Faster Identification of Mycobacteria Using Gas Liquid and Thin Layer Chromatography

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Gas liquid chromatography (GLC) and thin layer chromatography (TLC) analysis of cell wall content was used for identification of mycobacteria isolated in primary cultures. GLC permitted determination of the fatty acid and alcohol profiles of Mycobacterium simiae and Mycobacterium marinum and detection of a peak in Mycobacterium ulcerans formerly described for Mycobacterium malmoense. Using the data obtained to fill some of the gaps in the dichotomic trees of Tisdall et al. and Jantzen et al., GLC analysis allowed full identification of 8 of 22 mycobacterial species after 24 hours. The other 14 species could be divided into four groups on the basis of similar findings on GLC. TLC was used for full identification of three species. The identification results of conventional methods were concordant with those of GLC and TLC in 161 of 169 strains (93%) representing 21 different species. Using primarily chromatography for analysis of cell wall content, and in the case of some species complementary biochemical tests, the identification procedure could be shortened to a maximum of three days after primary culture.

Since serological tests remain controversial and lack specificity, the diagnosis of tuberculosis and mycobacteriosis is still based on isolation of the responsible agent from clinical specimens, which can take several weeks. Moreover, the majority of classical biochemical tests applied to primary cultures also require time (3 to 6 weeks) until results are available, so that alternative, more rapid techniques are needed. One possibility is gas liquid chromatography (GLC) and thin layer chromatography (TLC) analysis of primary cultures since the results are known within 24 hours. Using TLC it has been possible to classify mycobacteria according to their mycolic acid profiles and to clearly identify a number of species (1-3).

Tisdall et al. (4, 5) were the first to produce a scheme based on GLC determination of the membrane fatty acid profile for the identification of mycobacteria in a clinical laboratory. Subsequently, GLC profiles were used to identify mycobacteria such as Mycobacterium avium (6, 7), Mycobacterium tuberculosis (4), Mycobacterium gordonae (8–10), Mycobacterium gastri (7), Mycobacterium kansasii (10, 11), Mycobacterium szulgai (4, 12) and Mycobacterium flavescens (4). Later, the specific peak of Mycobacterium xenopi (13) and the two specific peaks of Mycobacterium malmoense (14) were identified.

Analysis of 182 strains by GLC (15) allowed us to identify several new components specific for some species or groups of mycobacteria and to fill in some gaps in the dichotomic trees developed by Tisdall et al. (4) and Jantzen et al. (16).

In this study we evaluated both GLC and TLC in the identification of mycobacteria using 169 strains formerly identified by conventional methods.

Materials and Methods

Bacterial Strains. The mycobacteria selected for this study included 19 reference strains and 150 clinical isolates submitted for identification purposes. They consisted of 21 different species.

Identification by Conventional Methods. The strains were identified according to conventional criteria (17, 18) including the growth rate at various temperatures, microscopic appearance, colonial morphology, pigmentation, growth on Lowenstein-Jensen medium containing iso-

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Table 1: Distribution of mycolic acid patterns in different mycobacterial species (adapted from references 1, 3 and 18).

<table>
<thead>
<tr>
<th>Pattern of mycolates</th>
<th>Mycobacterial species</th>
</tr>
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<tbody>
<tr>
<td>α</td>
<td>M. triviale</td>
</tr>
<tr>
<td>α, α’</td>
<td>M. chelonae</td>
</tr>
<tr>
<td>α, k</td>
<td>M. bovis type BCG</td>
</tr>
<tr>
<td>α, m, k</td>
<td>M. bovis, M. gastri, M. gordonae, M. kansasii, M. marinum, M. szulgai, M. tuberculosis</td>
</tr>
<tr>
<td>α, α”, k</td>
<td>M. malmoense, M.simiae</td>
</tr>
<tr>
<td>α, α”, e</td>
<td>M. fortuitum, M. smegmatis</td>
</tr>
<tr>
<td>α, w</td>
<td>M. nonchromogenicum, M. terrae, M. xenopi</td>
</tr>
<tr>
<td>α, k, w</td>
<td>M. avium-intracellulare, M. flavescens, M. phlei, M. scrofulaceum</td>
</tr>
</tbody>
</table>

α = α-mycolate (type I), long nonoxygenated mycolate.
α’ = α’-mycolate (type II), short nonoxygenated mycolate.
m = methoxymycolate (type III).
k = ketomycolate (type IV).
e = epoxymycolate (type V).
w = w-carboxymycolate (type VI) or dicarboxylic mycolate. It is always accompanied by a secondary alcohol (usually eicosanol, seldom docosanol) released upon saponification and coextracted with the fatty acids.

Thin Layer Chromatography (TLC). For extraction of mycolic acids and preparation of methyl esters, bacteria (30 mg wet weight) were scraped from the surface of Lowenstein-Jensen slants. Extraction, methylation and detection of the mycolic acid methyl esters were performed as described elsewhere (1, 3). Briefly, mycolic acids from mycobacteria were liberated by saponification at 100°C in 2 ml of 2-methoxyethanol containing 5 % (w/v) potassium hydroxide. After acidification with sulfuric acid, the fatty acids were extracted into ether and evaporated to dryness. Lipids were then methylated with freshly prepared diazomethane and spotted onto three aluminium sheets coated with silica gel 60F254 (Merck, Germany). The plates were eluted in one dimension in three different systems: dichloromethane (1 elution), petroleum benzine/diethyl ether (88:12[v/v]) (3 successive elutions) and petroleum benzine/acetone (95:5[v/v]) (2 successive elutions). The fatty acids were revealed by spraying the plates with a 0.01 % (w/v) solution of rhodamine B in 0.25 M sodium phosphate.

The six mycolate types and the secondary alcohol shown in Table 1 were identified by using three plates. As reported by Lévy-Frénault et al. (3) dichloromethane allowed good distinction between α’ and methoxymycolate, which were not clearly distinguished in the other two elution solvents. Petroleum benzine containing diethyl ether clearly resolved the methoxy and ketomycolates co-chromatographed as a single spot in dichloromethane, while petroleum benzine containing acetone allowed easy identification of the epoxymycolate and excellent differentiation between the dicarboxylic mycolate and the spot created by the medium. This is of particular importance in TLC analysis of Mycobacterium avium-intracellulare, which often grows into the culture medium, so that a small quantity of medium is present in the specimen. For each TLC run, mycolate profiles obtained from analyzed strains were compared with those from reference strains chosen to represent the various mycobacteria. Mycolic acid patterns for each mycobacterial species are shown in Table 1. Figure 1 shows typical examples of mycolic acid profiles.

Figure 1: Thin-layer chromatograms of methyl mycolates from mycobacteria. Lane 1: Mycobacterium chelonae; Lane 2: Mycobacterium marinum; Lane 3: Mycobacterium fortuitum; Lane 4: Mycobacterium avium; Lane 5: Mycobacterium scrofulaceum; Lane 6: culture medium. The elution system was petroleum benzine/acetone.