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Identification of Clinical Isolates of Mycobacteria with Gas-Liquid Chromatography Alone

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Identification of 18 mycobacterial species was performed by analysis of profiles obtained by using gas-liquid chromatography. Organisms were saponified in methanolic NaOH, and the reaction mixture was treated with BF_3 in methanol and extracted with a hexane-chloroform mixture. An identification scheme was developed from 128 stock strains and tested against a collection of 79 clinical isolates. By using gas-liquid chromatographic profiles alone, 58% of specimens were correctly identified to species level, and an additional 41% were correctly identified to a group of two or three organisms. Use in a clinical laboratory over a 2-month period proved chromatography to be as accurate as and more rapid than concurrent biochemical testing. Of 81 isolates tested, 64% were identified to species level by chromatography alone. An additional 35% were differentiated to the same groups of two or three organisms as found in our analysis of stock strains. These groups consisted of: *Mycobacterium tuberculosis*, *M. bovis*, and *M. xenopi*; *M. avium* complex, *M. gastri*, and *M. scrofulaceum*; or *M. fortuitum* and *M. chelonei*. Identification to species level from these groups could usually be done by colonial morphology alone and could always be done by the addition of one selected biochemical test. This study demonstrated the practical application of gas-liquid chromatography in the identification of mycobacteria in a clinical laboratory. In particular, all strains of *M. gordonae* and *M. kansasii* were identified to species level. *M. tuberculosis* was definitively identified in 85% of cases. When it could not be definitely identified, the only alternatives were *M. bovis* and *M. xenopi*, both of which are rare causes of infection.

When mycobacteria are cultured from human sources, appropriate therapy depends upon the species isolated. Identification of mycobacteria with biochemical testing is protracted by their slow growth rate, however, and thus there is need for more rapid methods to identify clinically important isolates. Chemical differentiation has been an attractive possibility since the demonstration by Anderson of the uniqueness and complexity of tubercle cell wall lipids (1). As early as 1954, Smith and associates characterized mycobacterial species by using column chromatography and infrared spectroscopy to detect species specific myococerenates of phthioceral (28-30). Later, several studies used thin-layer chromatographic analysis of lipids and glycolipids to classify many types of nontuberculous mycobacteria (4, 9, 10, 14-16, 31). This technique was developed further by Tsukamura and Mizuno (36), who differentiated mycobacterial species incubated with [^{35}S]methionine by the patterns of radioactive lipid spots. Pyrolysis gas-liquid chromatography of whole cells was successfully employed by Reiner and associates (21-27) to classify and prospectively identify many different strains of mycobacteria. Lucchesi and associates (13) were the first to show that mycobacteria could be differentiated by gas-liquid chromatography of methylated lipid extracts. More detailed work by Thoen et al. demonstrated that *M. kansasii* and *M. marinum* could be distinguished from certain other mycobacteria by characteristic branched-chain fatty acids (32-34). Larsson and Mårdh were able to show consistent differences of fatty acid methyl esters and trifluoroacetylated methyl glycosides among ten strains of *M. avium*, *M. kansasii* and *M. tuberculosis* by trifluoroacetylation of whole-cell methanolysates (12). It was the characterization of ten species of mycobacteria by Ohashi et al. (18), using reaction gas-liquid chromatography, that prompted the present study. Their single-step analysis of whole-cell hydrolysates with tetramethylammonium hydroxide initially allowed them to differentiate mycobacterial strains and demonstrate a unique peak for *M. tuberculosis*. With our, and subsequently their, inability to reproduce these results (T. Wade, personal com-

munication), a different technique for differentiating mycobacteria was developed and tested in our laboratory.

