Evaluation of PCR–RFLP analysis targeting *hsp65* and *rpoB* genes for the typing of mycobacterial isolates in Malaysia

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In this study, PCR–RFLP analysis (PRA) targeting *hsp65* and *rpoB* gene regions was evaluated for the identification of mycobacterial species isolated from Malaysian patients. Overall, the *hsp65* PRA identified 92.2% of 90 isolates compared to 85.6% by the *rpoB* PRA. With 47 rapidly growing species, the *hsp65* PRA identified fewer (89.4%) species than the *rpoB* PRA (95.7%), but with 23 slow-growing species the reverse was true (91.3% identification by the *hsp65* PRA but only 52.5% by the *rpoB* PRA). There were 16 isolates with discordant PRA results, which were resolved by 16S rRNA and *hsp65* gene sequence analysis. The findings in this study suggest that the *hsp65* PRA is more useful than the *rpoB* PRA for the identification of *Mycobacterium* species, particularly with the slow-growing members of the genus. In addition, this study reports 5 and 12 novel restriction patterns for inclusion in the *hsp65* and *rpoB* PRA algorithms, respectively.

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INTRODUCTION

There are at least 130 species in the genus *Mycobacterium*, of which more and more are being found to be associated with human diseases (Tortoli *et al.*, 2009). The timely diagnosis of mycobacterial infections is essential for early specific treatment and prevention of dissemination due to nosocomial (Bolan *et al.*, 1985; Astagneau *et al.*, 2001), occupational (Shelton *et al.*, 1999) or domestic exposure (Winthrop *et al.*, 2002; Lumb *et al.*, 2004). This is particularly important in view of the resistance of most non-tuberculous mycobacteria (NTM) to one or more first-line antituberculosis drugs (Brown-Elliott & Wallace, 2002).

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Abbreviations: MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria; PRA, PCR–RFLP analysis; UMMC, University Malaya Medical Centre.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *hsp65* gene sequences of M14, M15, M19, M26, M38, M41, M42, M44, M49, M60, M64, M65, M74, M81, M82 and M88 reported in this study are GQ184152–GQ184167, respectively, and GQ184168–GQ184183, respectively.

A table of isolate identification data is available as supplementary material with the online version of this paper.

Conventionally, mycobacterial species are identified by their growth characteristics (such as growth rate, colonial morphology, pigmentation and photoreactivity) and biochemical reactions. However, phenotypic tests are extremely time-consuming and laborious, and the interpretation of test results could be problematic owing to the presence of inter-assay and intra-species variations (Springer *et al.*, 1996). With the increasing diversity of mycobacterial species becoming known, conventional methods alone are no longer sufficient for diagnostic or research purposes. Mycolic acids analysis (Butler *et al.*, 1996) using HPLC and direct gene sequence analysis (Böddinghaus *et al.*, 1990; Ringuet *et al.*, 1999; Adékambi & Drancourt, 2004) have shown higher accuracy and discriminative power for mycobacterial identification. However, the application of these approaches is limited to reference laboratories owing to the requirement for sophisticated analytical instruments. A variety of probe-based assays (Reisner *et al.*, 1994; Kox *et al.*, 1997; Tortoli *et al.*, 2003) have been introduced, but their use is usually constrained by their relatively high running costs and the ability to identify only a limited number of mycobacterial species. However, PCR–RFLP assays (PRA) (Telenti *et al.*, 1993; Lee *et al.*, 2000) have been reported to provide rapid, accurate and cost-effective identification of a wide range of mycobacteria.

There is little information on the prevalence and distribution of mycobacterial species other than *Mycobacterium tuberculosis* in clinical settings in Malaysia. In this study, two PRAs, targeting *hsp65* (Telenti *et al.*, 1993) and *rpoB* (Lee

et al., 2000) genes were evaluated for the identification of local isolates. Conventional biochemical tests and DNA sequence analyses (of 16S rRNA and *hsp65* genes) were also performed to assist in the identification of these mycobacteria.

