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Identification of Major Slowly Growing Pathogenic Mycobacteria and *Mycobacterium gordonae* by High-Performance Liquid Chromatography of Their Mycolic Acids

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A rapid, reverse-phase high-performance liquid chromatography method was used to detect *p*-bromophenacyl mycolic acid ester patterns for strains of four major pathogenic *Mycobacterium* species and for the most commonly encountered saprophytic species, *Mycobacterium gordonae*. Mycobacteria in low numbers (2.5×10^6 CFU) were detected and identified to the species level. Standard chromatographic patterns characteristic of each species were established. Simple pattern recognition enabled rapid identification of *M. tuberculosis*, *M. kansasii*, *M. avium*, *M. intracellulare*, and *M. gordonae*.

Differential identification of *Mycobacterium* species has traditionally been based upon biochemical reactions (7). The time required to perform the battery of biochemical tests usually makes traditional taxonomic identification a time-consuming process. More rapid methods of differential identification are needed for the various *Mycobacterium* species.

Chemical analysis of cellular fatty acids, especially the high-molecular-weight, α-branched β-hydroxy mycolic acids, has been used as a rapid test in mycobacterial systematics (10). Extensive studies using two-dimensional thin-layer chromatography and mass spectrometry with mycolic acid methyl esters have revealed a variable species-related distribution of mycobacterial mycolic acid structural types (9–13). Recently, a study using thin-layer chromatography revealed the clinical usefulness of mycolic acid analysis for separating *Mycobacterium simiae* from *M. avium-M. intracellulare* in a mixed mycobacterial infection (8).

Other methods of mycolic acid analysis have combined absorption chromatography with high-performance liquid chromatography (HPLC) and demonstrated the chemical uniqueness of the cell wall-bound mycolic acids (15, 18). We previously used HPLC to separate the different mycolic acid-containing genera (1). In this study, we describe a simple, single-step HPLC analysis of the *p*-bromophenacyl esters of mycolic acids extracted from saponified whole cells of several species of mycobacteria and demonstrate the distinctly different mycolic acid "fingerprint" patterns produced by the different species. We examined four strictly or potentially pathogenic species (*M. tuberculosis*, *M. avium*, *M. intracellulare*, and *M. kansasii*) that account for almost 90% of the potentially pathogenic mycobacteria and 71% of the total (both pathogenic and saprophytic) mycobacteria recovered in state health laboratories in the United States (5). We compared the mycolic acid patterns of both reference culture strains and clinical isolates to establish standard species patterns. It was of special interest to determine if the mycolic acid patterns could be used as a rapid identification method for these slowly growing pathogenic mycobacteria. Although *M. gordonae* is not pathogenic, it was included in the study because it represents about 15% of the isolates recovered in public health laboratories (5). The five species together account for nearly 85% of all mycobacteria recovered in state health laboratories.

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MATERIALS AND METHODS

Microorganisms and growth conditions. *Mycobacterium* isolates were either from the Trudeau Mycobacterial Collection (TMC), formerly at the National Jewish Hospital and Research Center, Denver, Colo., or from the Mycobacteriology Laboratory, Centers for Disease Control (CDC), Atlanta, Ga., and were as follows: *M. tuberculosis* TMC 102, 106, 107, 110, 111, 112, 117, 119, 124, 205, 320, and 321 and CDC 3900, 3901, 3902, 3903, 3904, 3905, 3906, 3907, 3908 and 3909 (from a primary drug resistance study, i.e., isolates with no prior exposure to drug therapy); *M. kansasii* TMC 1203, 1204, 1214, and 1217 and CDC 769, 830, 946, 961, 983, 1010, 1069, 1087, 1210, and 1253 (these isolates were submitted from state health departments to the CDC reference laboratory for study); *M. gordonae* TMC 1319, 1324, 1325, and 1327 and CDC 911, 946, 986, 1196, 1215, 1231, 1245, and 1251 (these isolates were submitted from state health departments to the CDC reference laboratory for study); *M. avium* TMC 701, 702, 706, 714, 716, 724, 801, 1461, 1462, 1463, 1464, 1468, 1472, and 1479 and CDC 82-307, 82-905, and 82-993; *M. intracellulare* TMC 1403, 1405, 1406, 1419, 1466, 1469, and 1473 and CDC 81-641, 82-421, 82-625, 82-759, 82-1128, 82-1129, and 83-764. All of the CDC isolates were fresh clinical specimens. The pathogenic species included in this study were *M. tuberculosis*, *M. kansasii*, *M. avium*, and *M. intracellulare*. *M. gordonae*, a commonly nonpathogenic species, was also included. Isolates were grown on commercially prepared Löwenstein-Jensen slants (Carr-Scarborough Microbiologicals, Atlanta, Ga.) at 35°C for 3 weeks before analysis.