MATERIALS AND METHODS

Microorganisms. The majority of the 296 strains of mycobacteria used were identified biochemically by the method of Kubica (11). *M. szulgai* was identified by methods described by Marks and associates (15). *M. simiae* was identified by its photochromogenicity, positive niacin test, negative nitrate reduction test, and heat-stable catalase. All were recovered from clinical specimens in our laboratory or were specimens from the following centers: National Jewish Hospital and Research Foundation, Denver, Colo., 15 strains; Trudeau Institute mycobacterial culture collection, 21 strains; and College of American Pathologists microbiology proficiency testing specimens, 16 strains of mycobacteria and 2 strains of *Nocardia asteroides*. Cultures were grown on Lowenstein-Jensen and Middlebrook 7H10 media and were incubated at 37°C in an atmosphere containing 5 to 10% CO_2 until there was adequate growth for analysis. Two strains of *M. marinum* were incubated at room temperature. The incubation time ranged from 2 to 6 weeks.

Standard preparation. Two loopfuls (3-mm loop) of organism were suspended in 1 ml of NaOH (0.5 mol/liter) in absolute methanol. This suspension was incubated for 30 min at 70°C in a clean screw-capped test tube (16 by 125 mm). The screw caps were Teflon lined to prevent extraction of interfering substances from the standard rubber liner. After cooling, 2 ml of 14% (wt/vol) boron trifluoride-methanol (Applied Science Laboratories, Inc., State College, Pa.) was added, and the mixture was incubated at 70°C for 5 min. After cooling, 1 ml of saturated NaCl solution was added. A 4-ml amount of a chloroform-hexane mixture (1:4, vol/vol) was added, and the test tube was agitated for 5 min. The mixture was centrifuged for 5 min, and the upper phase was transferred to another test tube. Another 4-ml amount of the chloroform-hexane mixture was added to the remaining residue, and the extraction process was repeated. The combined upper phases were evaporated to dryness in a water bath at 50 to 60°C under a stream of nitrogen. The residue was redissolved in 0.2 ml of chloroform and kept at room temperature until the analysis of a 2-μl sample. During the course of our experiments, we found that extraction was as efficient with tipping of the tube as with vigorous agitation and that the second chloroform-hexane extraction was unnecessary. This shortened procedure saved 20 min and was used for approximately one-third of the isolates in the comparative studies of phase 3.

Scaled-down modification for comparative studies. The method was modified to permit interpretable chromatographic analysis with as little as a 1-mm colony (approximately one loopful of a 0.001-ml calibrated loop). Only the volumes and test tube sizes were modified, with temperatures and times remaining the same. Table 1 compares the standard method with the scaled-down methods. Two variations are given to accommodate different laboratory situations.

Chromatographic analysis. A Varian Aerograph 2700 gas chromatograph equipped with a flame-ionization detector was used for all analyses. A glass column 6 feet (ca. 1.82 m) long, with an internal diameter of 2 mm was packed with 3% OV-17 on 100- to 120-mesh Supelcoport (Supelco Inc., Bellafonte, Pa.). Although the column extended to the septum, it was packed only to the distal edge of the injector block. The injector port, and the detector were kept at 275 and 315°C, respectively. The column oven temperature was held at 170°C for 2 min after injection and then raised 6°C/min to a final temperature of 300°C, which was maintained for 15 min. Zero-grade helium (Union Carbide Corp., Linde Division, New York, N.Y.) was used as the carrier gas at a flow rate of approximately 17 ml/min. Detector attenuation was 1×10^{-10} A/mV. A dual-channel recorder was set at two sensitivities. At one sensitivity, a 10-mV signal produced a full-scale response which allowed approximately a full-scale response for the peak due to the methyl ester of palmitic acid ($C_{16:0}$). The other sensitivity gave approximately a full-scale response for one of the peaks due to either methyl tetracosanoate ($C_{24:0}$) or methyl hexacosanoate ($C_{26:0}$). The same recorder settings were used for all organisms. Mass spectra of the chromatographic peaks were measured with a 5805A Hewlett-Packard GC-MS system (Hewlett-Packard Co., Palo Alto, Calif.) under identical chromatographic conditions. Peaks were tentatively identified by mass-spectral patterns and, where possible, on the basis of identical retention time to standard fatty acid methyl esters. Mass-spectral patterns were interpreted by standard methods (5, 6, 38).

