JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1317–1320 Vol. 34, No. 5
0095-1137/96/$04.00+0

Direct Identification of *Mycobacterium* Species in Bactec 7H12B Medium by Gas-Liquid Chromatography

S. CHOU,[1] P. CHEDORE,[1] A. HADDAD,[2] N. R. PAUL,[2] AND S. KASATIYA[1]*

Public Health Laboratory, Ontario Ministry of Health, Ottawa, Ontario, K1G 5A4,[1] and Public Health Laboratory, Ontario Ministry of Health, Etobicoke, Ontario M9P 3T1,[2] Canada

Received 7 November 1995/Returned for modification 13 December 1995/Accepted 10 February 1996

Twenty-nine *Mycobacterium* reference strains representing 10 species and 60 mycobacterial cultures isolated from sputum specimens were studied. These cultures were grown in Bactec 7H12B medium (Becton Dickinson and Co., Paramus, N.J.) supplemented with oleic acid-albumin-dextrose-catalase enrichment broth (Becton Dickinson and Co., Cockeysville, Md.). The cultures were analyzed by gas-liquid chromatography for their fatty acids, secondary alcohols, and mycolic acid cleavage products. All of the clinical isolates could be identified by comparing their gas-liquid chromatography profiles with those of the reference strains. The data indicate that this method significantly shortens the turnaround time and could be used for the early detection and identification of mycobacterial species.

Identification and differentiation of mycobacterial species isolated from clinical specimens have traditionally been based on biochemical reactions. The time required to grow the organism and to perform the biochemical tests is usually extensive; therefore, the rapid and reliable identification of mycobacterial species continues to be an important and challenging task for the clinical mycobacteriology laboratory.

Gas-liquid chromatographic (GLC) analysis of cellular fatty acids, secondary alcohols, and mycolic acid cleavage products (MACP) has been used for the identification of mycobacterium isolates (4, 6–11, 13, 14). However, mycobacteria grow very slowly on conventional media and several weeks are required to obtain sufficient growth for identification. The use of Bactec medium (Becton Dickinson and Co., Paramus, N.J.) supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment broth (Becton Dickinson and Co., Cockeysville, Md.) has been shown to enhance the growth and biomass of mycobacterial species in cultures (12). The aim of this study was to use GLC for the early identification of mycobacterial species grown in Bactec medium supplemented with OADC. The fatty acid, alcohol, and MACP profiles of reference mycobacterial strains as well as those isolated from clinical specimens of patients with respiratory tract infections were studied.

(This study was presented in part at the 95th General Meeting of the American Society for Microbiology, 1995, Washington, D.C.)

Twenty-nine American Type Culture Collection reference strains representing 10 different mycobacterial species (*Mycobacterium tuberculosis*, five strains; *M. simiae*, three strains; *M. xenopi*, two strains; *M. malmoense*, two strains; *M. avium-M intracellulare* complex (MAIC), three strains; *M. flavescens*, three strains; *M. gordonae*, three strains; *M. szulgai*, three strains; *M. kansasii*, three strains; and *M. marinum*, two strains) were inoculated into Bactec medium supplemented with 0.5 ml of OADC enrichment broth and incubated at 37°C for 6 days.

Isolation of 60 mycobacterial strains (*M. tuberculosis*, 21 strains; *M. simiae*, 3 strains; *M. xenopi*, 2 strains; *M. malmoense*, 4 strains; MAIC, 25 strains; *M. gordonae*, 4 strains; and *M. szulgai*, 1 strain) from lower respiratory tract specimens of patients suspected of having tuberculosis was done as described by Cage (2). In summary, 0.5 ml of homogenized and NaOH-treated concentrate was inoculated into Bactec 7H12B medium and incubated at 37°C until the growth index reached >100, at which time 0.2 ml of culture was transferred to fresh Bactec 7H12B medium supplemented with 0.5 ml of OADC enrichment broth and reincubated at 37°C for 6 days.

After 6 days of incubation, all the contents of the Bactec vial were finally transferred to screw-cap centrifuge tubes (12 by 100 mm) and centrifuged at 3,900 × *g* for 40 min. The pellet was washed twice with phosphate buffer and centrifuged as previously described.

