occurred in two isolates, *M. malmoense* and the *M. terrae* complex. They were identified as *M. kansasii* by PCR-REA. This might be obtained from an incorrect preliminary Runyon's classification of the isolates resulting in the misidentification of the species. Unfortunately, these isolates were lost from the stock culture and could not be retrieved for repetition in the biochemical testings. In addition, 9 isolates of rapidly growing mycobacteria were discordantly identified. The misidentification of *M. chelonae* as *M. abscessus,* or vice versa, found in this study, has also been reported in previous studies (Conville and Witebsky, 1998; Yakrus *et al*, 2001).

The intraspecies phenotypic variation of an individual strain and the inexperience of laboratory personnel are all drawbacks for using the biochemical method to identify species. Nevertheless, 10 of 24 isolates of discordantly identified mycobacteria were submitted for 16S rDNA

sequencing, and the sequencing results showed that most of them (7 isolates) were identified concordantly with PCR-REA. The remaining three isolates, IWGMT 90236, IWGMT 90237, and MCRO 33, were problematic phenotypic clusters of slowly growing mycobacteria described previously (Springer *et al*, 1996; Wayne *et al*, 1996). Besides the identification purpose, PCR-REA could differentiate the rapid growing mycobacteria into *M. abscessus* type I and II, *M. chelonae* type I, and *M. fortuitum* type I and II. The correlation among species and subtypes of the *M. fortuitum* complex and clinical significance should be further analysed. Additionally, *M. kansasii* and *M. gordonae* were identified and divided into *M. kansasii* I, IV, *M. gordonae* III, IV, and VI, respectively. This suggests the genotypic heterogeneity of *hsp65* sequence in these groups and this may possibly be used in epidemiological studies.

In summary, PCR-REA showed good concordant results when compared to the conventional biochemical method. Besides the rapid and accurate results for unknown mycobacterial identification, the cost of PCR-REA is also inexpensive. This method needs only facilities for performing PCR and detecting of the amplified products, suggesting possible use in routine microbiological laboratories where a large number of samples are submitted for mycobacterial identification. In addition, the existence of a PRA database website for *hsp65* provides an easy guide to interpret the patterns and to prevent incorrect identification. This method is recommended for implemention in reference mycobacteriology laboratories in developing countries to aid their epidemiological data on mycobacteria.

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