A Comparison of Mycolic Acid Analysis for Nontuberculous Mycobacteria Identification by Thin-Layer Chromatography and Molecular Methods

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Abstract: The development of fast, inexpensive, and reliable tests to identify nontuberculous mycobacteria (NTM) is needed. Studies have indicated that the conventional identification procedures, including biochemical assays, are imprecise. This study evaluated a proposed alternative identification method in which 83 NTM isolates, previously identified by conventional biochemical testing and in-house M. avium IS1245-PCR amplification, were submitted to the following tests: thin-layer chromatography (TLC) of mycolic acids and PCR-restriction enzyme analysis of hsp65 (PRA). High-performance liquid chromatography (HPLC) analysis of mycolic acids and Southern blot analysis for M. avium IS1245 were performed on the strains that evidenced discrepancies on either of the above tests. Sixty-eight out of 83 (82%) isolates were concordantly identified by the presence of IS1245 and PRA and by TLC mycolic acid analysis. Discrepant results were found between the phenotypic and molecular tests in 12/83 (14.4%) isolates. Most of these strains were isolated from non-sterile body sites and were most probably colonizing in the host tissue. While TLC patterns suggested the presence of polymycobacterial infection in 3/83 (3.6%) cultures, this was the case in only one HPLC-tested culture and in none of those tested by PRA. The results of this study indicated that, as a phenotypic identification procedure, TLC mycolic acid determination could be considered a relatively simple and cost-effective method for routine screening of NTM isolates in mycobacteriology laboratory practice with a potential for use in developing countries. Further positive evidence was that this method demonstrated general agreement on MAC and M. simiae identification, including in the mixed cultures that predominated in the isolates of the disseminated infections in the AIDS patients under study. In view of the fact that the same treatment regimen is recommended for infections caused by these two species, TLC mycolic acid analysis may be a useful identification tool wherever molecular methods are unaffordable.

Key words: Mycolic acids, Nontuberculous mycobacteria, TLC, IS1245, PRA

Nontuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT), or simply atypical mycobacteria include those species not belonging to the Mycobacterium tuberculosis complex. It is widely acknowledged that as the incidence of tuberculosis decreased over the years, NTM infections have become more common (8). Even in developing countries like Brazil, where tuberculosis is still a major health care problem, NTM has emerged as the second most fre-

Abbreviations: AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; Hsp65, heat shock protein; IS, insertion sequence; LJ Löwenstein-Jensen culture medium; MAC, M. avium-intracellulare complex; MACu+, MAC pattern except for a positive urease activity; MAIS, M. avium-intracellulare-scrufu-

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quent disseminated mycobacterial infection among AIDS patients (11). Delays in an NTM diagnosis may

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have a serious impact on choosing the most adequate treatment so that the development of a simple, rapid, cost-effective and, above all, reliable alternative to the conventional identification procedures currently available has become an important public health care issue.

Traditionally, mycobacterial species identification has relied on the observation of time-consuming biochemical reactions (16, 20). Other methods, introduced to identify mycobacteria based on phenotypic characteristics, such as the mycolic acid composition analysis (4, 24), provided additional options. Mycolic acid methyl ester analysis by thin-layer chromatography (TLC) has also been used in several routine rapid laboratory tests to identify mycobacterial isolates (10, 22, 23) while high-performance liquid chromatography (HPLC) analysis of mycolic acids with high molecular weights (i.e., the α-branched β-hydroxylated chains) has for the most part been adopted in the more sophisticated reference centers (4).

Furthermore, the ready availability of nucleic acid-based methods has led to faster mycobacterial identification. Several mycobacterial genes are targets in assay systems, namely in commercial kits and in-house techniques capable of identifying both single and multiple mycobacterial species (5, 6, 13, 19, 25). Moreover, identification methods based on specific probes have been applied to *M. tuberculosis*, *M. avium*, and *M. intracellulare* (2, 12–14, 26).

Restriction enzyme analysis (REA) is now commonly applied after amplification of part of the 16S ribosomal RNA (17) and *hsp65* genes (PRA) that are typical of all mycobacteria (32). In addition to its high cost, however, this method also requires highly skilled personnel and sophisticated laboratory facilities, making it prohibitive for routine identification testing in developing countries.

The aim of this study was to evaluate the usefulness of TLC mycolic acid analysis in the routine identification of NTM isolates. The results of mycolic acid identification were compared to the IS1245-PCR amplification and hybridization and PRA genotypic techniques.

