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Short Communication

Gas chromatographic whole-cell fatty acid analysis as an aid for the identification of mixed mycobacterial cultures

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ABSTRACT

Gas chromatographic analysis of whole-cell fatty acids, secondary alcohols and mycolic acid cleavage products could be a useful technique in checking mixed mycobacterial cultures. The mixed cultures were confirmed when species-specific compounds of different mycobacterial species were detected in the same chromatogram.

INTRODUCTION

Identification of *Mycobacterium* species by conventional methods requires considerable time and enterprise. The development of rapid and simplified identification techniques has long been desired. Implementation of radiometric and probe technologies and analysis of lipid composition by various chromatographic methods have coincided with the marked increase in the incidence of disease due to *Mycobacterium tuberculosis* and *Mycobacterium avium* complex associated with the epidemic of acquired immunodeficiency syndrome (AIDS) [1]. Clinical samples sometimes yield more than one mycobacterial species, and in recent years mixed mycobacterial infections have been more frequently detected in AIDS patients [2].

Analysis of lipid composition by various chromatographic methods is recognized as a useful tool for differentiating mycobacterial species [3–12]. Analysis of the cellular fatty acids by gas chromatography (GC) appears to be a particularly suitable method for the identification of the mycobacteria, because it is capable of distinguishing between closely related species [6,8,11,12]. GC has been used to detect secondary alcohols, 2-octadecanol (2-OH-$C_{18:0}$) and 2-eicosanol (2-OH-$C_{20:0}$), stemming from wax-ester mycolates [5,6], and the $C_{22:0}$, $C_{24:0}$, $C_{26:0}$ fatty acids formed as methyl esters *in situ* upon pyrolysis of the complex branched-chain mycolic acid structures in the heated injector of the gas chromatograph [4,5].

As described in this study, gas chromatographic analysis of whole-cell fatty acids, secondary alcohols and mycolic acid cleavage products could also be a useful technique in checking some mixed mycobacterial cultures. We report the de-

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tection of three mixed mycobacterial cultures from respiratory samples from AIDS patients using the GC technique.

EXPERIMENTAL

Chemicals

Deionized, glass-distilled water was used for the preparation of the buffer solutions. Methanol, toluene and *n*-hexane were analytical reagent grade and purchased from Merck (Darmstadt, Germany). Methyl ester and alcohol standards were purchased from Supelco (Bellefonte, PA, USA) and Sigma (St. Louis, MO, USA).

Sample processing

Sputum and other respiratory specimens were homogenized and decontaminated with 0.5 g of N-acetyl-L-cysteine (Sigma) in 100 ml of 2% NaOH for 15 min at room temperature, neutralized with 0.067 *M* phosphate buffer, pH 6.8, and centrifuged at 2000 *g* for 20 min. The sediment was suspended in 2 ml of sterile 0.2% bovine serum albumin used for the inoculation of the media [13]. A 0.4-ml volume of the processed sediments was inoculated into Löwenstein-Jensen (L–J) slants and Middlebrook 7H12 radiometric broth vials (Bactec, Johnston Laboratories, Towson, MD, USA) with 0.1 ml of an antibiotic mixture containing polymyxin B (50 U/ml), amphotericin B (5 μg/ml), nalidixic acid (20 μg/ml), trimethoprim (5 μg/ml) and azlocillin (10 μg/ml) (Panta, Johnston Laboratories). Slants were incubated at 35°C and were examined for growth at weekly intervals for eight weeks. The Bactec vials were initially tested on the Bactec 460 instrument (Johnston Laboratories) to establish a 5–10% CO_2 atmosphere and incubated at 35°C. The Bactec vials were tested with the Bactec 460 twice a week for two weeks and weekly thereafter for the next six weeks. When a growth index (GI) of $\geqslant 100$ was detected, an aliquot of the broth was stained, and when acid-fast bacilli were present 0.1 ml was inoculated onto 7H11 agar plates. The L–J slants from bronchoalveolar lavage (sample 1) showed numerous colonies of slowly growing non-chromogenic mycobacteria. In the L–J slants from a sputum specimen (sample 2) there appeared to be more than 200 colony-forming units (c.f.u.) of a slowly growing photocromogenic mycobacteria. The L–J slants of another sputum specimen (sample 3) also showed confluent grow of slowly growing photocromogenic mycobacteria.

