Diagnostic application of genotypic identification of mycobacteria

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This study evaluated conventional methods, GLC and three molecular tests, including 16S rRNA sequencing, for the identification of mycobacteria, and the experiences of the authors with the integration of these methods into a diagnostic clinical laboratory were also recorded. Of 1067 clinical isolates of mycobacteria identified by conventional tests, 365 were tested by Accuprobe hybridization assays and PCRs specific for *Mycobacterium tuberculosis* (MTB) complex or *Mycobacterium avium* complex (MAC), 202 were tested by 16S rRNA sequencing, and 142 were tested by GLC. Three runs of all tests were performed on a weekly basis. The identifications for 209 MTB complex and 118 MAC isolates obtained by species-specific PCR were in complete agreement with AccuProbe hybridization and conventional test results. The 16S rRNA sequence-based identification, at a similarity of $\geq 99\%$, for 132 of 142 isolates was concordant with the identifications made by the biochemical methods, and for 134 isolates was concordant with the identifications made by GLC at species, group or complex level. 16S rRNA sequencing resulted in fewer incorrectly identified or unidentified organisms than GLC or conventional tests. For the slowly growing non-tuberculous mycobacteria, the mean turnaround times for identification were 4–5 days for 16S rRNA sequencing, 14–29 days for GLC and 22–23 days for conventional methods. Considering the large proportion of some species among clinical isolates, a strategy of initial screening with species-specific PCR (or AccuProbe assays) for the MTB complex and MAC, followed by direct sequencing of the strains that yield negative results, should make 16S rRNA sequencing more affordable for routine application in diagnostic laboratories.

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INTRODUCTION

Identification of mycobacteria to the species level is important for epidemiological, public health and therapeutic reasons. Traditionally, mycobacteria are identified by phenotypic traits, such as morphological features, growth rates, preferred growth temperature, pigmentation and biochemical profiles. These methods are well established and standardized. However, testing is laborious, difficult and time-consuming, and results are often not reported in a timely manner to guide clinical decisions. Furthermore, misidentification may occur, because different species may have indistinguishable morphological and biochemical profiles. This is especially true for the newly described species (Brown-Elliott *et al.*, 2002), for which the characteristic phenotypic patterns are either absent or unknown. Accordingly, it is necessary for the clinical laboratory to

consider alternative strategies to address these limitations of the conventional approach. Mycobacterial fatty acid analysis by GLC represents one such technique, and is commonly applied in regional and reference laboratories. The GLC method is well-established, and systems incorporating computer-assisted analysis of fatty acid identities have been commercially developed for routine application (Larsson *et al.*, 1985; Chou *et al.*, 1998; Torkko *et al.*, 2003). Nonetheless, GLC requires subculture of the primary growth, and 3–5 weeks are often necessary to obtain sufficient cells for analysis (Torkko *et al.*, 2003).

In the last decade, advances in molecular methods have facilitated the rapid and reliable identification of many mycobacterial species by molecular means. Nucleic acid probes, species-specific PCR, reverse hybridization and 16S rRNA sequencing have been evaluated for application in clinical laboratories (Cloud *et al.*, 2002; El Amin *et al.*, 2000; Goto *et al.*, 1991; Hall *et al.*, 2003; Kirschner *et al.*, 1993; Lebrun *et al.*, 1992; Patel *et al.*, 1997, 2000; Sarkola *et al.*,

Abbreviations: MAC, *Mycobacterium avium* complex; MTB, *Mycobacterium tuberculosis*.

2004). Due to its ease of use and commercial availability, an acridium ester-labelled probe assay (AccuProbe, GenProbe Inc.) is now incorporated into the workflow of many mycobacterial laboratories (Goto *et al.*, 1991; Lebrun *et al.*, 1992). Specific probes for the identification of *Mycobacterium tuberculosis* (MTB) complex, *Mycobacterium avium* complex (MAC), *Mycobacterium gordonae* and *Mycobacterium kansasii* have been developed. Probes for the differentiation of members of the MAC into *M. avium* and *Mycobacterium intracellulare* are also available. Due to its high analytical sensitivity, species-specific PCR has the added advantage that it may be applied directly to clinical specimens. For the MTB complex and MAC, studies have shown the possibility of nucleic acid detection in smear-negative clinical specimens (Li *et al.*, 1996; Yam *et al.*, 1998; Yuen *et al.*, 1997). The utility of nucleic acid probes and species-specific PCR is, however, limited by the range of mycobacteria which can be identified. Therefore, sequencing of the 16S rRNA was developed for identification of mycobacteria. The findings thus far suggest that this technique is rapid, highly discriminative and reliable (Cloud *et al.*, 2002; Hall *et al.*, 2003; Holberg-Petersen *et al.*, 1999; Kirschner *et al.*, 1993; Patel *et al.*, 2000).