The derivatization of mycobacterial cellular fatty acids, alcohols, and MACP was performed as described by Luquin et al. (9). In brief, the washed bacterial pellet was transferred to a Teflon-lined screw-cap tube and mixed with 1 ml of a mixture of methanol, toluene, and H_2SO_4. After vortexing, the mixture was heated at 80°C for 16 h and extracted twice with 2 ml of hexane. The hexane extracts were mixed with an equal amount of pH 11 phosphate buffer. The hexane layer was transferred to a V-vial and evaporated to 0.1 ml under a gentle stream of nitrogen gas. The final sample was analyzed by GLC or stored at −20°C.

The fatty acids, alcohols, and MACP were analyzed with a model 3700 gas-liquid chromatograph (Varian, Sunnyvale, Calif.) equipped with a flame ionization detector. The identification of the eluted substances was accomplished by comparing the retention time with those of known standards on both a nonpolar capillary DB-5 column and a polar capillary DB-225 column (3) (J & W Scientific Inc., Folsom, Calif.). The columns were 30 m long with an inner diameter of 0.25 mm and a film thickness of 0.25 μm. The trifluoroacetylation method was used to identify the secondary alcohols and unsaturated fatty acids. For sample analysis, the temperature for both the injector and the detector was maintained at 300°C. Helium was used as the carrier gas. The initial column temperature was 100°C and was increased to 300°C at the rate of 12°C/min. The chromatograms were integrated by a Varian 4400 integrator for identification and quantitation of each eluted substance. The fatty acid and alcohol standards were supplied by Supelco Inc. (Bellefonte, Pa.), Applied Science Laboratory (Rockwood, Ontario, Canada), Regis Chemical

* Corresponding author. Mailing address: 2380 St. Laurent Blvd., Ottawa, Ontario K1G 5A4, Canada. Electronic mail address: kasatidr@epo.gov.on.ca.

TABLE 1. Characteristic fatty acids, alcohols, and MACP of *Mycobacterium* species in this study

Bacterium	No. of reference strains	No. of specimens	Amt of compound (%)[a]											
			14:0	2-M-14:0	2,4-DM-14:0	2,4-DM-16:0	2-OH-18:0	TBSA	19:1	2-OH-20:0	2-M-20:0	2-OH-22:0	2,4,6-TM-22:0	24:0	2,4,6-TM-24:0	26:0
M. tuberculosis	5	21	0.8 (0.4–1.3)					9.5 (6.5–15.5)						1.7 (0.5–2.7)		7.2 (3.2–9.4)
M. simiae	3	3	4.8 (4.2–5.5)					12.5 (12.5–14.6)						1.5 (1.2–2.1)		6.2 (3.4–6.5)
M. xenopi	2	2	2.8 (2.1–3.5)					17.2 (15.4–19.0)		4.4 (4.2–4.6)		9.4 (8.8–10.0)		0.6 (0.5–0.7)		3.2 (2.8–3.4)
M. malmoense	2	4	1.8 (1.6–2.0)					10.4			2.8 (2.5–3.2)			1.1 (0.8–1.4)	3.2 (2.7–3.5)	5.2 (4.4–6.3)
MAIC	3	25					1.2 (0.9–1.4)	11.5 (7.5–12.5)		9.4 (8.5–13.4)				3.2 (2.7–3.6)		0.4 (0.2–0.6)
M. gordonae	3	2		3.4 (2.8–4.2)				1.3 (1.1–1.5)					3.5 (2.3–4.7)	2.4 (2.0–2.8)		0.4 (0.2–0.6)
M. szulgai	3	1		1.3 (1.1–1.5)				5.1 (3.3–6.9)						5.1 (4.2–6.0)		0.5 (0.2–0.8)
M. flavescens	3							6.7 (6.0–7.3)	3.1 (2.7–3.5)					3.3 (2.3–4.2)		0.4 (0.2–0.6)
M. kansasii	3				4.0 (3.5–4.5)			9.4 (7.8–11)						4.6 (3.7–5.5)		0.8 (0.8–1.2)
M. marinum	2					3.0 (2.5–3.5)		3.3 (3.3–3.3)						4.9 (3.8–6.0)		1.3 (1.2–1.4)

[a] See text for explanation of compound designations. Values are percentages of total amounts, given for each strain as the mean value and range.