Identification of mycobacteria. Mycobacteria were identified by conventional laboratory methods (6, 7).

Mycolic acid extraction and derivatization procedures. Mycobacteria were harvested either by washing the Löwenstein-Jensen slants with 2 ml of saponification reagent (25% KOH in 50% ethanol) if growth was scant or by suspending 1 loopful of cells from Löwenstein-Jensen slants with abundant growth in 2 ml of alcoholic KOH. Biomass saponification was performed at 85°C for 18 h in 13-mm glass tubes with Teflon-lined screw caps. Saponified cells were cooled to

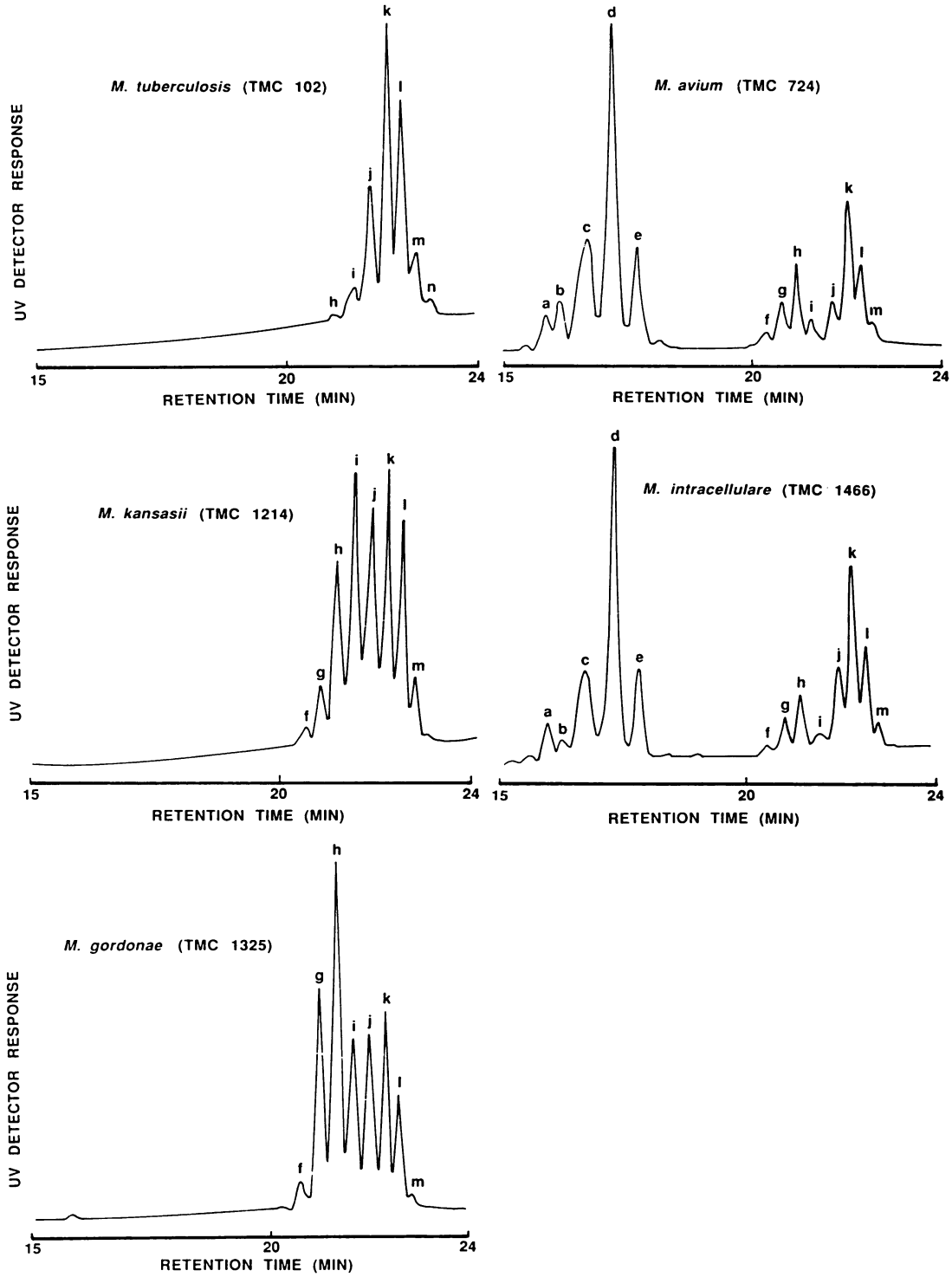

FIG. 1. High-performance liquid chromatograms of the *p*-bromophenacyl mycolic acid esters from saponified whole cells of mycobacteria.