METHODS

Mycobacterial isolates. A total of 90 clinical isolates (mostly from respiratory secretions but also from skin and other tissues) were obtained from the Microbiology Diagnostic Laboratory, University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. These isolates were from consecutive unique patients for whom a specific request for mycobacterial culture was made by their attending physicians to exclude tuberculous infections.

In addition, seven reference strains (*M. tuberculosis* H37Rv ATCC 27294, *Mycobacterium avium* ATCC 25291, *Mycobacterium intracellulare* ATCC 13950, *Mycobacterium kansasii* ATCC 12478, *Mycobacterium scrofulaceum* ATCC 19981, *Mycobacterium fortuitum* ATCC 6841, *Mycobacterium bovis* ATCC 19210) and five atypical mycobacterial isolates (*Mycobacterium gordonae*, *M. fortuitum*, *Mycobacterium chelonae*, *Mycobacterium marinum* and *Mycobacterium xenopi*) kindly provided by Professor Peter Hawkey, Public Health Laboratory, Heartlands Hospital, Birmingham, UK, were also included in the evaluation. All isolates were maintained on Löwenstein–Jensen (LJ) media.

DNA extraction. Colonies from LJ medium were suspended in 200 µl double-distilled water and boiled for 10 min. The supernatant collected after centrifugation at 8000 g for 10 min was used for PCR amplification.

PRA targeting *hsp65* and *rpoB* genes. The partial *hsp65* gene (439 bp) was amplified using primers Tb11 (5'-ACCAACGATG-GTGTGTCCAT-3') and Tb12 (5'-CTTGTGCAACCGCATACCCT-3') as described by Telenti *et al.* (1993). Amplification of the partial *rpoB* gene (360 bp) was performed using primers Rpo5' (5'-TCA-AGGAGAAGCGCTACGA-3') and Rpo3' (5'-GGATGTTGATCA-GGGTCTGC-3'), as described by Lee *et al.* (2000). A 10 µl aliquot of amplified *hsp65* products was digested with 5 units *Bst*EII (New England Biolabs) and *Hae*III (New England Biolabs) in separate reaction tubes. The reaction mixtures were incubated for 3 h at 60 °C for *Bst*EII and at 37 °C for the *Hae*III reaction. Similarly, amplified *rpoB* products were digested with 5 units *Msp*I (New England Biolabs) and *Hae*III (New England Biolabs) at 37 °C for 3 h. The digestion mixtures were analysed by Nusieve 3:1 agarose gel (3% w/v) electrophoresis. DNA size markers, pBR322-*Msp*I-digested DNA (New England Biolabs) and 100 bp DNA size markers (Fermentas), were used to enable estimation of the DNA fragment size.

For the *hsp65* PRA, mycobacterial isolates were identified by comparing their restriction patterns with those available in the online database PRAsite (<http://app.chuv.ch/prasite/index.html>); for the *rpoB* PRA, the restriction patterns described by Lee *et al.* (2000) were referred to. Isolates with discordant identification results by the PRAs were further studied by conventional biochemical tests and direct DNA sequence analysis of 16S rRNA and *hsp65* genes.

Growth and biochemical tests. The mycobacterial properties studied included growth rate and pigment production on LJ medium, ability to grow on MacConkey agar, sodium chloride tolerance, niacin accumulation, Tween 80 hydrolysis, tellurite reduction (Lutz, 1992), and the activity of nitrate reductase, urease, arylsulfatase and catalase (semiquantitative and heat-stable) enzymes.

Direct DNA sequence analysis. The 564 bp long 5'-region of the 16S rRNA gene was amplified from the isolates using primers 16MycF (5'-CGTGCTTAACACATGCAAGTCG-3') and 16MycR (5'-GTG-AGATTTTCACGAACA-ACGC-3') (Devulder *et al.*, 2005). Amplified *hsp65* and 16S rRNA gene products were purified using a MinElute PCR purification kit (Qiagen) and sequenced using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI-377 genetic analyzer (Applied Biosystems). The sequences were analysed by referring to public gene sequence repositories: the National Center for Biotechnology Information GenBank database (Altschul *et al.*, 1994; Benson *et al.*, 2007) and the Ribosomal Differentiation of Medical Micro-organisms (RIDOM) database (Harmsen *et al.*, 2002).