**Materials and Methods**

**Clinical isolates.** In this study, a total of 83 NTM clinical strains were considered, all of which were isolated in the Löwenstein-Jensen culture medium (LJ). The isolates had been previously submitted to conventional identification procedures and PCR amplification of a 427 bp fragment of IS1245, which, as described by Ferreira et al. (12), were used to identify the *M. avium* organism among MAC strains. Forty-nine of these strains revealed the classic *M. avium-intracellulare* complex (MAC) pattern concordance according to the following biochemical test results: positive for catalase activity and tellurite reduction and negative for nitrate reduction, tween hydrolyse, and urease activity. Twenty strains demonstrated the MAC pattern except for one positive urease activity (MACu+) whereas six strains were *M. scrofulaceum*, three were *M. terrae*, two were *M. gordonae*, one was *M. chelonae*, one *M. fortuitum*, and one remained unknown.

**Mycolic acids and nucleic acid based tests.** Mycolic acid extraction and methylation procedures: mycolic acid analysis was carried out according to the methodology proposed by Daffé et al. (2) and modified by Leite et al. (22). Approximately 25–50 mg of mycobacteria derived from the LJ medium were dispersed into a 5% potassium hydroxide solution in 2-methoxyethanol. The mixture was maintained at 110 C for 2 hr, cooled, and subsequently acidified with 1 ml of sulfuric acid solution (20%, w/w), after which the mycolic acids were released from their potassium salts by the sulfuric acid treatment. The mycolic acids were then extracted by shaking the mixture twice with diethyl ether (5 ml). The ether phase was decanted and washed three times with 2 ml of water. Ether was removed in a water bath, leaving a residue of mycolic acids that was methylated by adding 1 ml of a diazomethane ether solution. This reagent was prepared with nitrosomethylurea-potassium hydroxide.

**Analytical methods and identification of mycolic acids.** To carry out TLC, the methyl esters of the mycolic acids were spotted onto silica gel G (20×20 cm×0.25 mm plates). Mycolic acids of the reference strains spotted together served as a standard for the proper identification of the mycolic acids extracted from the strains under study. Using two different solvent systems, one-dimensional analysis was performed: diethyl ether/petroleum ether (12:88, v/v) with three chromatogram developments; and dichloromethane with a single development. The presence of separate components was revealed as a result of spraying the chromatograms with 0.1% (w/v) rhodamine in a phosphate buffer.

The PCR-restriction enzyme analysis (PRA) was performed as described by da Silva Rocha et al. (7).

*Mycobacterium* samples showing discrepancies in the identification results in one or more of the above-mentioned assay tests were sent to the Centers of Disease Control (CDC) (Atlanta, Ga., U.S.A.) to analyze the mycolic acids using the Beckman Gold System HPLC instrument and pattern recognition via Infometrix Pirouette software (4). For those strains with discordant results regarding *M. avium* IS1245-PCR and PRA, PCR was repeated and hybridized by
Southern blot analysis with the IS1245 427 bp sequence as described by Saad et al. (29). Mycobacterial DNA was extracted as previously mentioned (13). Approximately 1 µg of the extracted DNA was digested with 15 units of PvuII (Invitrogen Tech-Line, Rockville, Md., U.S.A.) overnight at 37 C and analyzed by the Southern blot method on a nylon membrane. The 427 bp PCR-amplified DNA (13) was labeled with digoxigenin and used as a probe to hybridize the IS1245 sequences in the membrane. The bands were visualized by a color substrate detection system (Roche Molecular Biochem., Mannheim, Germany). Digoxigenin-labelled markers II and VII (Roche Molecular Biochem.) were used as molecular size markers in all of the membranes. The DNA fingerprint patterns on the membrane were detected visually.