Experimental design. The study was divided into three phases. In phase one, the procedure was developed and stock cultures of mycobacteria were analyzed. A decision tree was made from the chromatographic patterns of these stock organisms for the identification of unknowns. In phase two, 79 unknown strains were identified, using only their chromatograms and the decision tree from phase one. In phase three, the chromatographic method was run in parallel to conventional biochemical methods for a 2-month period in the laboratory.

RESULTS

Chromatographic characteristics. The chromatogram of *M. tuberculosis* in Fig. 1 demonstrates a typical mycobacterial pattern obtained by our method. The identity of each peak as determined by mass spectroscopy is shown. Those peaks that were present in all species but were of no aid in identification are marked with an asterisk. Table 2 shows the elution order and identifications for standards and for peaks of taxonomic importance. We stress that the identifications of branched-chain fatty acids are based only on their mass spectra and retention times relative to straight-chain standards. Branching at the 2 position of a methyl ester gives characteristic ions at m/e 88 and 101 (38).

TABLE 1. *Comparison of volumes used in lipid extraction and derivatization*

Method	Amt of organism (loopfuls)	Test tube size (mm)	Amt of NaOH in methanol[a]	Amt of BF_3 in methanol[b]	Saturated NaCl (ml)	Chloroform-hexane (ml)[c]	Amt of chloroform used to redissolve (μl)
Standard	2[d]	16 by 125	1	2	1	4	200
Scaled down							
A	1[d]	14 by 100	0.5	1	0.5	1	100
B	1[e]	6 by 50[f]	0.05	0.1	0.05	0.2	20

[a] Values indicate milliliters of NaOH at 0.5 mol/liter.
[b] Values indicate milliliters of BF_3 at 14% (vol/vol).
[c] Values indicate milliliters of chloroform-hexane at 1:4 (vol/vol).
[d] Each loop was 3 mm.
[e] Each loop was 1 mm.
[f] A 200-μl polypropylene centrifuge tube (Bio-Rad Laboratories, Richmond, Calif.) was used as a cork.

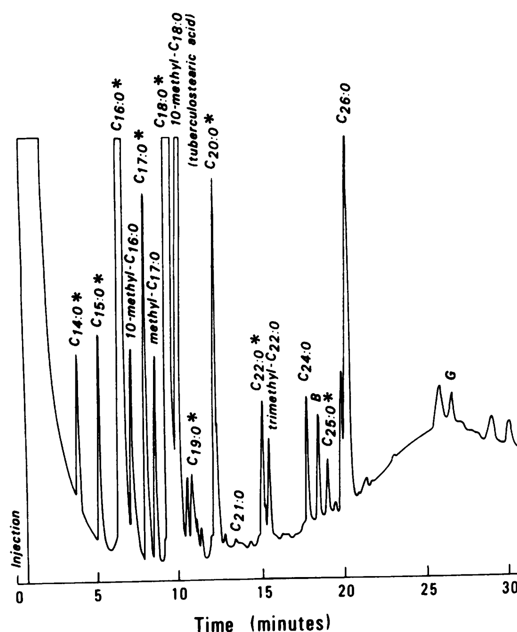

FIG. 1. *Typical chromatogram of fresh isolate of M. tuberculosis.*

This branching assignment is easily and definitively made by mass spectroscopy. Our assignments of branching at other positions depended upon the relatively greater abundance of ions due to fracture of the alkyl chain on either side of the branch compared with fragmentation at other locations in the chain. These assignments should be considered tentative, because pure compounds which could be used for comparison were not available. Nevertheless, these assignments serve a useful function for discussion of the various peaks.

Effects of media. Analysis of cultures grown on Lowenstein-Jensen medium gave few chromatographic differences compared with Middlebrook 7H10 medium. Lowenstein-Jensen medium was preferred because organisms grown on it gave more intense peaks, although either medium could be used without adversely affecting accuracy. Although extracts of Middlebrook 7H10 medium showed no significant chromatographic peaks, those of Lowenstein-Jensen did, and thus care was taken not to contaminate the organism with small pieces of this medium.

