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# Direct Identification of *Mycobacterium* Species in BACTEC 7H12B Medium by High-Performance Liquid Chromatography

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**Primary cultures of mycobacteria grown in BACTEC 7H12B medium (Becton Dickinson and Co., Paramus, N.J.), with and without the addition of oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson and Co., Cockeysville, Md.), were analyzed for their mycolic acid patterns by high-performance liquid chromatography. Of the 126 isolates grown in medium to which OADC was added, 117 (93%) were successfully identified to the species level. The time to identification of *Mycobacterium tuberculosis* ($n$ = 65) averaged 19 days, and the average time was 21 days for nontuberculosis mycobacteria ($n$ = 52) from initial specimen processing. None of the 10 isolates cultured without OADC were identified. The mycolic acid patterns were considered reliable for identification if the height of the tallest peak in the chromatogram was at least 50% of the internal standard peak height.**

Mycolic fatty acid analysis of mycobacteria by high-performance liquid chromatography (HPLC) has been shown to be a useful tool in the identification of these organisms to the species level. The high-molecular-weight, long-chain, α-branched, β-hydroxylated fatty acids present in the cell wall form unique, reproducible chromogenic patterns when separated by HPLC. The method is rapid in comparison with conventional biochemical tests, and unlike genetic probes, it is not limited to the identification of a few species.

*Mycobacterium tuberculosis* can be isolated in BACTEC medium 1 to 2 weeks earlier than it can in conventional medium, while the nontuberculosis mycobacteria often can be isolated in even less time (1, 12, 17). BACTEC 7H12B medium (Becton Dickinson and Co., Paramus, N.J.) does not provide the ideal source for maximizing growth of the organism; therefore, the addition of oleic acid-albumin-dextrose-catalase (OADC) enrichment can supplement the medium to provide an increase in biomass (15). OADC is added after the BACTEC vial is determined to contain acid-fast bacilli, because if it is added at the time of specimen inoculation, the oleic acid would compete for the [$^{14}$C]palmitic acid and retard the measurement of the $^{14}CO_2$ given off as the organism utilizes the substrate. This measurement of $^{14}CO_2$ is translated as the growth index (GI), of which 999 is the maximum value.

A total of 126 isolates were obtained from clinical specimens submitted to the Arizona State Laboratory. The specimens were processed by conventional methods (14) and were subcultured onto the following media: Lowenstein-Jensen slant, Middlebrook 7H11, and Mitchison's agar plates and a BACTEC 7H12B vial containing 0.1 ml of PANTA (polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin) reconstituted with POES (polyoxethylene sterate) Reconstituting Fluid (Becton Dickinson and Co.). Acid-fast smears were made and read at this time, and 10 random duplicate (non-OADC-enriched) BACTEC vials were inoculated from specimens showing the presence of 2+ or more acid-fast bacilli on the smear.

The BACTEC vials were incubated at 37°C and read three times a week for the first 2 weeks and then once a week thereafter for a total of 6 weeks. If the GI was 50 or more, an acid-fast stain was made to determine the presence and characteristics (e.g., cording, clumping, or size) of the acid-fast bacilli. If mycobacteria were present, 0.1 ml of the medium was subcultured to each of the three solid media mentioned above, after which 0.5 ml of OADC enrichment (Becton Dickinson and Co., Cockeysville, Md.) was added aseptically to the vial. When a GI of 50 or more was reached for the 10 duplicate BACTEC vials, OADC enrichment was not added. All vials were reincubated at 37°C.

BACTEC vials were held at 37°C after the addition of OADC for an average of 6 days, with a range of 5 to 7 days. An attempt was made to hold the vials for only 5 days, but the time range was dependent on the laboratory's hours of operation. A 5-ml syringe was used to remove the contents of the BACTEC vial (in a biological safety hood), and the removed medium was transferred to a 13-by-100-mm screw-cap tube. The tube was placed into a 50-ml Falcon screw-cap centrifuge tube (Becton Dickinson Labware, Lincoln Park, N.J.) and centrifuged 3,900 × $g$ in a nonaerosol-generating sealed cup (International Equipment Co., Needham Heights, Mass.) for 15 min at 10°C. The tube was opened in the biological safety hood, and the supernatant was decanted into a 5% Amphyl solution (Sterling Drug, Inc., Montvale, N.J.) by use of a Pasteur pipette. If the pellet began to break up, some of the supernatant was left in the tube.

Saponification was performed by the addition of 2.0 ml of methanolic potassium hydroxide (25% potassium hydroxide dissolved in methanol-water [1:1; vol/vol]), mixed well for 30 s, and placed into a 100°C heating block for 2 h. Derivatization of the mycolic acids was performed as described previously (10).