In the present study, we report our evaluation of phenotypic methods, GLC and three genotypic tests for identification of mycobacteria, as well as our experience of their integration into a clinical laboratory.

METHODS

Specimens and isolates. Between July 2001 and May 2004, a total of 1067 *Mycobacterium* strains were isolated from respiratory and non-pulmonary specimens of 932 patients in Hong Kong, including 342 patients in Queen Mary Hospital, 437 patients in Grantham Hospital, 68 patients in Haven of Hope Hospital and 85 outpatients in chest clinics of the Department of Health. All mycobacteria were identified to species, group or complex level according to growth rate, growth at temperatures of 25, 30, 35 and 42 °C, colony morphology, pigmentation, photoreactivity, and a battery of biochemical tests (niacin accumulation, nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, tellurite reduction, arylsulfatase and urease production, NaCl tolerance, and carbohydrate-utilization tests), as described previously (Witebsky *et al.*, 1996; Vincent *et al.*, 2003; Brown-Elliott *et al.*, 2002). The selection of biochemical tests was made according to growth rate, pigment production and colony morphology. Mycobacteria forming visible colonies within 7 days upon subculture were classified as rapid growers (Runyon group IV). Based on production of pigments, the slowly growing organisms were classified into three groups: photochromogen, scotochromogen and nonchromogen, corresponding to Runyon groups I, II and III respectively. The nomenclature of mycobacterial species, complex and group in the latest edition of the *Manual of Clinical Microbiology* was adopted in the present study (Pfyffer *et al.*, 2003).

GLC analysis of mycobacterial cellular fatty acids. Selected mycobacterial isolates were subcultured onto Middlebrook 7H10 agar supplemented with oleic acid/albumin/dextrose/catalase (OADC) and incubated at 35 °C for 1–5 weeks until heavy growth of colonies was observed. A mass of mycobacterial cells sufficient for analysis was saponified, methylated and extracted according to the instructions provided by the manufacturer (MIDI). Whole-cell fatty acid analysis

was performed by a Sherlock Microbial Identification System-Agilent model 6890 gas chromatograph equipped with a flame-ionization detector controlled by Sherlock MIS software (version 4.5). The chromatograms were integrated automatically, and fatty acid identities and percentages were determined using Sherlock pattern-recognition software. The identification of the eluted substances was accomplished by comparing the retention times with that of an external calibration standard provided by the manufacturer. The standard provided was a mixture of straight-chain saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and of 5-hydroxy fatty acids. A reference strain, *Stenotrophomonas maltophilia* (ATCC 13637), was run in parallel as control.

AccuProbe assay. Selected isolates were subjected to AccuProbe assay for MTB complex, MAC, *M. gordonae* or *M. kansasii*, depending on the pigmentation and growth rate of the isolates. This assay uses acridium ester-labelled DNA probes and the principle of a hybridization protection assay (Arnold *et al.*, 1989). Testing was performed in accordance with the manufacturer's instructions. After lysis, a 100 µl sample was transferred to the corresponding AccuProbe tube. Hybridization results, expressed as relative light units (RLUs), were determined with a Leader 50 luminometer (GenProbe). A positive reaction was a result greater than the cutoff value of 30 000 RLU, with a repeat range of 20 000 to 29 999 RLU.

DNA extraction for in-house PCR and partial 16S rRNA gene sequencing. Mycobacterial DNA extraction was modified from our previously published protocol (Woo *et al.*, 2003). Briefly, inocula of mycobacterial colonies on LJ medium were washed with 500 µl 0.1 M Tris/HCl (pH 7.5), and the pellet was suspended in 100 µl lysis solution containing 0.1% NaOH and 0.025% SDS. The mixture was incubated at 60 °C for 45 min, followed by addition of 100 µl 0.1 M Tris/HCl (pH 7.5). The DNA extract was stored at –20 °C before PCR.