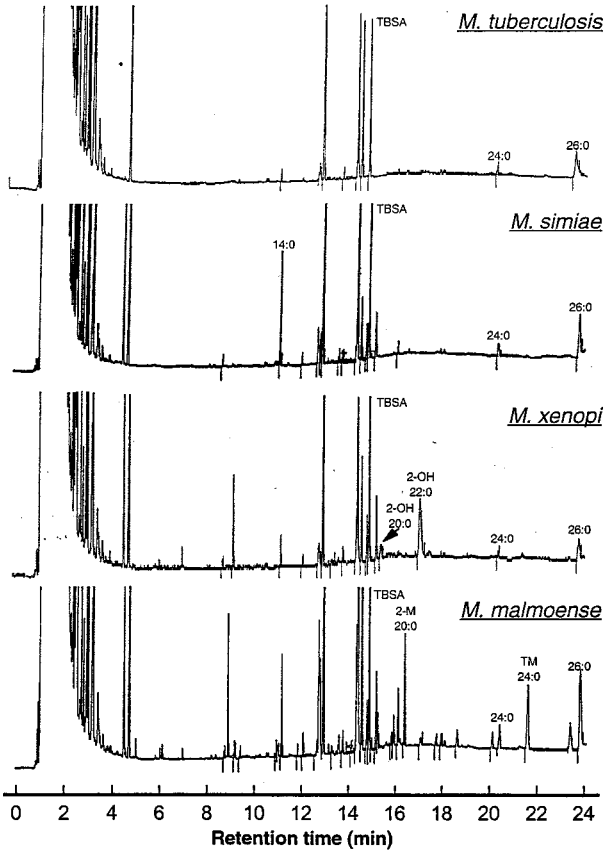

FIG. 1. Gas chromatograms of *Mycobacterium* species in group 1. See text for fatty acid and alcohol designations.

Co. (Morton Grove, Ill.), and Sigma Chemical Co. (St. Louis, Mo.). The standards for branched-chain fatty acids 2-M-14:0 (2-methyltetradecanoic acid), 2,4-DM-14:0 (2,4-dimethyltetradecanoic acid), 2,4-DM-16:0 (2,4-dimethylhexadecanoic acid) 2-M-20:0 (2-methyleicosenoic acid), 2,4,6-TM-22:0 (2,4,6-trimethyldocosanoic acid), and 2,4,6-TM-24:0 (2,4,6-trimethyltetracosanoic acid) were not available. Their identities were determined by comparison of their retention times with the profiles of previously analyzed known mycobacterial extracts (6, 9, 11) and the profiles of our reference strains.

The study showed that the chromatographic profiles of the mycobacterial species from the clinical specimens were identical to those of their reference type strains. In general, the major cellular fatty acids in all studied mycobacterial species were 16:0 (hexadecanoic acid), 18:1 (octadecenoic acid), tuberculostearic acid or 10-methyloctadecanoic acid (TBSA) and 16:1 (hexadecenoic acid). This is generally in agreement with those reported by Luquin et al. (9), Tisdall et al. (13), and Valers-Guillén et al. (14). Table 1 shows the percentages of distinguishing components in the examined species. These distinguishing components included MACP 24:0 (tetracosanoic acid) and 26:0 (hexacosanoic acid) and secondary alcohols 2-OH-18:0 (2-octadecanol), 2-OH-20:0 (2-eicosanol), and 2-OH-22:0 (2-docosanol), as well as branched-chain fatty acids 2-M-14:0, 2,4-DM-14:0, 2,4-DM-16:0, 2-M-20:0, 2,4,6-TM-22:0, and 2,4,6-TM-24:0).

The ratio of the percentage of 24:0 to that of 26:0 was used to separate all the studied mycobacterial species into two groups. For the species in the first group, which included *M.*