The mycolic acid samples were reconstituted with 100 μl of HPLC-grade dichloromethane, and 5 μl of Ribi High-Molecular-Weight Internal Standard for HPLC (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) was added. The standard was diluted to a concentration of 0.5 μg/μl with HPLC-grade chloroform before use. A total of 10 μl of the

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TABLE 1. Average validity index values and times for HPLC analysis of mycobacteria from OADC-enriched BACTEC 7H12B vials

| Organism (no.) | Time (days [range]) | | | | Validity index[a] |
|---|---|---|---|---|---|
| | From specimen processing to a GI of ≥50 | OADC added after a GI of ≥50 | Incubation with OADC | Total from setup to HPLC analysis | |
| *M. avium* complex (20) | 8 (2–20) | 0 (0–2) | 7 (5–7) | 15 (9–29) | 6.20 (2.20–13.91) |
| *M. flavescens* (2) | 6 (5–6) | 0 | 7 | 13 (12–13) | 54.81 (40.29–69.34) |
| *M. gordonae* (13) | 27 (20–35) | 0 (0–1) | 6 (5–7) | 33 (26–40) | 3.97 (0.53–9.57) |
| *M. simiae* (13) | 11 (7–22) | 0 | 7 (5–7) | 18 (13–28) | 4.83 (1.77–11.89) |
| *M. szulgai* (4) | 28 (27–29) | 0 | 6 (5–7) | 34 (34–35) | 2.60 (1.01–5.22) |
| *M. tuberculosis* (65) | 13 (2–29) | 0 (0–1) | 6 (5–7) | 19 (7–35) | 3.45 (0.55–12.35) |

[a] The validity index is calculated by dividing the tallest peak height of the isolate's pattern by the height of the internal standard peak (see text). Values in parentheses show the range for the particular isolate.

sample and standard mixture was injected into the HPLC system as described previously (10).

Chromatograms were analyzed as described previously (10) and compared with those of species described in the literature as the source for identification (2–7, 9–11, 13, 18–20). Of the 126 isolates grown in medium to which OADC enrichment was added, 117 (93%) were successfully identified to the species level. Isolates of *M. tuberculosis* required an average of 13 days from the time of initial specimen processing to the time that they achieved a GI of 50 or greater. The BACTEC 7H12B vials were then incubated with OADC for an average of 6 days before HPLC analysis