Precision. Chromatograms obtained by repeat analyses of the same preparation and of separate preparations of the same subculture were superimposable. Separate subcultures of $H_{37}Rv$ strain ranging in age from 3.5 to 9 weeks were analyzed on six occasions. All peaks required for identification were present with only slight changes in relative heights. Identification was not affected by these changes. Similarly, separate subcultures of *M. chelonei* and *M. fortuitum* analyzed after 1.5 and 6 weeks of incubation gave chromatograms that were virtually unchanged.

Identification scheme. Our scheme for identification was developed and then tested in three phases of experiments. In the first phase, 128 stock strains, representing 17 species of mycobacteria, were tested to determine which similarities and differences were reproducible and could be used for identification. Table 3 presents the incidence of peaks found in each species, with the peaks most important for identification of a given species in parentheses. It includes peak patterns of *M. tuberculosis* obtained for both fresh isolates from phase 2 and stored isolates from phase 1. This was done because of differences found during the testing of fresh clinical isolates. Organisms that could be identified as *M. tuberculosis* by other peaks consistently demonstrated a new peak, B, that had not been found in the original, stored organisms used

TABLE 2. *Elution order for mycobacterial hydrolysis products on 3% OV-17*[a]

Standard[b]	Mycobacterial component	RT[c]
$C_{14:0}$	$C_{14:0}$	3.0
	2-Methyl $C_{14:0}$	3.2
	2,4-Dimethyl $C_{14:0}$	3.3
$C_{15:0}$	$C_{15:0}$	4.4
	2,4-Dimethyl $C_{15:0}$	4.7
$C_{16:0}$	$C_{16:0}$	5.6
	8-Methyl $C_{16:0}$	5.9
$C_{17:0}$	$C_{17:0}$	7.0
	Methyl $C_{17:0}$	7.4
$C_{18:0}$	$C_{18:0}$	8.3
	10-Methyl $C_{18:0}$	8.9
	$C_{19:1}$	9.9
$C_{19:0}$	$C_{19:0}$	9.9
$C_{20:0}$	$C_{20:0}$	11.5
	Trimethyl $C_{20:0}$	11.8
$C_{21:0}$		12.6
$C_{22:0}$	$C_{22:0}$	14.1
	Trimethyl $C_{22:0}$	14.4
$C_{23:0}$	$C_{23:0}$	15.6
$C_{24:0}$	$C_{24:0}$	16.7
	A	16.8
	B	17.0
Myristyl laurate		17.4
	$C_{25:0}$	18.1
	C	18.5
	D	18.9
	$C_{26:0}$	19.2
	$C_{28:0}$	21.5
Myristyl palmitate		22.2
	E	22.8
	F	23.9
Palmityl palmitate		25.2
	G	25.5
	H	27.4
	I	31.8
Stearyl stearate		32.6

[a] The following fatty acid designations are used: the number before the colon indicates number of carbon atoms in the longest unbranched chain; the number after the colon indicates the number of double bonds or rings; the number before the word methyl or dimethyl refers to the position of the group on the chain. Assignment of a peak name without a corresponding standard was made by mass spectroscopy, and thus is tentative.

[b] All fatty acids were methyl esters, except as noted.

[c] RT, Retention time in minutes.

to characterize this species. Because peak B proved to be of importance in identifying *M. tuberculosis* to species level, but was not detected in stored specimens from phase 1, the peak pattern of fresh isolates is included in a separate row for completeness. Although most identifications could be made by the presence or absence of particular peaks, differences in peak heights proved of value in two situations. First, the methyl hexacosanoate peak was always greater than the methyl tetracosanoate in *M. bovis*, *M. tuberculosis*, *M. xenopi*, and *M. simiae* and served to separate them as a group from the other species. Second, a methyl docosanoate ($C_{22:0}$) peak greater than 25% of the methyl hexacosanoate peak was never found in *M. bovis*; therefore, a large methyl docosanoate peak would exclude *M. bovis* as a possible identification. Figure 2 presents a flow chart for identification based on the data in Table 3. In the second phase, we analyzed by chromatography 79 organisms collected in our laboratory over a 3-month period. An identification was made based solely on the chromatographic results and without knowledge of the biochemical test results. A total of 44% of all the organisms were identified to species level correctly by using only the chromatographic results generated by stock cultures (Table 4). With the knowledge that fresh isolates of *M. tuberculosis* express a new peak, B, identification to species level was increased to 60%. It should be noted (Table 3) that one of ten strains of *M. bovis* demonstrated a peak B. In view of the medical importance of this species and *M. tuberculosis*, these identifications must be confirmed by biochemical testing. An additional 39% were differentiated to a group of two or three other organisms with similar chromatographic patterns. For example, one unknown of *M. tuberculosis* could not be differentiated from *M. bovis* and *M. xenopi*, and seven *M. avium* complex could not be differentiated from *M. gastri* and *M. scrofulaceum*. The *Nocardia* isolate was clearly not mycobacteria because it lacked peaks due to methyl tetracosanoate and methyl hexacosanoate. In no case was a pure isolate of a biochemically typical organism misidentified.