In-house PCR for MTB complex and MAC. Each PCR reaction contained 10 µl DNA extract. A manual one-tube nested PCR for IS6110 in MTB complex was performed as described previously (Chan *et al.*, 1996; Drancourt *et al.*, 2000; Yam *et al.*, 1998; Yuen *et al.*, 1997). PCR for MAC was performed according to the protocol published by Li *et al.* (1996), with minor modifications. Of the two forward primers, MAC1 (5'-GGACCTCAAGACGCATGTCTTCTG-3') was derived from positions 138–161 of the 16S rRNA gene of *M. avium*, and MAC2 (5'-GGACCTTAGGCGCATGTCTTTAG-3') was derived from positions 128–151 of the *M. intracellulare* 16S rRNA gene. The design of the reverse primer MACR (5'-GCTCTTACGCCCAGTAATTCCGG-3') was based on a sequence which is common to both species. A 100 µl reaction mixture consisted of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTP, 0.4 µM each primer, and 2 U AmpliTaq Gold polymerase (Perkin Elmer). To activate the *Taq* polymerase, the mixture was first incubated at 94 °C for 10 min. The reaction mixture was then subjected to 50 cycles of amplification (94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min), with a final single extension at 72 °C for 10 min. A 10 µl aliquot of PCR product was electrophoresed for 1 h through a 2% agarose gel in 1 × TBE, and the target band of 390 bp was visualized under UV illumination.

Partial 16S rRNA gene sequencing. The broad-range primer 285 (5'-GAGAGTTTGATCCTGGCTCAG-3') and the mycobacteria-specific primer 264 (5'-TGCACACAGGCCACAAGGGA-3') were used for amplification, corresponding to *Escherichia coli* 16S rRNA positions 9–30 and 1027–1046, respectively (Kirschner *et al.*, 1993). The size of the amplicon was about 1040 bp, depending on species. PCR was carried out in a 50 µl reaction mixture containing 5 µl DNA template, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTP, 0.4 µM each primer, and 2 U AmpliTaq Gold polymerase. To activate the *Taq* polymerase, the mixture was

first incubated at 94 °C for 10 min. The reaction mixture was then subjected to 35 cycles of amplification (94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min), with a final single extension at 72 °C for 10 min. A 5 µl aliquot of the PCR product was electrophoresed for 1 h through a 2% agarose gel in 1× TBE. Subsequently, PCR products were purified using the QIAquick PCR Purification kit (Qiagen) to remove the unpolymerized primers and deoxynucleoside triphosphates before cycle sequencing. Nucleotide sequences from both DNA strands were determined using the same forward primer 285 and reverse primer 259 (5'-TTTCACGAACAACGCGACAA-3') corresponding to *E. coli* 16S rRNA position 590–609 (Kirschner *et al.*, 1993). The purified fragment was subjected to cycle sequencing by the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (version 3.0), at a quarter of the recommended reaction volume, and an ABI 377 Genetic Analyser (Applied Biosystems). For all samples, the sequences of both strands of the amplicons were determined. The generated sequences were assembled and edited using the EDITSEQ version 4.0 program in the DNASTAR software (Lasergene), followed by a FASTA search in Ribosomal Differentiation of Medical Micro-organisms (RIDOM) (<http://www.ridom-rdna.de/>). A sequence match of ≥99% with that of the prototype strain sequence in a repository was used as the criterion for species, group or complex level identification (Drancourt *et al.*, 2000). A sequence was considered adequate if the edited sequence had 1% or less ambiguity (Drancourt *et al.*, 2000). A reference strain, *Mycobacterium smegmatis* (ATCC 700084), was run in parallel as control. The person performing the sequence protocol was not informed of the identity of the strains by the conventional methods and GLC.

RESULTS AND DISCUSSION

Identification by conventional method

Of the 1167 isolates isolated during the study period, the conventional method identified 1157 to the species, group or complex level. They included 895 MTB complex, 118 MAC, 57 *Mycobacterium fortuitum* group, 32 *Mycobacterium chelonae/Mycobacterium abscessus*, 20 *M. gordonae*, 18 *M. kansasii*, 12 *Mycobacterium marinum*, three *Mycobacterium terrae* complex, one *Mycobacterium asiaticum* and one *Mycobacterium szulgai*. The remaining ten unidentified isolates comprised four rapidly growing mycobacteria, three scotochromogens, two nonchromogens and one photochromogen. The conventional tests that we routinely used in our laboratory were unable to differentiate the species within the

MTB complex, the MAC, the *M. terrae* complex, the *M. fortuitum* group, and the group containing *M. chelonae* and *M. abscessus*.