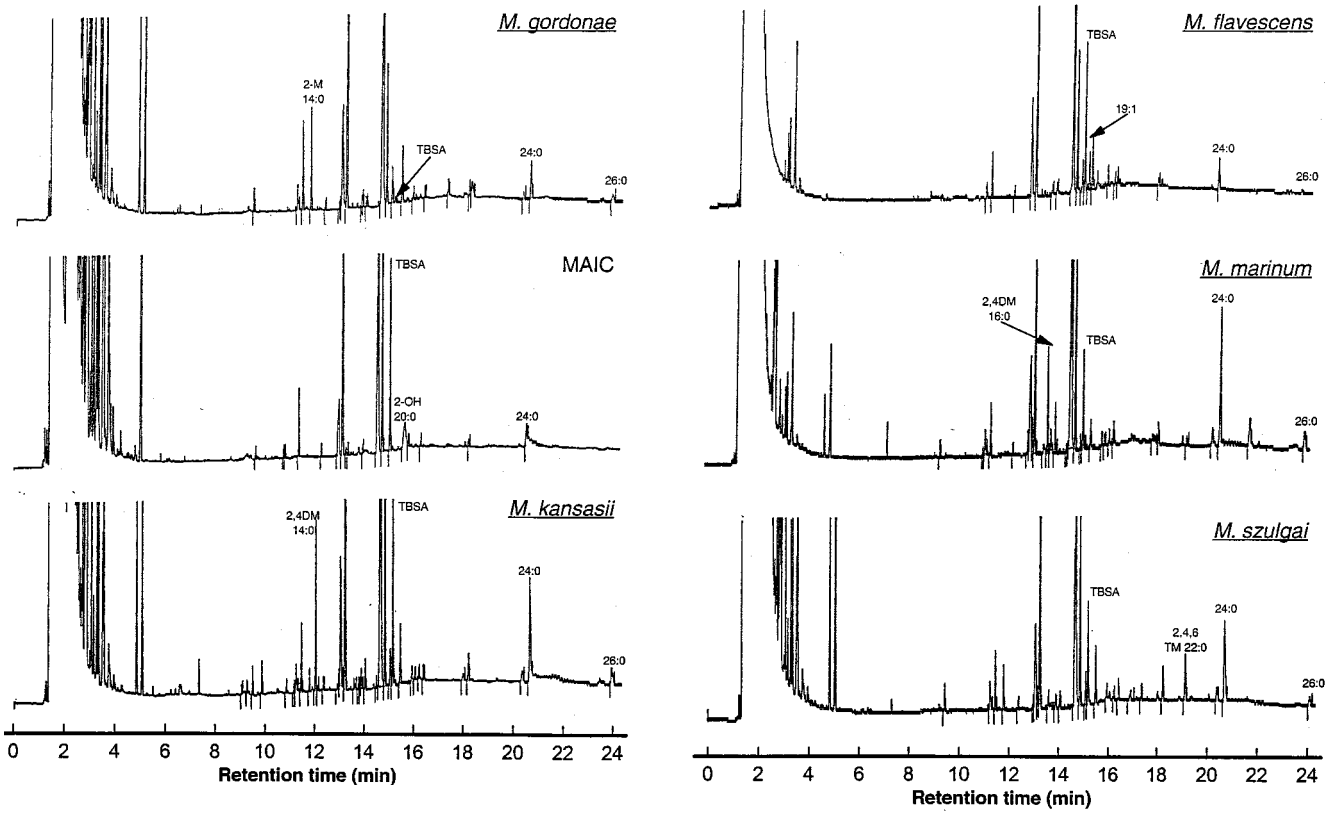

FIG. 2. Gas chromatograms of *Mycobacterium* species in group 2. See text for fatty acid and alcohol designations.

tuberculosis, *M. simiae*, *M. xenopi*, and *M. malmoense*, this ratio was <1. *M. simiae* contained a relatively high content of C14:0 (3 to 5%) (Fig. 1). This could be used to differentiate *M. simiae* from *M. tuberculosis*. *M. xenopi* contained 6 to 7% 2-OH-22:0, and *M. malmoense* contained 2.5 to 3.5% 2-M-20:0 and 2,4,6-TM-24:0 as specific markers. The mycobacterial species MAIC, *M. gordonae*, *M. szulgai*, *M. flavescens*, *M. kansasii*, and *M. marinum* were placed in the second group (Fig. 2), on the