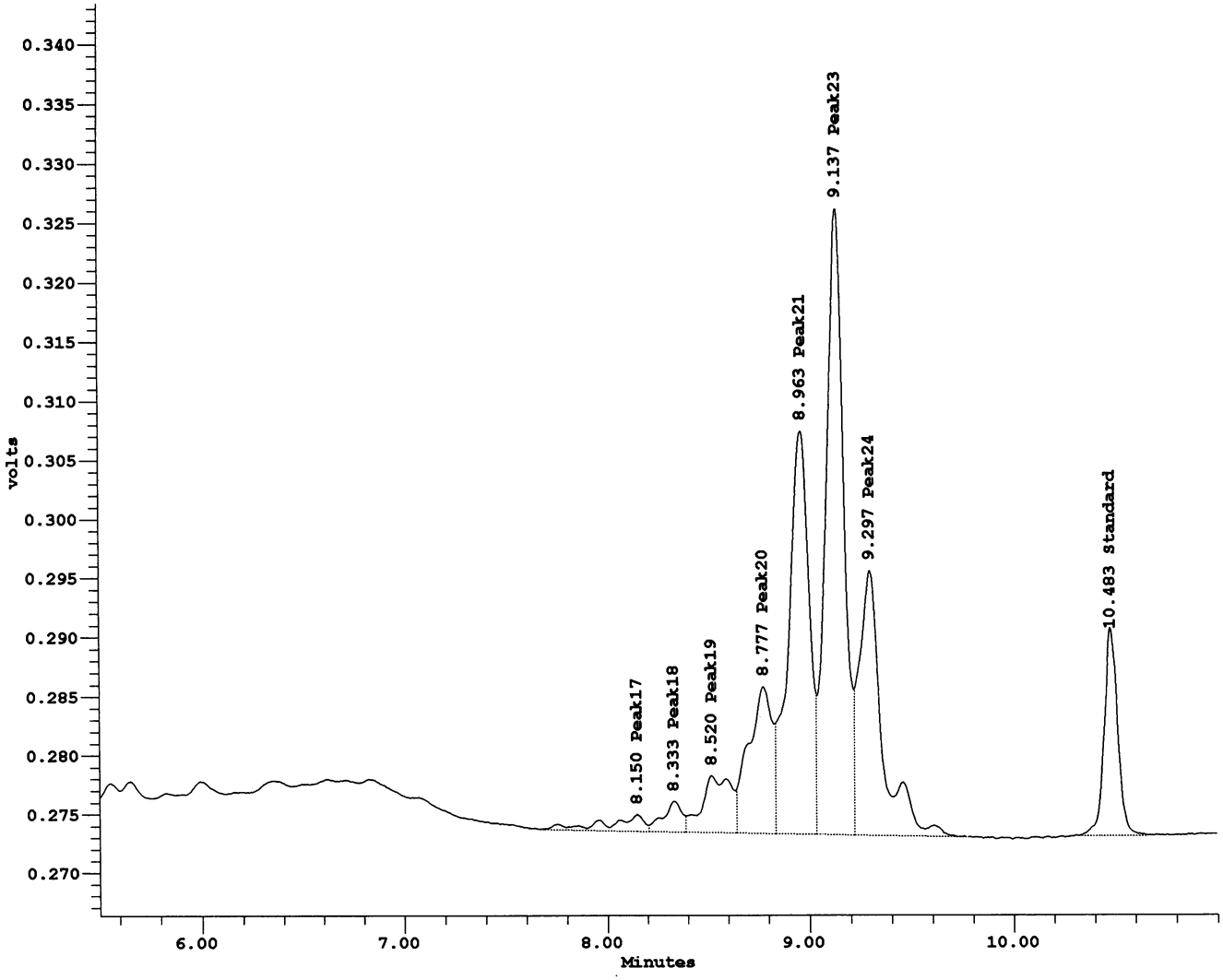


FIG. 1. HPLC chromatogram of the *M. tuberculosis* isolate obtained from BACTEC 7H12B medium containing OADC enrichment (see text).