TABLE 1. Reproductibility of mycolic acid patterns of mycobacteria

Species (no. of strains)	Δt (±SEM)[a] of peak:					
	a	b	c	d	e	f
M. tuberculosis (22)						
M. kansasii (14)						−0.62 (0.006)
M. gordonae (12)						−0.69 (0.009)
M. avium[b] (17)	−5.07 (0.02)	−4.78 (0.02)	−4.27 (0.03)	−3.75 (0.02)	−3.21 (0.01)	−0.62 (0.008)
M. intracellulare[b] (14)	−5.00 (0.04)	−4.69 (0.04)	−4.27 (0.04)	−3.68 (0.03)	−3.18 (0.03)	−0.64 (0.008)

room temperature and acidified to pH 1 with 6 N HCl. The mycolic acids were extracted by adding 2 ml of chloroform, mixing for 20 s on a test tube mixer, and centrifuging to assist separation of the chloroform layer. The extracted mycolic acids were evaporated to dryness and resuspended in chloroform for derivatization. The mycolic acids were derivatized to *p*-bromophenacyl esters by the method of Durst et al. (4). Briefly, a mixture containing the extracted mycolic acids in 0.5 ml of chloroform–2 mg of potassium carbonate–25 μl of *p*-bromophenacyl bromide in acetonitrile–25 μl of the catalyst dicyclohexyl-18-crown-6 ether in acetonitrile was heated for 45 min at 85°C in Teflon-capped 5-ml Reacti-Vials (Pierce Chemical Co., Rockford, Ill.) with constant stirring with Teflon-coated magnetic stirrers. The reagents were included a kit obtained from Alltech Associates, Inc., Applied Science Div., State College, Pa. Chloroform was used instead of the acetonitrile recommended for shorter-chain fatty acid derivatization because the long-chain, high-molecular-weight mycolic acids were more soluble in chloroform. After derivatization, the *p*-bromophenacyl esters of the mycolic acids were filtered through 0.45-μm-pore nylon 66 membrane filters, evaporated to dryness, and resuspended in 100 μl of chloroform for analysis.

HPLC conditions. HPLC of the *p*-bromophenacyl esters of the mycolic acids was done with an HPLC model 450 data system controller (Beckman Instruments, Inc., Fullerton, Calif.). The UV light-absorbing *p*-bromophenacyl esters were detected with a variable-wavelength detector (Laboratory Data Control, Milton Roy Co., Riviera Beach, Fla.) set at 254 nm. A reverse-phase C-18 cartridge column (22 cm by 2.1 mm; Pierce) with a 5-μm spherical particle size was used to separate the mycolic acid esters. A solvent gradient system of chloroform and methanol was controlled by two model 110A solvent delivery pumps (Beckman) adjusted to a constant flow rate of 0.6 ml/min. The column was equilibrated to 10% chloroform–90% methanol over a 13-min period. After the injection of 5-μl samples, the solvent concentration was changed linearly over a 1-min period to 25% chloroform–75% methanol; over the next 20-min period, the solvent concentration was changed linearly to 70% chloroform–30% methanol.

Standards for the identification of the mycolic acids. Mycolic acid standards were not available commercially. Authentic *p*-bromophenacyl esters of the mycolic acids from *M. tuberculosis* ($H_{37}Ra$) and *M. smegmatis* CDC 8 (derived from ATCC 607), confirmed by HPLC and mass spectrometry and provided by Kuni Takayama, Veterans Administration Hospital, Madison, Wis., were used as standards for comparing the elution times and patterns of our derivatized mycolic acids. The mycolic acid peaks were arbitrarily designated by letters corresponding to the order of their elution.

RESULTS AND DISCUSSION

The elution patterns of the *p*-bromophenacyl esters of the mycolic acids were determined by HPLC for 85 isolates representing five *Mycobacterium* species (Fig. 1). Each analysis was completed in 34 min, from the initial column equilibration to the end of the program run. The mycolic acid esters of these mycobacteria eluted between 15 and 23 min.

The *p*-bromophenacyl esters of "standard," purified mycolic acids from *M. tuberculosis* and *M. smegmatis* corresponded directly in retention times and overall patterns to the derivatized mycolic acids that we obtained from whole organisms, and these standards were used as a presumptive identification of the mycolic acids. We found that the derivatized products were stable for months at an ambient temperature. Components of Löwenstein-Jensen medium, excess derivatization reagents, acetonitrile (from the reagent kit), and short-chain cellular fatty acids (C_{18} or less) all eluted within 5 min and did not interfere with the identification of the mycolic acids. Quantitation of the method by use of an *M. kansasii* culture revealed that definitive mycolic acid fingerprint patterns could be achieved with as few as 2.5 $\times$ 10^6 CFU.