Comparison of conventional method with Accuprobe assays and in-house PCRs

Among the 1167 clinical isolates, 365 mycobacteria (209 MTB complex, 118 MAC, 20 *M. gordonae* and 18 *M. kansasii*) were tested with the Accuprobe assays and in-house PCRs for MTB complex and MAC. The results showed that the results for the conventional method and the two genotypic tests were in complete concordance (Table 1).

Comparison of conventional method with GLC and 16S rRNA gene sequencing

Among the 1167 clinical isolates, 142 mycobacteria were tested by both GLC and 16S rRNA gene sequencing. This subset included a random sample of 127 isolates chosen to represent the common mycobacteria (MTB complex, MAC, *M. fortuitum* group, *M. gordonae*, *M. chelonae/M. abscessus*, *M. kansasii* and *M. marinum*); all five infrequent mycobacteria (*M. szulgai*, *M. asiaticum* and *M. terrae* complex), and all ten unidentified isolates (Table 2). All isolates were subjected to identification by conventional tests, GLC and 16S rRNA gene sequencing simultaneously. Of the 142 isolates, GLC and 16S rRNA sequencing (at ≥99% sequence match) identified 137 (96.4%) and 139 (97.9%) isolates, respectively, to species, complex or group level. Table 2 shows that concordant results at species, complex or group level were obtained by GLC and 16S rRNA sequencing for all 132 isolates that could be identified by conventional tests. For the ten isolates that could not be identified in the conventional tests, several problematic and discrepant results were obtained by the three methods. Firstly, the fatty acid profiles by GLC of *Mycobacterium interjectum* were similar to those of *Mycobacterium scrofulaceum*. Since the former species is not included in the GLC software that we used, both scotochromogens were identified as *M. scrofulaceum* by GLC, whereas 16S rRNA analysis identified both as *M. interjectum/Mycobacterium simiae*. Secondly, despite the fact

Table 1. Comparison of Accuprobe assay and two in-house PCRs with conventional tests, for 365 clinical isolates of mycobacteria

Abbreviations: +, positive; –, negative; ND, not done.

Identification by conventional tests	In-house PCR				Accuprobe assay							
	MTB complex		MAC		MTB complex		MAC		<i>M. gordonae</i>		<i>M. kansasii</i>	
	+	–	+	–	+	–	+	–	+	–	+	–
MTB complex	209	0	0	209	209	0	0	97	ND	ND	ND	ND
MAC	0	118	118	0	0	52	118	0	ND	ND	ND	ND
<i>M. gordonae</i>	ND	ND	ND	ND	ND	ND	ND	ND	20	ND	ND	ND
<i>M. kansasii</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	18	ND

Table 2. Comparison of identification by GLC and 16S rRNA sequencing with conventional methods, for 142 mycobacterial isolates

The number of isolates is shown in parentheses.

Organism(s) identified by phenotypic methods	No. of isolates	Mycobacterial identification method	
		GLC	16S rRNA sequencing*
MTB complex	36	MTB complex (36)	MTB complex (36)
MAC	29	MAC (29)	<i>M. avium</i> (2), <i>M. intracellulare</i> (27)
<i>M. fortuitum</i> group	19	<i>M. fortuitum</i> (19)	<i>M. fortuitum</i> (19)
<i>M. goodii</i>	9	<i>M. goodii</i> (9)	<i>M. goodii</i> (9)
<i>M. chelonae/M. abscessus</i>	19	<i>M. chelonae</i> complex (19)	<i>M. chelonae/M. abscessus</i> (19)
<i>M. kansasii</i>	10	<i>M. kansasii</i> (4), <i>M. gastri</i> (6)	<i>M. kansasii/M. gastri</i> (10)
<i>M. marinum</i>	5	<i>M. marinum</i> (5)	<i>M. marinum/M. ulcerans</i> (5)
<i>M. szulgai</i>	1	<i>M. szulgai</i> (1)	<i>M. szulgai</i> (1)
<i>M. asiaticum</i>	1	<i>M. asiaticum</i> (1)	<i>M. asiaticum</i> (1)
<i>M. terrae</i> complex†	3	<i>Mycobacterium nonchromogenicum</i> (3)	<i>M. terrae</i> (3)
Unidentified			
Scotochromogens	3	<i>M. scrofulaceum</i> (2), no match (1)	<i>M. interjectum/M. simiae</i> (3)
Photochromogens	1	No match (1)	<i>Mycobacterium lentiflavum</i> (1)
Nonchromogens	2	<i>M. terrae</i> complex (2)	<i>M. poriferae</i> -like (2)‡
Rapid growers	4	<i>M. terrae</i> complex (1),† no match (3)	<i>Mycobacterium chlorophenolicum</i> (1), <i>Mycobacterium mucogenicum</i> (1), <i>Mycobacterium neoaurum</i> (1), <i>Mycobacterium wolinsky</i> -like (1)‡