In phase 3 of developing and testing our identification scheme, the chromatographic method was evaluated concurrently with biochemical methods of identification. Colonies were picked directly off the primary 7H10 isolation media when there was adequate growth. This was always possible before growth was adequate to start biochemical testing. Chromatographic identification was compared with biochemical identification of 81 organisms collected in our laboratory over a 2-month period. The data in Table 5 demonstrate that complete identification to species level was possible for 64% of the isolates by chromatography alone. The groups of two and three organisms were the same as shown in Table 4 and in Fig. 2. Only 33% of the cultures could not be differentiated to a single species. Of particular importance, however, 2 of the 4 *M. tuberculosis* specimens that could not be identified to species level were survey samples. As such, they were stored organisms, ex-

TABLE 3. *Characteristic lipids in the identification of mycobacteria*

Species	Strains tested	Peaks[a]																		
		2-Methyl $C_{14:0}$	2,4-Dimethyl $C_{14:0}$	2,4-Dimethyl $C_{15:0}$	10-methyl $C_{16:0}$ 10-methyl $C_{18:0}$	$C_{19:1}$	Trimethyl $C_{22:0}$	Methyl $C_{24:0}$	A	B	C	D	$C_{24:0} > C_{26:0}$	$C_{24:0} < C_{26:0}$	E	F	G	H	I
M. tuberculosis	8	0	0	0	100	0	0	88	0	0[b]	0	0	0	100	0	0	83[c]	0	0
M. tuberculosis (phase 2)	16	0	0	0	100	0	0	100	0	(100)	0	0	0	(100)	0	0	(83)[c]	0	0
M. bovis	10	0	0	0	100	0	0	50	0	(10)[d]	(80)	0	0	(100)	0	0	(90)[c]	0	0
M. xenopi	9	0	0	0	100	0	0	33	0	(0)	0	0	0	(100)	33	0	0	0	0
M. szulgai	7	(100)	0	0	100	0	(71)	(100)	0	(86)	0	(86)	(100)	0	0	0	0	0	0
M. gordonae	10	(100)	0	0	(0)	0	0	0	0	0	0	0	(100)	0	0	0	0	0	0
M. flavescens	2	50	0	0	100	(100)	0	50	0	50	0	0	(100)	0	50	0	0	0	0
M. kansasii	10	80	(100)	0	100	0	0	10	0	10	0	0	(100)	0	0	0	0	0	0
M. marinum	6	(100)	(83)[e]	(83)[e]	100	0	17[e]	50	0	0	0	0	(100)	0	33	0	0	0	0
M. simiae	3	0	0	0	100	0	0	33	0	0	0	0	0	(100)	(100)	(100)	(100)	(100)	0
M. scrofulaceum	10	0	0	0	100	0	10[e]	30[e]	0	10[e]	0	0	(100)	0	0	(80)	(100)	(90)	0
M. avium complex	10	0	0	0	100	0	0	10	0	0	10	0	(100)	0	(90)	(100)	(100)	(100)	0
M. gastri	10	0	0	0	100	0	0	0	0	10[e]	0	0	(100)	0	(100)	(100)	(100)	(100)	0
M. triviale	3	0	0	0	100	0	0	100	0	(67)	0	(67)	(0)	(0)	33	0	0	0	0
M. terrae	5	0	0	0	100	0	0	(100)	(100)	(100)	(80)	(100)	(100)	0	0	0	0	0	0
M. fortuitum	10	0	0	0	100	0	0	60	0	10	20	0	(100)	0	50	0	(40)[f]	0	0
M. chelonei	10	0	0	0	100	0	10	70	0	30	10	0	(100)	0	70	0	0	0	0
M. chelonei subsp. *abscessus*	1	0	0	0	100	0	0	100	0	0	0	0	(100)	0	0	0	0	0	0
M. smegmatis	4	(50)	0	0	100	0	25	25	0	0	0	0	(100)	0	0	0	0	0	(100)