M. tuberculosis had the least complicated mycolic acid pattern, with a cluster of seven peaks that eluted between 21 and 23 min and one major, central peak (designated k). *M. kansasii* and *M. gordonae* both produced clusters of eight peaks that eluted over the same 21- to 23-min range, but the patterns for all three species were distinctly different. *M. kansasii* and *M. gordonae* both had mycolic acid peaks that eluted 1 min before (at 20.6 min) the mycolic acid peak of *M. tuberculosis* (at 21.6 min). *M. kansasii* had two major peaks, i and k, while *M. gordonae* had one major peak, h (Fig. 1).

M. avium and *M. intracellulare* both produced an early cluster of five more polar mycolic acids (peaks a, b, c, d, and e) that eluted between 15 and 17.5 min and a later cluster of less polar mycolic acids (peaks f to m) that eluted between 20 and 23 min. The two groups together comprised 13 peaks that were characteristic for the two species, as was the 2.5-min interval (17.5 to 20 min) between the two groups during which no peaks eluted (Fig. 1). The differences between *M. avium* and *M. intracellulare* were only minor (note especially peaks a, b, and i in Fig. 1).

Initially, the reproducibility of the patterns was studied by analyzing mycolic acid peak retention times for 22 different isolates of *M. tuberculosis* (12 TMC isolates and 10 CDC clinical specimens). Cultures were selected to represent differences in old isolates (>19 years in cultures) and fresh isolates (<1 year old). Standard methods of statistical analysis were used to determine the mean and standard error of the mean for the retention times and the time differences in minutes between each peak and peak h. The average stan-

TABLE 1—*Continued*

Δt (±SEM)[a] of peak:							
g	h	i	j	k	l	m	n
	0	+0.30 (0.003)	+0.64 (0.005)	+0.97 (0.006)	+1.26 (0.008)	+1.53 (0.008)	+1.80 (0.01)
−0.32 (0.003)	0	+0.31 (0.002)	+0.68 (0.005)	+1.00 (0.007)	+1.29 (0.008)	+1.56 (0.01)	
−0.34 (0.003)	0	+0.34 (0.004)	+0.71 (0.006)	+1.03 (0.01)	+1.33 (0.01)	+1.60 (0.01)	
−0.30 (0.004)	0	+0.29 (0.004)	+0.72 (0.01)	+1.02 (0.01)	+1.29 (0.02)	+1.54 (0.03)	
−0.31 (0.005)	0	—[c]	+0.75 (0.02)	+1.00 (0.02)	+1.26 (0.02)	+1.50 (0.02)	

[a] Δt represents the mean time difference (in minutes) ± the standard error of the mean between key peak h (designated 0) and all other mycolic acid ester peaks. A negative value indicates that the peaks appeared before peak h, while a positive value indicates that the peaks followed peak h. See Fig. 1 for peak letter designations.

[b] Identification was confirmed by standard serotyping and by DNA-DNA hybridization.

[c] The shrewd nature of peak 1 for this species would not allow accurate retention times to be recorded by the instrument.

dard error of the mean deviation from the mean time differences for all seven peaks was 0.007 min (Table 1).

Since increased incubation time has been noted to have a quantitative effect on the production of mycolic acids in mycobacteria (3) and because this could affect the reproducibility of the patterns, we examined the mycolic acid patterns of *M. tuberculosis* $H_{37}Rv$ (TMC 102) harvested during a period from 3 weeks to 4 months and found that the HPLC pattern did not change. Although slight variations in retention times and minor peak height fluctuations were occasionally noted between freshly isolated (CDC) specimens and TMC specimens that had been maintained for years on artificial media, the basic patterns of elution remained the same. The reproducibility of the patterns of all five species studied was demonstrated by examining relative time differences between peak h and all the other mycolic acid peaks for each species (Table 1).

All the mycobacteria studied had overlapping patterns because of similar mycolic acids (2, 14–18). The pattern of *M. tuberculosis*, the organism most often isolated from patients with pulmonary tuberculosis, was significantly different from those of the other pathogens and from that of the common laboratory contaminant *M. gordonae*. The patterns of *M. kansasii* and *M. gordonae* also were similar in retention times, but the relative heights of peaks g to l were consistently and sufficiently different to permit differentiation of the two species. Species differentiation between *M. avium* and *M. intracellulare* was less distinct.

Sample preparation was easy and required only 20 h from receipt of a mycobacterial culture to final identification. Overnight saponification (unattended) accounted for 90% of this time. Shorter saponification times (2 and 4 h) were also effective but less convenient. The method described provided a rapid, simple, and direct analysis of the mycolic acids found in mycobacteria, and the results suggest that the procedure could be used for preliminary species identification of the slowly growing pathogenic mycobacteria.

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