*Result obtained when organisms were identified to species, group or complex level by using $\geq 99\%$ sequence match.

†*M. terrae* complex consists of three species: *M. terrae*, *M. nonchromogenicum* and *Mycobacterium triviale*. Members in this complex are slow-growing nonchromogens. *M. poriferae* is a rapid grower.

‡Match at $< 99\%$ level: strain HKM159 (98.1% match with *M. wolinsky*), strain HKM160 (95.4% match with *M. poriferae*), strain 158 (95.6% match with *M. poriferae*).

that the two nonchromogens were identified as *M. terrae* complex by GLC, no sequence matches at $\geq 99\%$ were obtained in the 16S rRNA sequencing. These two isolates had the closest sequence match with *Mycobacterium poriferae* (95.4–95.6% similarities), and in fact may represent a novel species. Thirdly, one rapidly growing mycobacterium was identified as *M. terrae* complex by GLC. This is likely to be erroneous, as all members of the *M. terrae* complex are slow growing. Of the 118 MAC, the 16S rRNA sequencing identified 116 as *M. intracellulare* and two as *M. avium*.

Comparison of turnaround times

During the present study, inoculated LJ medium was examined daily in the first week, and weekly thereafter. Upon detection of visible growth, Ziehl–Neelsen staining was performed to confirm mycobacterial growth. Depending on the tests and amount of growth, phenotypic and genotypic tests were conducted either directly or after subculture. During the study period, all the genotypic tests were performed two to three times per week. The turnaround time from detection of visible growth on LJ medium to mycobacterial identification is shown in Table 3.

To the best of our knowledge, this study is unique in evaluating mycobacterial identification by several methods under routine operational conditions. This is an important issue, because workflow and the frequency of testing have an important influence on the length of time required for mycobacterial identification. Due to manpower and resource constraints, many laboratories, including ours, routinely perform only two or three runs of genotypic tests per week. Upon the special request of a clinician, individual testing will be conducted on the same day as growth detection. Therefore, in considering the turnaround time for mycobacterial identification, it is important to take into account the lag time imposed by operational limitations. Therefore, although the AccuProbe hybridization and PCR assays could both be completed within one day, the actual turnaround time was between 2 and 3 days.

This study found 16S rRNA sequencing to be an excellent tool for mycobacterial species identification. Overall, sequence-based identification provided more rapid results and fewer unidentified organisms than conventional methods, corroborating the findings of a recent study (Hall *et al.*, 2003). Furthermore, our findings showed that GLC was largely unhelpful for resolving the ten isolates

Table 3. Comparison of the actual turnaround time for mycobacterial identification by five methods

Mycobacterium	Turnaround time (days), mean $\pm$ SD*				
	Conventional method†	GLC‡	Accuprobe‡	In-house PCR‡	16S rRNA sequencing‡
MTB complex	16 $\pm$ 7	23 $\pm$ 4	3 $\pm$ 1	2 $\pm$ 1	4 $\pm$ 2
Non-tuberculous mycobacteria					
MAC	23 $\pm$ 10	29 $\pm$ 5	3 $\pm$ 2	2 $\pm$ 1	5 $\pm$ 2
Other slow growers	22 $\pm$ 9	14 $\pm$ 5	3 $\pm$ 2§	—	4 $\pm$ 2
Rapid growers	7 $\pm$ 2	6 $\pm$ 3	2 $\pm$ 1	—	3 $\pm$ 2

*Time required for completion of tests for the bacterial identification of an isolated mycobacterium. This did not include the time employed in transport and in culture isolation from clinical specimens.

†For all 1167 mycobacterial isolates.

‡For the isolates tested as detailed in Tables 1 and 2.