[a] Large peaks that are found in all strains are excluded. Designation as branched-chain fatty acid is tentative. Values represent percentages, and parentheses indicate peaks most important for identification.

[b] Fresh and stored isolates showed different patterns, see text. Data from fresh isolates prepared in phase 2 are included for clarity.

[c] These peaks are of low amplitude in these species and are less than 20% of the $C_{26:0}$ peak.

[d] Peaks B and C are not seen together in *M. bovis*.

[e] Denotes that statistics include exceptional strain, see text.

[f] *M. fortuitum* contained a subset of four strains that could be differentiated from *M. chelonei*. Each strain in this subset had peaks E and G and unique peaks at effective chain lengths $C_{14.5}$, $C_{20.8}$, and $C_{22.7}$.

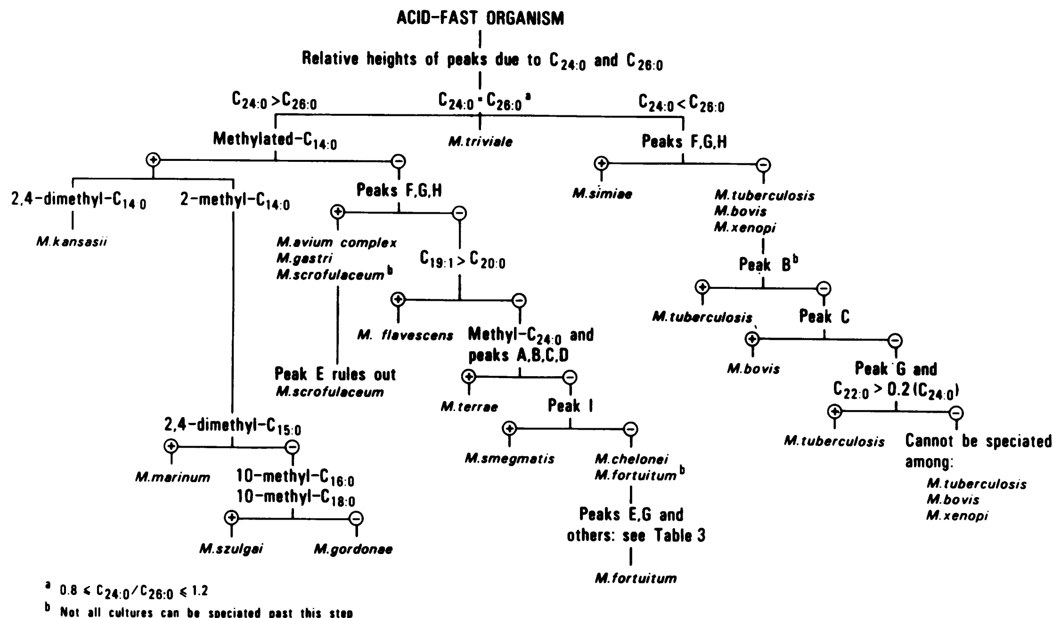

FIG. 2. *Identification scheme of mycobacteria by chromatographic pattern.*

TABLE 4. *Identification of blind unknowns*

Species	Degree of differentiation				
	Strains tested	Identified to species level	Group of 2	Group of 3	Not identified
M. tuberculosis	20	18 (6)[a]	1 (7)	1 (7)	
M. xenopi	1			1	
M. avium complex	19		12	7	
M. scrofulaceum	2			2	
M. gastri	1				1[b]
M. terrae	1	1			
M. kansasii	6	6			
M. szulgai	2	2			
M. gordonae	19	19			
M. fortuitum	5		5		
M. chelonei	2		2		
Nocardia	1	1[c]			
Total	79	47 (35)	20 (26)	11 (17)	

[a] Numbers in parentheses denote identification without knowledge of fresh culture effects on *M. tuberculosis* lipid pattern; see text.
[b] Identified as *M. fortuitum*, but biochemically atypical; see text.
[c] Identified as "not a mycobacterium."