RESULTS

Identification of mycobacteria by *hsp65* PRA and *rpoB* PRA

All seven American Type Culture Collection reference strains were identified correctly by the two PRAs. With the five reference strains from the UK Public Health Laboratory, however, there were discrepancies between the two assays. The *hsp65* PRA identified *M. fortuitum* as *Mycobacterium peregrinum*, a closely related species in the *M. fortuitum* complex. *M. chelonae* was identified as *Mycobacterium abscessus* in the *hsp65* assay but as the *M. chelonae*/*M. abscessus* complex in the *rpoB* PRA, which does not distinguish between the two species (Lee *et al.*, 2000). *M. gordonae* was assigned to different subtypes (type IX by the *hsp65* PRA and type IV by the *rpoB* PRA). Neither *M. marinum* or *M. xenopi* were identified in the *rpoB* PRA.

Among the 90 clinical isolates examined, there were 20 strains of the *M. tuberculosis* complex (MTBC), 47 NTM rapid growers and 23 slow growers (Table 1). All MTBC isolates were concordantly identified by the two assays. Among the rapid growers, the *rpoB* assay identified 28 *M. fortuitum* (21 type I and 7 type II) and 17 strains classified as *M. chelonae*/*M. abscessus* complex because the restriction patterns generated for the two species are indistinguishable. In contrast, the *hsp65* assay identified only 24 *M. fortuitum* (type I) but was able to differentiate *M. abscessus* (8 type I and 9 type II) from *M. chelonae*, and *M. peregrinum* (type III) from *M. fortuitum*. One isolate (M15) was unidentifiable in both assays and four isolates (one *Mycobacterium austroafricanum* and three *M. fortuitum*) were identified by the *rpoB* assay but not by the *hsp65* assay.

Among the slow-growing NTM species, the *rpoB* assay performed dismally compared to the *hsp65* assay, identifying only 12 of 23 isolates even with the use of two restriction enzymes. The 11 isolates not identifiable included *M. marinum*, *Mycobacterium simiae*, *M. gordonae*, *Mycobacterium parascrofulaceum*, *M. avium* and *M. intracellulare*. However, with the *hsp65* PRA, there were only two unidentifiable patterns, both of which were also unidentifiable with the *rpoB* assay.

Table 2 summarizes the results obtained for all 90 clinical isolates. Overall, the *hsp65* PRA identified 83 (92.2%)