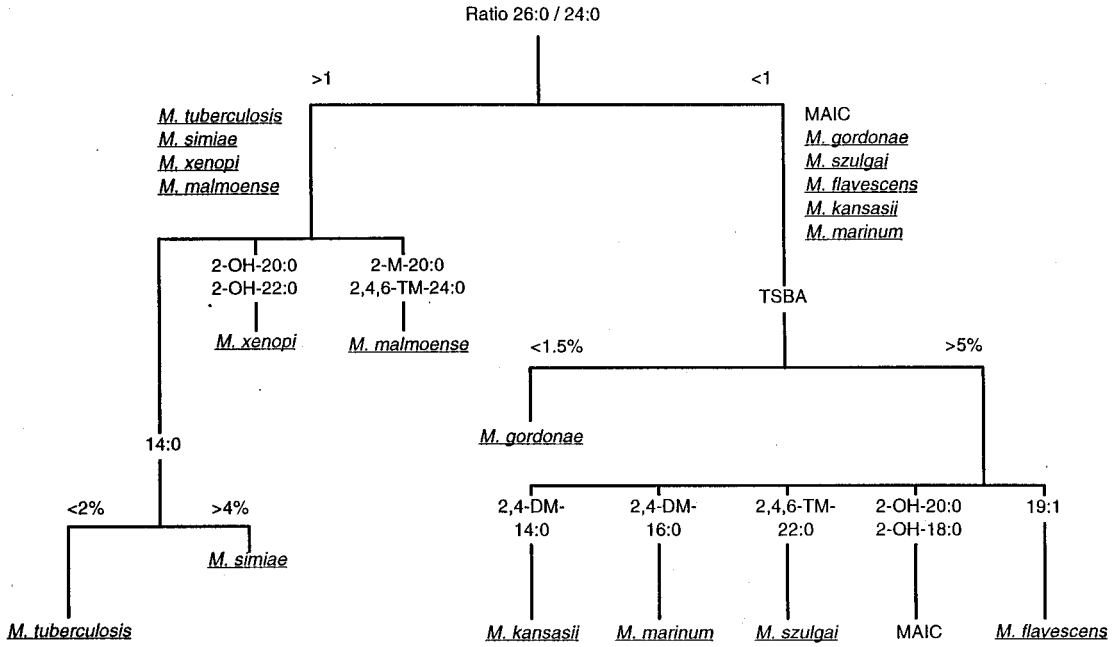

FIG. 3. Dichotomic tree for identification of mycobacteria on the basis of GLC profile. See text for fatty acid and alcohol designations.

basis of the ratio of the percentage of 24:0 to that of 26:0, which for these species is >1. *M. gordonae* was the only species in this group that contained <2% TBSA, and thus it could be easily differentiated from other species, as reported earlier (6, 10, 13). Although 4% 2-M-14:0 was detected from *M. gordonae* cultures, minor amounts, 1.6 and 0.8%, were also detected from *M. szulgai* and *M. kansasii*, respectively. Therefore, this acid could not be used as a discriminating factor in our culture conditions. The presence of branched-chain fatty acids 2,4-DM-14:0, 2,4-DM-16:0, and 2,4,6-TM-22:0 could be used to identify *M. kansasii*, *M. marinum*, and *M. szulgai*, respectively. MAIC was characterized by the presence of a significant amount of 2-OH-20:0 (9 to 13%) and a trace amount of 2-OH-18:0 (0.5 to 1.5%). *M. flavescens* contained 3% nonadecenoic acid (19:1) and could be easily differentiated from other species in this group. The retention time of 19:1 overlapped with that of 2-OH-20:0 but was not modified after trifluoroacetylation as reported earlier (11). Fig. 3 represents the data in a dichotomic-tree format.

Since the differentiation of mycobacterial species is based on the contents of the specific cellular constituents, the purity of the culture plays a critical role. The occurrence of contamination of a Bactec culture is often indicated by turbidity or a sudden increase in growth index values. Contamination can be further confirmed by a stained smear or by subculture onto a blood agar plate. A reliable chromatogram from a pure mycobacterial culture would be characterized by a relatively high (>5%) TBSA content, except for *M. gordonae*, and by the presence of 26:0 and 24:0 fatty acids with no unexpected peaks in high percentages. In mycobacterium-negative or contaminated cultures, the TBSA content would be variable, with unnoticeable 26:0 and 24:0 fatty acids. This also occurs when the culture is not supplemented with OADC.