Results

The comparative results after analyzing the mycolic acids using TLC, IS1245, and PRA are shown in Table 1. Sixty-two strains yielded the mycolic acid TLC profile of I, IV, and VI, characteristic of a *M. avium-intracellulare-scrofulaceum* complex (MAIS). Species of this complex, but predominantly *M. avium*, were identified by the IS1245-PCR and PRA methods, except for one strain in which IS1245 amplification failed despite a positive IS1245 hybridization and PRA-*M. avium* identification. Three other strains were IS1245-PCR negative, including one identified as *M. scrofulaceum* and a second as *M. intracellulare*, both by PRA. The third culture showed a mixed profile of *M. avium* and *M. intracellulare* by PRA and had a positive IS1245 hybridization. PRA also determined polyclonal infection in 7 out of 15 (47%) patients with multiple isolates of *M. avium* PRA-profiles I and II (n=18 and 16 samples, respectively). Among the 62 strains identified by TLC as a MAIS complex, 47 (75.8%) were isolated from sterile body sites (n=33), mainly from the blood. The 6 remaining concordantly identified strains showed *M. marinum*, *M. gordonae*, and *M. simiae* PRA patterns. The profiles resulting from the TLC mycolic acid analysis were in accordance with these species, respectively I.III, I.III.IV, and I.II.IV. All of these strains were isolated from non-sterile body sites and none were found to have IS1245.

Mycolic acid analysis by HPLC was performed on the 12 samples (14.5%) showing discrepancies in one or more assayed tests. The identification results of all tests are shown in Table 2. All strains except two were isolated from a non-sterile site. Strains 5, 6, 7, 8, and 10 were multiple isolates of patient 10, four of which were isolated on the same day and identified as *M. simiae* by
Table 2. Nontuberculous mycobacterial strains with a discrepancy in one or more identification tests

<table>
<thead>
<tr>
<th>Strain</th>
<th>Patient ID</th>
<th>HIV status</th>
<th>Clinical isolate</th>
<th>Culture date</th>
<th>Mycolic acid analysis by TCL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPLC</th>
<th>Molecular typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>P</td>
<td>Sputum</td>
<td>28/11/96</td>
<td><em>M. simiae</em> (I.II.IV)</td>
<td></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>P</td>
<td>Sputum</td>
<td>28/11/96</td>
<td><em>M. simiae</em> (I.II.IV)</td>
<td></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>P</td>
<td>Sputum</td>
<td>28/11/96</td>
<td><em>M. simiae</em> (I.II.IV)</td>
<td></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>P</td>
<td>Blood</td>
<td>28/11/96</td>
<td><em>MAIS</em> (I.IV.VI)</td>
<td></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>P</td>
<td>Sputum</td>
<td>01/02/97</td>
<td><em>M. simiae</em> (I.II.IV)</td>
<td></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>P</td>
<td>Blood</td>
<td>18/12/96</td>
<td><em>MAIS</em> (I.IV.VI)</td>
<td></td>
<td><em>M. gordonae</em> III</td>
</tr>
<tr>
<td>14</td>
<td>35</td>
<td>P</td>
<td>Sputum</td>
<td>28/07/97</td>
<td><em>M. gordonae</em> (I.III.IV)</td>
<td></td>
<td><em>M. avium</em> I</td>
</tr>
<tr>
<td>16</td>
<td>34</td>
<td>P</td>
<td>Sputum</td>
<td>21/07/97</td>
<td><em>M. gordonae</em> (I.III.IV)</td>
<td></td>
<td><em>M. avium</em> I</td>
</tr>
<tr>
<td>17</td>
<td>15</td>
<td>N</td>
<td>Sputum</td>
<td>08/06/96</td>
<td><em>MAIS</em> (I.IV.VI)</td>
<td></td>
<td><em>M. terrae</em></td>
</tr>
<tr>
<td>18</td>
<td>17</td>
<td>N</td>
<td>BAL</td>
<td>05/06/97</td>
<td><em>MAIS</em> (I.IV.VI)</td>
<td></td>
<td><em>M. terrae</em></td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>UK</td>
<td>Sputum</td>
<td>17/09/96</td>
<td><em>M. terrae Complex</em> (I.IV)</td>
<td></td>
<td><em>M. tuberculosis</em></td>
</tr>
<tr>
<td>21</td>
<td>39</td>
<td>P</td>
<td>Sputum</td>
<td>06/10/97</td>
<td><em>M. simiae</em> (I.II.IV)</td>
<td></td>
<td><em>M. abscessus</em> I</td>
</tr>
</tbody>
</table>

<sup>a</sup> Thin-layer chromatography identification patterns.