TABLE 5. *Identification of unknowns concurrent with biochemicals over a 2-month period*

Species	Degree of differentiation				
	Strains tested	Identified to species level	Group of 2	Group of 3	Not identified
M. tuberculosis	9	5	3[a]	1	
M. bovis	2	2			
M. xenopi	1			1	
M. kansasii	7	7			
M. gordonae	32	32			
M. marinum	1				1[b]
M. szulgai	1	1			
M. avium complex	14		4	10	
M. scrofulaceum	1			1	
M. terrae	1	1			
M. fortuitum	7	2	5		
M. chelonei	2		2		
Not mycobacteria	2	2[c]			
Not identified	1				1[d]
Total	81	52	14	13	2

[a] This included two stored strains from survey specimens and thus did not have peak B of fresh strains for identification to species level.
[b] Identified as *M. fortuitum*; see text.
[c] Identified as "not a mycobacterium." Both later proved to be *Nocardia* species.
[d] Could not be clearly identified by biochemical pattern.

hibited no peak B, and thus would not be representative of the typical clinical laboratory experience.

DISCUSSION

Chromatographic methodology. Three other methods of derivatization were evaluated in preliminary experiments. Reaction gas-liquid chromatography was applied to three strains of each of the species described in this study by the method of Ohashi et al. (18). Sample preparation was easy, and chromatograms showed some re-

producible differences, but these qualities were outweighed by the excessive number of peaks from pyrolysis products. The method was difficult to standardize and would not lend itself to interlaboratory reproducibility. An alternative method for preparation of methyl esters, using tetramethylammonium hydroxide hydrolysis followed by CH_3I methylation (7), did not have the sensitivity of the BF_3 method. Chromatograms obtained after derivatization with trifluoroacetylated methyl glycosides to detect hydroxy acids did not exhibit any new peaks useful for identification.

Stationary-phase polarity is known to affect some separations, in particular the separation of fatty acids with different degrees of unsaturation. We therefore evaluated various stationary phases with a range in polarity (8, 14). Silar 10C (McReynolds Index, 736), diethylene glycol succinate, as used by Ohashi et al. (18) (McReynolds index, 709), OV-17 (McReynolds index, 177), and SP-2100 (McReynolds index, 46) all gave satisfactory peak resolution. The choice of OV-17 was made because of its intermediate polarity and high maximum temperature of 350°C.

Unusual strains. Each of the species *M. marinum*, *M. scrofulaceum*, and *M. gastri* from phase 1 (Table 3) had one very unusual strain. This strain was included in its species statistics, although further investigation revealed that it was also atypical biochemically and had been identified on a best-fit basis. The atypical *M. marinum* was nonchromogenic and had an identical biochemical and chromatographic profile to the atypical *M. gastri*. The species designations of these two strains were based on the culture sites from which each was obtained. In no case would the chromatographic profiles of these atypical strains have been mistaken for another species. With these exceptions, the pattern for each species was very consistent.

Of the 79 unknown specimens in phase 2, only one was identified incorrectly. This was a *M. gastri* misidentified as a *M. fortuitum*. The organism was biochemically atypical, however, with a negative urease test, and had been speciated on a best fit basis.