§For *M. kansasii* and *M. goodii* only.

unidentified by conventional methods. Unlike GLC, which requires heavy growth of mycobacteria in standard medium, sequencing of PCR-amplified 16S rRNA products can be performed with less than a single colony. Thus, colonies on LJ medium can be tested directly without subculture if growth is pure. With this direct approach, however, one should note that mixed growth could lead to ambiguous 16S rRNA data (Drancourt *et al.*, 2000).

16S rRNA sequencing-based identification has limitations. Firstly, it could not always distinguish between closely related species. The following examples were found in our evaluation: *M. chelonae* and *M. abscessus*; *M. kansasii* and *Mycobacterium gastri*; *M. marinum* and *Mycobacterium ulcerans*; and *M. interjectum* and *M. simiae*. Secondly, 16S rRNA sequencing is unable to discriminate members of the MTB complex: *M. tuberculosis sensu stricto*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium canettii*. For differentiation of the above closely related species, or members of the MTB complex, additional biochemical or other tests are required. For example, *M. chelonae* can be distinguished from *M. abscessus* by its ability to utilize citrate, by susceptibility to tobramycin (MIC of $\leq 4 \mu\text{g ml}^{-1}$), or by the RFLP pattern of the *hsp* gene (Yakrus *et al.*, 2001). *M. kansasii* and *M. marinum* can be readily distinguished from the other two related species by their pigmentation. Unfortunately, no single phenotypic or genotypic test is able to readily distinguish all members within the MTB complex. In most diagnostic laboratories, the great majority of isolates are likely to be *M. tuberculosis sensu stricto*. Therefore, it has been suggested that specific tests, such as *oxyR/pncA* allele-specific PCR, may be limited to strains with atypical features, such as not yielding rough, cream, cauliflower-like colonies (Vincent *et al.*, 2003). Recently, the GenoType MTBC assay (Hain Lifescience) has also been reported to be useful for differentiation of subtypes within the MTB complex (Richter *et al.*, 2004). Since the assay allows MTB complex identification and subtyping in a single test, it has superseded Accuprobe in some clinical laboratories. Secondly, reliable sequence

identification depends on the accuracy of the obtained data as well as that of the reference sequence in the database. Therefore, the obtained sequence should contain no more than 1 % ambiguity and should be analysed against a peer-reviewed database such as Ribosomal Differentiation of Medical Micro-organisms (RIDOM).

Despite the many advantages of sequence-based identification, the cost of DNA sequencing is still too expensive (US \$80–130 per specimen) to be used routinely for all samples in diagnostic laboratories (Clarridge, 2004). This has prompted us to explore strategies for the more cost-effective use of 16S rRNA sequencing. While almost 100 members of the mycobacteria are now recognized (Pfyffer *et al.*, 2003), a small proportion of them account for the great majority of clinical isolates: MTB complex, MAC, *M. fortuitum* group, *M. chelonae*, *M. abscessus*, *M. kansasii* and *M. marinum*. In the present study, MTB complex and MAC provided 95 % (1013/1067) of the total isolates. If the cultured isolates were screened by specific in-house PCR (or AccuProbe assays) for the two complexes, a large proportion of isolates could be readily identified and very few isolates would require sequencing (Fig. 1). With this algorithm, subculture and growth for a short period of time may be necessary to ascertain pure growth and also for the designation of the mycobacterium as a rapid or slow grower. Thereafter, the great majority of isolates could be identified and reported within one week. As a cost-reduction measure, our present experience demonstrates that an adequate result could be obtained by using one-quarter reaction volumes for DNA sequencing. We sought to develop the in-house PCR in lieu of AccuProbe assays for identification of MTB complex and MAC as a means of cost containment. Our in-house PCR for MTB complex targets IS6110 as a highly specific marker for identification purposes. This marker is present in virtually all members of the MTB complex (Vincent *et al.*, 2003). The specificity and sensitivity of this in-house PCR have been extensively evaluated by our group (Yuen *et al.*, 1997; Yam *et al.*, 1998, 2004; Chan *et al.*, 1996). However, it should be noted that some populations have a small number of

Fig. 1. Algorithm for rapid species identification of *Mycobacterium* by an integrated approach. *Three runs per week. †In this study, further differentiation was required to differentiate *M. chelonae* and *M. abscessus*, *M. kansasii* and *M. gastri*, and *M. marinum* and *M. ulcerans*. +, Positive; -, negative.