Table 3. Thin-layer chromatography method in comparison with other identification methods for NTM mixed cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Patient ID</th>
<th>HIV status</th>
<th>Clinical isolate</th>
<th>Culture date</th>
<th>Mycolic acids</th>
<th>Molecular typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>P</td>
<td>Blood</td>
<td>04/10/97</td>
<td>MAIS/<em>M. simiae</em> (I.II.IV.VI)</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>P</td>
<td>Blood</td>
<td>24/09/96</td>
<td>MAIS/<em>M. gordonae</em> (I.II.IV.VI)</td>
<td>P</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>P</td>
<td>Sputum</td>
<td>17/12/96</td>
<td>MAIS/RGM (I.IV.VI)</td>
<td>N</td>
</tr>
</tbody>
</table>

nationwide, particularly among patients with acquired immunodeficiency syndrome (AIDS) (1, 12, 28).

The biochemical tests used to identify *Mycobacterium* spp. present a challenge because they basically rely on only a few morpho-biochemical characteristics. In addition to being labor intensive and time consuming, these tests may elicit ambiguous or even erroneous results due to the convergence of a number of characteristics.

In this study, the isolates were submitted to an alternative phenotypic test based on the analysis of mycolic acids by TLC and then compared with the results of the genotypic identification method. Most of the MAIS strains harbored mycolic acid types I, II, IV, VI. Members of this complex (predominantly *M. avium*) were concordantly identified by genotypic methods using IS1245, the specific insertion element for *M. avium*, and PRA, which can distinguish MAIS from *M. avium* and *M. scrofulaceum*. The TLC method also identified other NTM species corroborated by PRA, such as *M. marinum* and *M. gordonae*. Both, isolated from non-sterile sites, were, therefore, probably only colonizing (Table 1). Our results suggested that TLC mycolic acid analysis may be an effective yet simple, less costly screening alternative method to identify NTM isolates in routine mycobacterial laboratory practice in developing nations. PRA is rarely adopted at present due to the high cost of carrying out species-specific genotypic methods. Although differentiation of MAIS at the species level is important for taxonomical and epidemiological studies, in MAC drug resistance is more isolate specific than species specific (3, 15, 18) so a species-specific diagnosis is not necessarily an aid in choosing the most adequate treatment (4). It is noteworthy that, in this study, the use of TLC as a mycobacterial identification tool was found to be useful for roughly two thirds of the participating patients with mycobacterial disease. PRA showed that 37% of the AIDS patients had *M. avium*-disseminated polyclonal infections. This finding is in agreement with a previous report indicating that MAC polyclonal infection may not be such a rare event among Brazilian HIV patients (29, 30). Since the detection of polyclonal infection by MAIS cannot actually be considered beneficial to the patient for the above-stated reasons, multidrug therapy remains the standard treatment.

While mixed infections with different *Mycobacterium* species were detected in three patients by TLC mycolic acid analysis, both HPLC and PRA identified a single species (Table 3). Detection of both a mixed MAC/*M. simiae* and *M. fortuitum* infection in AIDS patients using TLC mycolic acids has been previously described (22, 23). In our study, the first case (patient 2, Table 3)
under investigation was a *M. simiae*/*M. avium* infection that was initially detected as a single species by PRA and HPLC, respectively. Together with a positive hybridization by IS1245, this finding was indicative of a mixed culture involving these two species. According to both the TLC and HPLC results, the second case (patient 16) was an *M. avium*/*M. gordona e* infection that presented a PRA-*M. lentiflavum* I profile as well. The latter species was described in 1996 (31) as, after mycolic acid analysis, demonstrating a pattern similar to the one found in *M. simiae*. Although some PRA patterns are shared among the different species (7), such was not the case for the species found in this particular culture. So, it might be that, in this HIV-positive patient, there was a mixed infection involving three different species even though *M. lentiflavum* could not be isolated. The presence of *M. avium* was further supported by a IS1245-positive hybridization. Contrary to the other two cases, however, the isolate from patient 9 originated from a non-sterile body site. In addition, patient 9, whose RGM was identified by mycolic acids, was HIV-positive and had a history of non-compliance when under treatment for pulmonary tuberculosis. Indeed, isolation of RGM is a common occurrence. But despite the presence of *M. avium*, as attested to by both PRA and TLC, no positive PCR or hybridization for IS1245 was obtained. The cultures described in Table 3 were subcultured for colony isolation and, except for *M. lentiflavum*, the strains identified above were all found.