We performed the lipid extraction as follows. One spadeful of mycobacteria was scraped from the surfaces of the L–J slants. The mycobacterial lipids were extracted and derivatized to methyl esters by a modification of the method of Minnikin *et al.* [9]. The cells were mixed with 1 ml of a reagent composed of 30 ml of methanol, 15 ml of toluene and 1 ml of concentrated H_2SO_4 in a screw-cap test tube fitted with a PTFE-lined cap. The mixture was heated in a covered bath at 80°C for 16 h (overnight). After being cooled at room temperature, the samples were extracted twice with 2 ml of *n*-hexane. The hexane extracts were combined, transferred to another test tube and mixed with an equal volume of 0.3 *M* phosphate buffer pH 12 [8]. The hexane upper layer was then removed, placed in a clean tube and evaporated to dryness in a water bath at 40°C in a stream of nitrogen. The residue was dissolved in *n*-hexane.

Chromatography

Mycolic acid cleavage products (MACPs), secondary alcohols and fatty acids were analysed on a fused-silica capillary column (15 m × 0.25 mm I.D.) with cross-linked methyl silicone (film thickness 1 μm) (SPB-1; Supelco) as the stationary phase, inserted in a Hewlett-Packard 5890 A chromatograph equipped with a flame ionization detector. The temperature of the column was programmed to increase from 175 to 300°C at 8°C/min and was maintained at 300°C for 15 min. The injector and detector temperatures were 275 and 315°C, respectively. The carrier gas was helium at a flow-rate approximately 1 ml/min at 175°C; the sample size was 1 μl, with a split ratio of approximately 1:50. The chromatograms were integrated by using a Hewlett-Packard 3390A integrator.

RESULTS AND DISCUSSION

The gas chromatogram of sample 1 (Fig. 1A)