IS6110-negative strains (Park *et al.*, 2000). Under the proposed scheme, these IS6110-negative strains would be identified at the 16S rRNA sequencing stage. Likewise, the specificities of the primers MAC1, MAC2 and MACR have previously been studied by Li *et al.* (1996). Depending on the local requirement, both species-specific PCR and AccuProbe assays can be adapted for identification to MAC or species level. The hands-on time for both AccuProbe and in-house PCR are similar (2-3 h) and both can be efficiently completed on the same day. Under operational conditions, this study found that both methods were also equivalent in terms of accuracy and actual turnaround time. With respect to reagents and supplies, the cost of the in-house PCR (approx. US \$5 per test) is lower than that of the AccuProbe test (approx. US \$35 per test). With this integrated scheme (Fig. 1), the majority of the phenotypic tests could be abandoned and the resources channeled into genotypic testing. The main purpose of the remaining phenotypic tests (e.g. growth rate, chromogenicity and citrate utilization) would be to resolve closely related species or members of a complex or group with highly similar 16S rRNA sequences. A difficulty with the AccuProbe test is that a decision needs to be made about which probe to use, according to the morphology of the organism, the time to positive growth, and clinical factors. For sequence-based identification, our experience approximates the earlier estimation of Clarridge (2004) that for three runs of 20 samples per week, the total labour time is about 40-50 h. In our laboratory, we estimate that there would be no overall increase in the laboratory budget, while manpower would be saved by approximately 15%.

We used an in-house protocol for the sequence-based identification. This could limit applicability in other laboratories because of the technical demands of manual DNA

extraction, PCR, cycle sequencing and output analysis. The turnaround time for the testing and analysis was about 2 days, which is comparable to that of the commercial MicroSeq 500 system (Patel *et al.*, 2000). Understandably, the required expertise and resources for molecular testing in the present scheme may be lacking in some developing countries. Our scheme is also limited in that the similarity match was relatively low for a small number of isolates. However, this problem is common to all identification based on 16S rRNA sequencing, and has also been reported by other investigators (Cloud *et al.*, 2002; Hall *et al.*, 2003; Patel *et al.*, 2000). The question arises of when to consider a sequence as novel and when it should be considered as closely related to that of an existing species. At present, there are no accepted guidelines available to establish cutoffs for computer-assisted analysis of sequence similarity for 16S rRNA-based bacterial identification. Difference rates of ≤ 0.5 , ≤ 1 and ≤ 3 % have been recommended for delineation at the species level (Clarridge, 2004). Since bacterial genera do not evolve at the same speed, it may be necessary to use different cutoff values for different bacterial genera. We used a ≤ 1 % difference as a suitable cutoff, as suggested by Drancourt *et al.* (2000). In terms of commercially available options, assays have been developed that can rapidly identify a broad range of mycobacterial species by amplification of culture material and subsequent reverse hybridization onto strips (Padilla *et al.*, 2004).

It might be argued that for patients with typical presentation, the clinical benefit of speeding up identification of MTB complex by 2 weeks is small, because these patients are already on empirical therapy. However, tuberculosis is well known to have atypical presentations. The management of this patient subset and of those with extra-pulmonary non-tuberculous mycobacteria (NTM) infections is likely to

benefit from the shortened turnaround time for identification of culture material. In terms of practical utility, it is important that genotypic testing be integrated with rapid culture. In many developed countries, automated liquid-based systems are increasingly used for routine mycobacterial culture and sensitivity testing. These systems provide faster speed and greater sensitivity for primary isolation of mycobacteria from clinical specimens. A further advantage is that culture materials are available on a daily basis for genotypic testing; thus, the integration of automated liquid-based systems with genotypic testing will further add to the rapidity of the total laboratory investigation. In our laboratory, the BACTEC Myco/F Lytic system (Becton Dickinson) has also been available since the mid-1990s. Due to budgetary constraints, we have not been able to replace all LJ culture by this liquid-based system. Instead, its use is prioritized to immunocompromised patients, persons with risk factors for drug-resistant tuberculosis, and upon request by the attending clinician.

In conclusion, 16S rRNA sequencing offers a rapid and reliable means for mycobacterial identification. Our findings provide a model for how this technique can be integrated with species-specific PCR tests, AccuProbe hybridization assays and conventional tests in diagnostic laboratories in a cost-conscious manner. Interpretation of the sequences without a good match in the database remains problematic. As 16S rRNA sequences become more widely used in diagnostic laboratories, guidelines for and standardization of the delineation of cutoffs for sequence interpretation are required.

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