Table 1. *hsp65* and *rpoB* PRA results for mycobacterial clinical isolates

<i>hsp65</i> PRA	No. of isolates	<i>rpoB</i> PRA	Remark
MTBC	20	MTBC	–
Rapid growers (n=47)			
<i>M. abscessus</i> I	8	<i>M. chelonae</i> / <i>M. abscessus</i>	–
<i>M. abscessus</i> II/ <i>M. massiliense</i> I/ <i>M. bolletii</i> I	9	<i>M. chelonae</i> / <i>M. abscessus</i>	–
<i>M. fortuitum</i> I/ <i>M. fortuitum</i> subsp. acetamidolyticum I/ <i>M. farcinogenes</i> I/ <i>M. conceptionense</i> I/ <i>M. houstonense</i> I/ <i>M. neworleanense</i> I/ <i>M. senegalense</i> I	21	<i>M. fortuitum</i> I	–
<i>M. peregrinum</i> III	3	<i>M. fortuitum</i> II	–
Novel pattern (<i>Bst</i> EII: 240/115/80; <i>Hae</i> III: 140/125/100/50)	1	<i>M. fortuitum</i> II	M38, M42, M65
Novel pattern (<i>Bst</i> EII: 240/210; <i>Hae</i> III: 130/115/60/40/35)	1	<i>M. austroafricanum</i>	M49
Novel pattern (<i>Bst</i> EII: 240/115/100; <i>Hae</i> III: 140/90/60)	1	Novel pattern (<i>Msp</i> I: 135/105/60/50; <i>Hae</i> III: 110/90/80)	M15
Slow grower, photochromogenic (n=4)			
<i>M. kansasii</i> I	2	<i>M. kansasii</i> I	–
<i>M. marinum</i> I/ <i>M. ulcerans</i> I/ <i>M. malmoeense</i> II	1	Novel pattern (<i>Msp</i> I: 105/70/60/45/40; <i>Hae</i> III: 210/80/60)	M19
<i>M. simiae</i> I/ <i>M. genavense</i> II	1	Novel pattern (<i>Msp</i> I: 105/95/75/45/40; <i>Hae</i> III: 180/115)	M14
Slow grower, scotochromogenic (n=11)			
<i>M. gordonae</i> III	6	<i>M. gordonae</i> I/ <i>M. celatum</i> I	–
<i>M. szulgai</i> I	1	<i>M. szulgai</i>	–
<i>M. gordonae</i> V/ <i>M. interjectum</i> I/ <i>M. heidelbergense</i> I	1	Novel pattern (<i>Msp</i> I: 165/60/40; <i>Hae</i> III: 210/100)	M44
<i>M. parascrofulaceum</i> II/ <i>M. lentiflavum</i> IV	1	Novel pattern (<i>Msp</i> I: 120/95/70; <i>Hae</i> III: 210/100)	M81
<i>M. parascrofulaceum</i> II/ <i>M. lentiflavum</i> IV	1	Novel pattern (<i>Msp</i> I: 95/80/70; <i>Hae</i> III: 210/100)	M82
Novel pattern (<i>Bst</i> EII: 240/115/100; <i>Hae</i> III: 130/115/70/40/35)	1	Novel pattern (<i>Msp</i> I: 145/80/40; <i>Hae</i> III: 205/90)	M74
Slow grower, nonchromogenic (n=8)			
<i>M. avium</i> subsp. <i>avium</i> I/ <i>M. avium</i> subsp. <i>silvaticum</i> I/ <i>M. avium</i> subsp. <i>paratuberculosis</i> I	3	<i>M. avium</i>	–
	1	Novel pattern (<i>Msp</i> I: 180/95/40; <i>Hae</i> III: 180/90)	M26
	1	Novel pattern (<i>Msp</i> I: 180/135/40; <i>Hae</i> III: 180/90)	M60
<i>M. avium</i> II/ <i>M. colombiense</i> I	1	Novel pattern (<i>Msp</i> I: 105/95/80; <i>Hae</i> III: 180/90)	M88
<i>M. intracellulare</i> I/ <i>M. chimaera</i> I	1	Novel pattern (<i>Msp</i> I: 175/95/80; <i>Hae</i> III: 185/115)	M41
Novel pattern (<i>Bst</i> EII: 440; <i>Hae</i> III: 140/100/90/40/35)	1	Novel pattern (<i>Msp</i> I: 120/80/55/45; <i>Hae</i> III: 180/60)	M64

M., *Mycobacterium*.

isolates, while the *rpoB* PRA identified only 78 (86.7 %). The two assays gave concordant results in 73 (81.1 %) isolates. Among the 70 NTM isolates, there was only 1 discordant result (a *M. peregrinum*/*M. fortuitum* difference that was not considered a significant discrepancy); however, there were 16 (22.9 %) unidentifiable PRA patterns, 9 from the *rpoB* assay, 4 from *hsp65* and 3 from both assays.

Resolving undeterminable results

The 16 NTM isolates that gave unidentifiable PRA patterns with either PRA were further analysed by taking into consideration their growth characteristics, the results of biochemical tests and DNA sequence information. Nucleotide sequences 389–535 bp in length were obtained from the direct sequence determination of the 16S rRNA gene and sequences 365–407 bp long from the *hsp65* gene.

All the DNA sequences obtained for these gene sequences of M14, M15, M19, M26, M38, M41, M42, M44, M49, M60, M64, M65, M74, M81, M82 and M88 have been deposited in the National Center for Biotechnology Information GenBank with accession numbers GQ184152 to GQ184183.