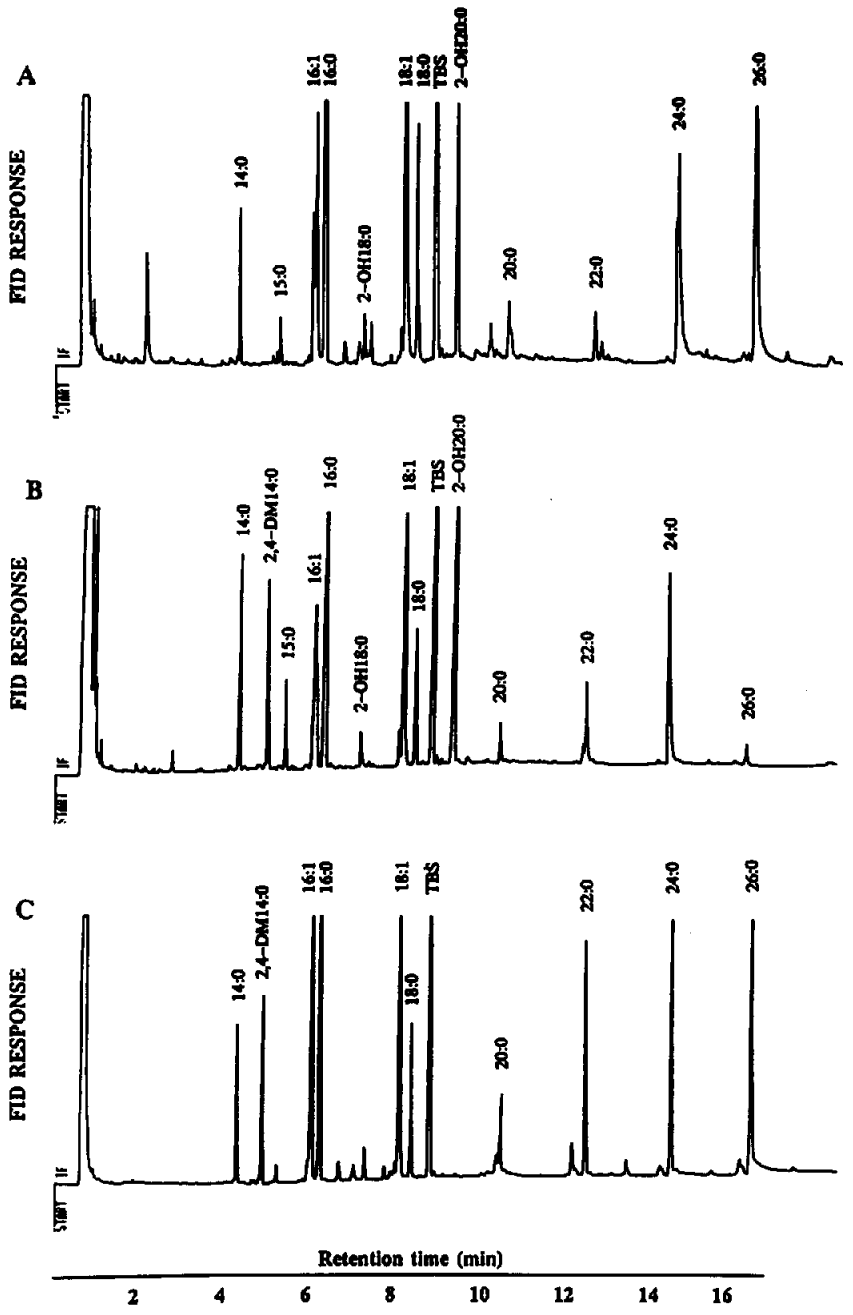

Fig. 1. Gas chromatograms of fatty acid methyl esters and MACPs from mixed mycobacterial cultures of sample 1 (A), sample 2 (B) and sample 3 (C). The paired numbers indicate the number of carbon atoms followed by the number of double bounds. 2-M and 2,4-DM indicate methyl groups at the 2 and at the 2 and 4 positions, respectively. 2-OH indicates a secondary alcohol. TBSA, tuberculostearic acid (10-methyloctadecanoic acid).

presented as interesting peaks: hexacosanoate ($C_{26:0}$), as a major mycolic acid cleavage product, which is characteristic of *M. tuberculosis* complex, *M. simiae*, *M. malmoense* and *M. xenopi* [4,8]; the secondary alcohols, 2-octadecanol (2-OH-$C_{18:0}$) and 2-eicosanol (2-OH-$C_{20:0}$), which are not found in the species mentioned above with the exception of 2-OH-$C_{20:0}$ in *M. xenopi*.