Widely used for species identification, several insertion sequences and other genes have been described as species specific. The IS1245 sequence, for example, has been applied as a specific marker for the identification and strain relatedness of *M. avium* (9, 27, 29). On the other hand, *M. intracellulare* seems to lack this sequence (13). Among the strains with discrepant identification results, 7/12 showed an IS1245-positive amplification (Table 2). The presence of this marker was not confirmed by hybridization and, except for two strains (strains 14 and 16), none were identified as *M. avium I* by PRA. Six strains were characterized as *M. simiae* (5/7) and *M. terrae* (1/7) by PRA and/or mycolic acids. These results may suggest that strains such as *M. simiae* harbor sequences similar to those found in IS1245, but, obviously, this observation must be validated or not by future experiments. It is possible that *M. simiae* may, in fact, be a mixture of two or more specific species or subspecies (15). Springer et al. (31) also observed this type of diversity on NTM analyzed by phenotypic methods and concluded that final identification results could only be reached by 16S rRNA sequence determination.

As commented above, these discrepant identification results could be explained by the presence of a mixed culture in the isolate tubes, a plausible suggestion when keeping in mind that 71% of these samples were isolated from non-sterile sites and that most of the patients were immune compromised (HIV-infected). Other possible explanations may be related to the high allelic variation in the NTM species about which, to date, little is known. Leão et al. (21) reported that a variant strain of *M. avium* is sometimes misidentified by PRA as *M. simiae II* or *M. kansasii I*. Although allelic variations of some NTM species together with genotype sharing among many different species could be another reason for at least part of the findings in this study (7, 21), it is unlikely due to the large numbers of PRA types analyzed by our group in an earlier study (7).

The identification procedures that are most widely performed to determine mycobacterial species are based on cultural and biochemical tests. The results of these procedures may be affected by variations in the reagents used and by the size, age, and physiological condition of the inoculum, all of which may at least partially explain the results obtained for strain 21 (Table 2). After biochemical and HPLC mycolic acid testing, a *M. chelonae* profile cannot be distinguished from a *M. abscessus* or *M. simiae* profile. By the TLC method, however, these species, respectively, harbor I.II and I.II.IV mycolic acid patterns. It appeared that PRA correctly identified strain 21 as being *M. abscessus*. Assuming this identification was incorrect, the growth could perhaps be a mixed culture and the TLC-mycolic acid profile was misinterpreted. In this case, then, I.II.IV could be the profile of a *M. simiae-M. abscessus* mixed culture.

The clinical characteristics of each patient may help to understand the results found, for instance, for strain 20—patient 5 (Table 2), a pulmonary tuberculosis patient with a positive culture for *M. tuberculosis* and NTM who was clinically doing well with an antituberculosis-specific treatment regimen. This case involved the previously mentioned mixed culture in which PRA recognized a *M. tuberculosis* profile and a mycolic acid analysis recognized a *M. terrae* profile. The fact that PRA was capable of identifying *M. tuberculosis* in a mixed culture at an early stage of growth is an important contribution toward the minimization of diagnostic delays. Although Leite et al. (22) have reported that TLC might be able to distinguish both *M. tuberculosis* and MAC in a mixed culture, it clearly failed to do so in this study. Regardless, this apparent failure may be more closely related to an inoculum problem favoring species selection over subcultures.

In the end, it was concluded that each of the identifi-
cation procedures under study had its own particular advantages. Notwithstanding the fact that mycolic acid analysis by TLC cannot identify isolates at the species level, it, nevertheless, appeared to be a good method for screening NTM since it is relatively inexpensive, fast, reliable, and easy to perform. It, therefore, may prove to be highly suitable in public health care laboratories in low-income communities. Many laboratories do not routinely identify mycobacteria at the species level because medical therapy is not always carried out in response to identification results. Furthermore, the treatment regimen is the same for both MAC and M. simiae infections, which predominated in the isolates of the AIDS patients under study. On the other hand, while PRA genotyping does rapidly identify isolates at the species level, the procedure requires highly qualified personnel and adequate laboratory facilities to successfully do the job. In this regard, it may be more appropriately adopted by reference centers and epidemiological studies.

Finally, it became clear that the isolates submitted to the techniques described above should have been clonally isolated to make it possible to associate differences in results with the performance of each technique. When dealing with mixtures of species, different fractions could contain several species at different ratios. It is recommended that studies be carried out on clonal isolates to better understand discordant results and the strength of TLC in its ability to rapidly identify mycobacterial strains.

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