Based on the results of conventional tests and direct DNA sequence analysis, each of the 16 NTM isolates could be assigned to a *Mycobacterium* species (Supplementary Table S1 available with the online journal). Nine of these (M14, M19, M26, M41, M44, M60, M81, M82 and M88) were consistent with the species identified by the *hsp65* PRA and three (M38, M42 and M65) were similar to the *rpoB* PRA identification. M49, which was identified as *M. austroafricanum* by the *rpoB* PRA, was redesignated *M. gordonae* based on the sequence analysis, although phenotypically, it was a rapid growing scotochromogen more like *M.*

Table 2. Summary of mycobacterial species identification by *hsp65* and *rpoB* PRAs

Mycobacterial clinical isolate	No. (%) of isolates with:		No. (%) of isolates unidentifiable by:		
	Concordant PRA results	Discordant PRA results	<i>hsp65</i> PRA only	<i>rpoB</i> PRA only	<i>hsp65</i> and <i>rpoB</i> PRAs
MTBC (<i>n</i> =20)	20 (100 %)	0	0	0	0
NTM					
Rapid growers (<i>n</i> =47)	41 (87.2 %)	1 (2.1 %)	4 (8.5 %)	0	1 (2.1 %)
Slow growers, photochromogen (<i>n</i> =4)	2 (50.0 %)	0	0	2 (50.0 %)	0
Slow growers, scotochromogen (<i>n</i> =11)	7 (63.6 %)	0	0	3 (27.3 %)	1 (9.1 %)
Slow growers, nonchromogen (<i>n</i> =8)	3 (37.5 %)	0	0	4 (50.0 %)	1 (12.5 %)
Total	73 (81.1 %)	1 (1.1 %)	4 (4.4 %)	9 (10.0 %)	3 (3.3 %)

austrorfricanum (Tsukamura *et al.*, 1983). The remaining three isolates (M15, M64 and M74) exhibited novel restriction patterns that have not been reported before in the published algorithms of both PRAs. They were identified as *Mycobacterium insubricum*, *Mycobacterium terrae* and *M. gordonae* based on sequence analysis and, in the case of the latter two, on biochemical reactions as well.

Intraspecies variation

The intraspecies genetic variation was roughly assessed by the number of banding patterns noted for each species of NTM studied. As the number of isolates was small, only *M. abscessus* (17 strains), *M. fortuitum* (28 strains), *M. avium* (6 strains) and *M. gordonae* (7 strains) were analysed. The number of banding patterns observed for these isolates were 1, 2, 4 and 3, respectively, in the *rpoB* PRA, and 2, 3, 2 and 3, respectively, in the *hsp65* PRA.

DISCUSSION

Current knowledge on the complexity and diversity within the genus *Mycobacterium* has come largely from the use of molecular techniques for the differentiation of species and subspecies. Phenotypic characteristics often fail to provide a definitive identification, as illustrated in this study where biochemical tests were inconclusive in 7 of 16 isolates eventually identified by DNA sequence analysis. Nevertheless, these tests can still be useful for the differentiation of certain clusters of mycobacteria that are genetically similar but phenotypically distinct (such as *M. marinum*–*Mycobacterium ulcerans* and *M. kansasii*–*Mycobacterium gastri* clusters).

In Malaysia, few laboratories have facilities for mycobacterial speciation. Reference and state hospital laboratories use liquid culture for isolation and commercial tests such as the AccuProbe system (Gen-Probe) for the identification of *M. tuberculosis*, *M. kansasii* and the *M. avium*–*M. intracellulare* complex. In teaching hospitals, commercial line probe assays such as GenoType Mycobacterium CM/AS (Hain Lifescience) and INNO-LiPA Mycobacteria (Innogenetics) are sometimes used to identify NTM