The misidentified *M. marinum* in phase 3 was a survey specimen picked at a young age, and the error was recognized as soon as photochromogenicity was demonstrated. Its lack of production of 2,4-dimethylmyristic acid (2,4-dimethyl $C_{14:0}$) and 2,4-dimethylpentadecanoic acid (2,4-dimethyl $C_{15:0}$) may have been due to the fact that, as with *M. tuberculosis*, storage had altered its lipid pattern. The *M. szulgai* was identified chromatographically 9 days before growth was adequate for biochemical testing. With this presumptive identification to species level, the initially negative nitrate test (11) was prolonged an additional 4 hours to prove its positivity. This prolongation of the nitrate test would not be done normally and vindicates the advice of Marks and associates (15) that identification of this organism should be confirmed by lipid analysis.

Chromatography was a more rapid method of identification because it required less growth and gave a result 1.5 h from the start of analysis. Identification took an average of 2 to 3 days less than biochemical testing for *M. kansasii*, 8 to 10 days less for *M. gordonae*, and 2 to 3 weeks less for *M. tuberculosis*. In one example, a biochemically unusual scotochromogen was chromatographically identified as *M. scrofulaceum* 10 days before additional tests confirmed this answer.

Those isolates differentiated only to a group of two or three species presented little further work because most groups contained some morphologically different members. Identification to species level was often possible by using cultural characteristics, such as growth rate and color, and was always possible with the addition of one selected biochemical test. At the very least, the chromatographic method identified 15 of 16 atypical mycobacteria as belonging to species other than *M. tuberculosis*.

The possibility that *M. tuberculosis* could be chemically differentiated from the atypical mycobacteria has existed since the early 1950s, when it was first demonstrated that saprophytic strains synthesized tetracosanoic acid ($C_{24:0}$) as the longest carbon chain of their fatty acid series, whereas *M. tuberculosis* and *M. bovis* synthesized hexacosanoic acid ($C_{26:0}$) (3). The presumed reason for this lies in the method of mycolic acid synthesis (20). The 88 carbon atoms of the mycolic acids in *M. tuberculosis* and *M. bovis* may be produced by the condensation of two molecules of stearic acid and two molecules of hexacosanoic acid ($C_{26:0}$), whereas the mycolic acids of most saprophytic strains, having 84 carbons, involve condensation of two molecules of stearic acid with two molecules of tetracosanoic acid ($C_{24:0}$).

With few exceptions, the chromatographic lipid patterns we found for various mycobacterial species concur with the findings of other workers using different techniques. Neither Reiner et al. and Reiner and Kubica (26, 27) nor Jenkins et al. (10) could differentiate among *M. avium*, *M. intracellulare*, and *M. scrofulaceum*. The opinion of Jenkins et al. that *M. scrofulaceum* should be considered a pigmented form of

M. avium is also corroborated by the biochemical studies of Tsukamura et al. (37) and Portaels (19). Tsukamura and co-workers also included *M. gastri* as a member, biochemically, of the *M. avium* complex, which was substantiated here in our studies by their related lipid profiles. All members of this group demonstrated the presence of marked amounts of peaks E, G, and H. Adansonian techniques (35) have shown the relatedness of *M. fortuitum* to *M. chelonei*, including *M. chelonei* subsp. *abscessus*. They have been grouped together as a species complex (39). In accord with this, we could not easily differentiate *M. fortuitum* from *M. chelonei*, because only a small subset of our strains had a lipid profile distinctive for *M. fortuitum*.

This study demonstrates the practical application of gas-liquid chromatography for the identification of mycobacteria. The methodology has minimal complexity and gives a result in less than 1.5 h from the start of analysis. We have identified reference compounds that can be used as chromatographic markers for both interlaboratory and intralaboratory standardization. Chromatographic identification is accurate, and only 2 of the 288 strains tested in this study gave atypical chromatographic patterns that led to misidentification. Of these two organisms, one was biochemically atypical and one was a photochromogen in which the error was recognized by gross examination of the colony. The method is easily standardized by comparison of extracted peaks with those of known fatty acid ester standards. Chromatography identified all isolates of *M. gordonae* and *M. kansasii* to species level without error. It could consistently differentiate *M. tuberculosis* from other mycobacteria. In the 15% of cases in which *M. tuberculosis* could not be definitively identified, the only alternatives were *M. bovis* and *M. xenopi*. Both of these organisms are rare causes of infection in the United States.

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