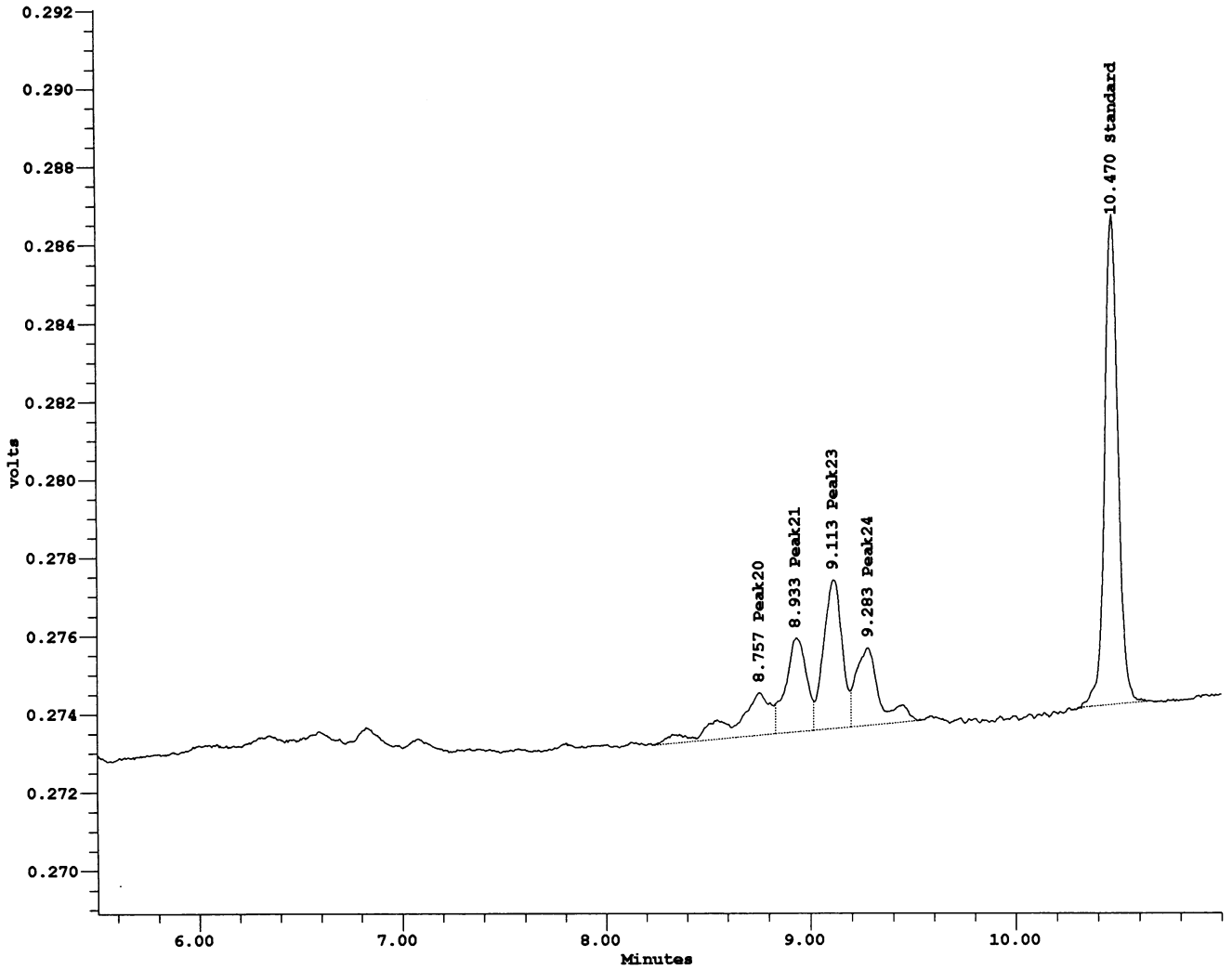


FIG. 2. HPLC chromatogram of the *M. tuberculosis* isolate obtained from BACTEC 7H12B medium without the addition of OADC enrichment (see text).

was performed. The total time from initial specimen processing to identification was an average of 19 days (Table 1). Results for mycobacteria other than *M. tuberculosis* were about the same. The average total time from the time of initial specimen processing to the time that a GI of 50 or more was obtained was 15 days. The BACTEC 7H12B vials were incubated with OADC for an average of 6 days, making the overall time from processing to identification an average of 21 days. The times varied for different species; e.g., *M. flavescens* required an average of 13 days for complete identification and *M. szulgai* required an average of 34 days (Table 1).

Of the nine (7%) chromatograms that could not be identified, six (5%) lacked adequate or key peaks, preventing a reliable interpretation. One isolate (1%) was overgrown with mold, which prevented further isolation. Two isolates (2%) presented chromatograms with adequate peaks but unrecognizable patterns. Upon examination of the BACTEC subcultures, one was found to be a mixed culture, later identified as *M. avium* complex and *M. simiae*. The other isolate was a scotochromogen that could not be identified even by conventional biochemical tests (14).

A method that can be used to distinguish between reliable and unreliable chromatograms was examined. The height of the tallest peak in the specimen chromatogram was divided by the height of the internal standard peak. The resulting ratio (a validity index) was then used to establish the acceptability of the identification. For successfully identified *M. tuberculosis* isolates, the average validity index was 3.45 (range, 0.55 to 12.35). For those species other than *M. tuberculosis*, the average validity index was 6.89 (range, 0.53 to 69.34). Again, the validity index varied from species to species (average, 2.60 for *M. szulgai* to 54.81 for *M. flavescens*) (Table 1). In contrast, the six isolates showing poor peaks had a validity index range of from 0.00 to 0.49. Values below 0.50 constituted a gray area and depended on the characteristics of the species' pattern. Therefore, a value of at least 0.50 appeared to be needed before identification could be considered reliable.

The 10 duplicate specimens that grew in medium to which OADC enrichment was not added were also prepared for HPLC analysis 6 days after they reached a GI of 50 or greater. None of the specimens produced sufficient peaks to be reliable for identification, and all specimens had a validity

index of less than 0.40. An example of one such specimen shows the chromatograms obtained with and without the addition of OADC enrichment. Peak 23 showed nearly a 400% increase in height when OADC was added (Fig. 1) compared with that for the vial that did not contain OADC (Fig. 2). A word of caution is presented here. Commercially prepared OADC may not be of the highest quality needed to support the growth of mycobacteria. When the present study was initially begun, HPLC chromatograms of those isolates grown with and without OADC enrichment did not seem to differ greatly (unpublished data). This inability to support better growth was later found to be due to the OADC enrichment itself (16). Therefore, it is important that the additive be tested for its ability to support the growth of mycobacteria. A simple method of assaying its suitability prior to use has been described (8).

The present study has shown that reliable identification of *Mycobacterium* spp. can be made from HPLC analysis of mycolic acids in BACTEC 7H12B medium in which mycobacteria have grown if certain guidelines are followed. (i) OADC enrichment is added to the BACTEC vial after the GI has reached 50 or greater. (ii) The vial is then incubated for at least 5 days at the appropriate temperature. (iii) A chromatogram shall be considered valid if it produces a recognizable pattern in accordance with the published literature and has a validity index of $\geq 0.50$.

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