species. None of these commercial tests have been adequately compared for test performance and value for money. This study has shown that the PRA [at a cost of £5.33 (US \$ 8) per test compared with about £23.33 (US \$ 35) for a line probe assay] could be an inexpensive alternative to the commercial tests in laboratories with smaller budgets. It requires only basic facilities for DNA amplification and gel electrophoresis, but is capable of identifying a wide spectrum of mycobacteria, especially when multiple targets and digestion enzymes are used. Many of the NTM species identified in this study are reported for what is believed to be the first time from Malaysian patients, for instance, *M. simiae*, *Mycobacterium szulgai*, *Mycobacterium interjectum*, *M. terrae*, *M. parascrofulaceum* and *M. insubricum*. The role they played in the clinical conditions they were associated with is not known, but their identification is expected to lead to an increased awareness and interest that will lead to more studies and a better understanding of their epidemiology and pathogenicity. A multi-country retrospective survey conducted by the International Union Against Tuberculosis and Lung Disease found *M. avium* complex, *M. gordonae*, *M. xenopi*, *M. kansasii* and *M. fortuitum* to be the five species most frequently isolated from patient samples, and concluded that NTM diseases appeared to be on the rise, with a changing pattern of infecting species over time and in different geographical areas (Martín-Casabona *et al.*, 2004). The UMMC is a teaching hospital that also serves as a major referral hospital for patients from all over Malaysia. Each year, the laboratory cultures about 8000 specimens for mycobacteria and identifies *M. tuberculosis* and NTM in 7–8 % and 2–3 % of specimens, respectively. The 70 NTM isolates examined in this study would represent close to 35 % of all NTM strains isolated in the medical centre in 1 year. The paucity of reports on NTM infections in the country does not allow us to estimate how representative our isolates are of all isolates circulating in the country. Our data suggest that in Malaysia *M. fortuitum* is by far the most frequently encountered NTM in clinical samples, followed by *M. abscessus*, *M. avium*–*M. intracellulare* and *M. gordonae*. However, as in other parts of the world, a different pattern may emerge following the introduction and wider use of

better diagnostic techniques for the identification of NTM species.

Telenti *et al.* (1993) and Lee *et al.* (2000) showed that *hsp65* and *rpoB* PRAs provided sufficient information to identify most clinically important *Mycobacterium* species and subspecies. The *hsp65* PRA has been evaluated by many other workers (Brunello *et al.*, 2001; Häfner *et al.*, 2004; Leão *et al.*, 2005) and there are 135 restriction patterns described for 174 mycobacterial species and subspecies in the PRA site database (<http://app.chuv.ch/prasite>). There are fewer restriction patterns documented in the *rpoB* PRA algorithm. Lee *et al.* (2000) used 43 species and subspecies for the initial evaluation of this PRA and described only 36 restriction patterns. This smaller database could have contributed to the larger number of unidentifiable slow-growing NTM species obtained with the *rpoB* PRA in this study. In both assays, intraspecies genetic variation gives rise to more than one restriction pattern for a single mycobacterial species, and this, together with similarity in band sizes that are critical for species discrimination, has caused difficulty in the interpretation of restriction patterns for some mycobacteria.

There is no major disagreement in the results obtained from the sequence analysis of 16S rRNA and *hsp65* genes for the 16 NTM isolates with different *hsp65* and *rpoB* PRA results. Both assays contributed novel restriction patterns, 5 (7.1 %) by the *hsp65* PRA (2 for *M. gordonae* and 1 each for *M. fortuitum*, *M. terrae* and *M. insubricum*) and 12 (17.1 %) by the *rpoB* PRA (3 for *M. avium*, 2 for *M. parascrofulaceum*, 1 each for *M. gordonae*, *M. marinum*, *M. simiae*, *M. intracellulare*, *M. interjectum*, *M. terrae*, and *M. insubricum*) (Supplementary Table S1 available with the online journal). These novel patterns illustrate the immensity of intraspecies variation that can be revealed with the testing of mycobacterial species from different geographical areas.

With an increasing recognition of mycobacterial species as human pathogens, mycobacterial diagnostic laboratories must be able to establish a minimum set of tests that will allow them to classify and correctly identify the most commonly found mycobacteria in clinical practice. Where facilities exist for molecular testing, a cost-effective protocol would be to apply the PRA for first line identification and reserve DNA sequencing for isolates not identifiable by PRA patterns. Using representative strains in the country, the construction of a national database (to include clinical information, PRA restriction patterns and other laboratory test findings) will facilitate species identification and eventually improve the epidemiological surveillance for these important micro-organisms.

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