In order to prevent contamination, the initial decontamination procedure as well as the addition of the antimicrobial agent PANTA to each subculture is essential. In the present study, mycobacterial species achieved a growth index of 100 or greater in approximately 12 days in initial Bactec medium followed by an additional 6 days in OADC-supplemented medium before identification by GLC. These isolates required an average of 18 days from the time of initial specimen processing to the time of GLC identification, compared with 43 days by conventional methods (5). Bactec cultures supplemented with OADC enrichment broth have been used to identify mycobacterial species by high-performance liquid chromatography (2). In this study, we used capillary GLC in conjunction with flame ionization detection, which provided greater resolution and sensitivity in detecting fatty acids, secondary alcohols, and MACP.

In summary, this report describes a rapid, sensitive, and inexpensive method for the isolation and identification of mycobacterial species from clinical sputa.

We thank A. Laszlo and the staff of the Laboratory Centre for Disease Control, Ottawa, Canada, for provision of ATCC mycobacterium reference strains. We also thank M. Antoine for clerical assistance.

REFERENCES

1. **Butler, W. R., G. Warren, G. P. Kubica, and J. O. Kilburn.** 1990. Modified method for testing the quality of albumin-containing enrichments used in growth media for mycobacteria. J. Clin. Microbiol. **28:**1068–1070.
2. **Cage, G. D.** 1994. Direct identification of *Mycobacterium* species in Bactec 7H12B medium by high-performance liquid chromatography. J. Clin. Microbiol. **32:**521–524.
3. **Chou, S., E. Aldova, and S. Kasatiya.** 1991. Cellular fatty acid composition of *Plesiomonas shigelloides*. J. Clin. Microbiol. **29:**1072–1074.
4. **Guerrant, G. O., M. A. Lambert, and C. W. Moss.** 1981. Gas chromatographic analysis of mycolic acid cleavage products in mycobacteria. J. Clin. Microbiol. **13:**899–907.
5. **Huebner, R. E., R. C. Good, and J. I. Tokars.** 1993. Current practices in mycobacteriology: results of a survey of state public health laboratories. J. Clin. Microbiol. **31:**771–775.
6. **Jantzen, E., T. Tangen, and T. Eng.** 1989. Gas chromatography of mycobacterial fatty acids and alcohols: diagnostic applications. Acta Pathol. Microbiol. Immunol. Scand. **97:**1037–1045.
7. **Juliak, J., F. Turecek, and Z. Mikova.** 1980. Identification of characteristic branched-chain fatty acid of *Mycobacterium kansasii* and *Mycobacterium gordonae* by gas-chromatography-mass spectrometry. J. Chromatogr. **190:** 183–187.
8. **Lambert, M. A., C. W. Moss, V. A. Silcox, and R. C. Good.** 1986. Analysis of mycolic acid cleavage products and cellular fatty acids of *Mycobacterium* species by capillary gas chromatography. J. Clin. Microbiol. **23:**731–736.
9. **Luquin, M., V. Ausina, F. López Calahorra, F. Belda, M. García Barceló, C. Celma, and G. Prats.** 1991. Evaluation of practical chromatographic procedures for identification of clinical isolates of mycobacteria. J. Clin. Microbiol. **29:**120–130.
10. **Minnikin, D. E., G. Dobson, M. Goodfellow, P. Draper, and M. Magnusson.** 1985. Quantitative comparison of the mycolic and fatty acid composition of *Mycobacterium leprae* and *Mycobacterium gordonae*. J. Gen. Microbiol. **131:** 2013–2021.
11. **Parez, J. J., M. Fauville-Dufaux, J. L. Dossogne, E. de Hoffmann, and F. Pouthier.** 1994. Faster identification of mycobacteria using gas-liquid and thin layer chromatography. Eur. J. Clin. Microbiol. Infect. Dis. **13:**717–725.
12. **Siddiqui, S. H.** 1989. Bactec TB system: product and procedure manual. Becton Diagnostic Instrument System, Towson, Md.
13. **Tisdall, P. A., G. D. Roberts, and J. P. Anhalt.** 1979. Identification of clinical isolates of mycobacteria with gas-liquid chromatography alone. J. Clin. Microbiol. **10:**506–514.
14. **Valers-Guillén, P. L., F. Pacheco, and F. Martin-Luengo.** 1985. Fatty acid composition and mycolic acid patterns of some chromogenic mycobacteria. J. Appl. Microbiol. **59:**113–126.