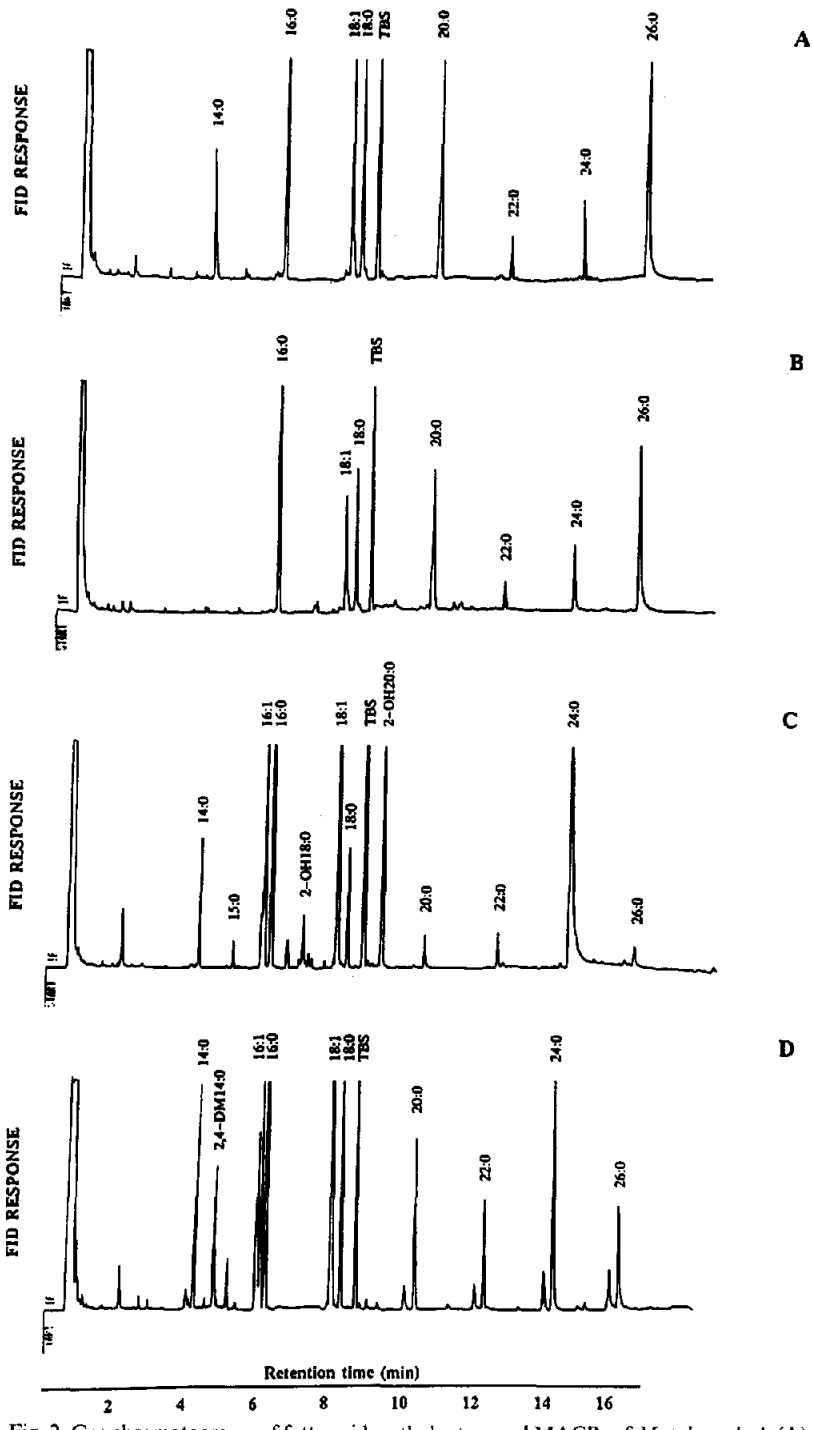

Fig. 2. Gas chromatograms of fatty acid methyl esters and MACPs of *M. tuberculosis* (A), *M. simiae* (B), *M. avium* (C) and *M. kansasii* (D). See caption to Fig. 1 for an explanation of acid designations.

These alcohols are regularly detected in gas chromatograms of *M. scrofulaceum*, *M. terrae*, *M. avium* complex and *M. flavescens* [6,8]. Specific compounds of *M. malmoense* (2-M-$C_{20:0}$, 2-

methyleicosanoate, and 2,4,6-TM-$C_{24:0}$, 2,4,6-trimethyltetracosanoate) and *M. xenopi* (2-OH-$C_{22:0}$, 2-docosanol) were not detected [14,15].

The gas chromatogram of sample 2 (Fig. 1B) presented as interesting peaks: tetracosanoate ($C_{24:0}$) as a major mycolic acid cleavage product; 2,4-dimethyltetradecanoate (2,4-DM-$C_{14:0}$), which is a specific compound of *M. kansasii* [8]. Secondary alcohols, 2-octadecanol and 2-eicosanol, were also detected. These alcohols are not found in *M. kansasii* [8].

The gas chromatogram of sample 3 (Fig. 1C) presented as interesting peaks: 2,4-dimethyltetradecanoate and hexacosanoate, as a mycolic acid cleavage product, in unusual amounts for *M. kansasii*. Secondary alcohols were not detected. These lipid analyses showed a mixed mycobacterial fatty acid pattern. 7H11 agar plates from L–J slants were inoculated in order to check the colony's morphology. Like the plates from the Bactec broth bottles, they showed mixed mycobacterial cultures. Further isolation and identification by conventional [13] and gas chromatographic procedures [8] (Fig. 2) revealed in each case two different mycobacterial species. We isolated from sample 1, *M. tuberculosis* and *M. avium* complex; from sample 2, *M. kansasii* and *M. avium* complex; and from sample 3, *M. kansasii* and *M. simiae*. Sometimes is not easy to differentiate mixed cultures even from 7H11 agar plates, when, as in the third case, there are two photochromogenic species.

In recent years, several methods have been described as an aid for the rapid detection and identification of *Mycobacterium* spp. The introduction of radiometric techniques, computer-assisted gas chromatography and nucleic acid probes has greatly reduced this identification time. Several specific DNA probes have been developed for the identification of the *M. tuberculosis* group, *M. avium*, *M. intracellulare*, *M. gordonae* and *M. kansasii* [16–19]. However, these DNA probes have been designed only to identify certain species. For the remaining pathogenic species, such probes are not available. In fact, many laboratories in large hospitals combine radiometric detection and DNA probe analysis technologies for the rapid detection and identification of mycobacteria [20–22], missing in the process possible mixed infections or mixed cultures. The present study shows that through the detection of species-specific lipidic compounds, gas chromatography permits the identification of some mixed mycobacterial cultures.

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