JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1993, p. 610–614
0095-1137/93/030610-05$02.00/0

Vol. 31, No. 3

Differentiation of Mycobacteria on the Basis of Chemotype Profiles by Using Matrix Solid-Phase Dispersion and Thin-Layer Chromatography

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Received 17 September 1992/Accepted 9 December 1992

Because of the rising incidence of clinical mycobacterial infections and the difficulty in identification and characterization of mycobacteria at the subspecies and serovar levels, a technique for differentiation that could be performed quickly and with relatively little equipment and expense was developed. Lysis and fractionation of mycobacteria by matrix solid-phase dispersion followed by thin-layer chromatography were used to produce chemotype profiles of the lipid and glycolipid components of each isolate. Organisms tested included *Mycobacterium scrofulaceum*, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium flavescens*, *Mycobacterium kansasii*, *Mycobacterium bovis*, 11 isolates of *Mycobacterium gordonae*, 10 serovars of *Mycobacterium avium*, and four strains of *Mycobacterium paratuberculosis*. A relative retention (R_x) value was established for each visible band on the chromatographs by comparison with a band common to all organisms tested. The chemotype profiles produced were different for every separate species and serovar sampled. These findings suggest that matrix solid-phase dispersion and subsequent thin-layer chromatography may have the sensitivity and flexibility to characterize and identify mycobacteria at the species and subspecies levels and potentially to identify clinical isolates of mycobacteria that have been difficult to identify by standard means. The technique has certain advantages over current biochemical, immunologic, and genetic methods because it is relatively simple to perform, is inexpensive, and requires a small amount of bacterial sample.

Mycobacterial organisms are considered one of the more common causes of disseminated bacterial infections in AIDS patients and are increasing in the frequency of isolation (18). These include organisms of the *Mycobacterium avium* complex (*M. avium*, *M. intracellulare*, and *M. scrofulaceum*), *Mycobacterium gordonae*, and other mycobacterial species. Because many mycobacteria are difficult to identify quickly or with precision by standard criteria, especially at the subspecies level, presumptive identification of mycobacterial species has historically relied upon biochemical test profiles (3, 10). Schaefer developed the seroagglutination test, which was successful in distinguishing 31 serovars in the *M. avium* complex (16). However, because of cross-reactivity between antigens and autoagglutination, the results were sometimes confusing (10, 16). A number of techniques have since been developed for use in the characterization and identification of mycobacterial species, including thin-layer chromatography (TLC), gas chromatography, high-performance liquid chromatography (HPLC), large restriction fragment analysis of DNA, gene amplification and analysis by the polymerase chain reaction, and enzyme-linked immunosorbent assay (ELISA) testing (1–8, 10, 12–15, 18, 19). The majority of these techniques are based on the uniqueness of cell wall-bound glycolipid antigens characterized as glycopeptidolipids, lipooligosaccharides, or phenolic glycolipids (5, 11, 14). Particular phenolic glycolipids are specific for certain mycobacterial species, such as *Mycobacterium bovis*, *Mycobacterium leprae*, and others, and these species can be distinguished by biochemical methods. In addition, serovar-specific alterations in other glycolipid side chains of *M. avium*-complex organisms result in distinct mobility patterns by TLC and often a characteristic spectrum of sugars in gas chromatograms (5). The term "chemotype" has been given to strain- and serovar-specific differences in lipid and glycolipid profiles between mycobacterial organisms, when they are characterized by TLC and other biochemical methods (13). Although techniques are improving, HPLC, gas chromatography, polymerase chain reaction, and ELISA all continue to have inherent weaknesses in differentiating certain species and strains within a given species (5, 6, 10, 12, 13). Combinations of these procedures have solved many of the problems, but the expense and limited number of laboratories able to do several techniques with one sample have limited their clinical applicability.

TLC is a relatively inexpensive technique which has been used to differentiate mycobacterial species (6, 10, 12, 19). Limitations of standard TLC include its inability to clearly distinguish between serovars which have type-specific glycopeptidolipids with identical TLC mobilities because of similar molecular weights or polarities (6). This has resulted in overlapping and smearing of bands and, therefore, the inability to distinguish between many serovars in the *M. avium* complex and between other related species (6). In addition, extracting alkali-stable whole lipids from clinical isolates, in order to perform TLC for mycobacterial components, has historically been a tedious, time-consuming process (10). The great variability between TLC plates from different laboratories has made interpreting R_f values difficult, and the lack of standardization of the technique has further detracted from the procedure's practicality. For these reasons, TLC has rarely been utilized as the sole means for mycobacterial characterization.

Matrix solid-phase dispersion (MSPD) has been recently demonstrated to be an effective technique for lysis and

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partial fractionation of mycobacterial components and a quick, relatively inexpensive method for obtaining samples of various mycobacterial lipids and glycolipids (9). In this study, the MSPD technique was combined with TLC to establish a procedure for identifying mycobacterial chemotypes that could be performed easily and cost effectively in clinical or research laboratories. The two-dimensional nature of the MSPD-TLC technique provided a means of overcoming the problem of overlapping bands that is associated with TLC alone. In addition, the time-consuming step of lipid extraction that is necessary with TLC of mycobacteria was circumvented with the combined MSPD-TLC technique, and comparison of relative retention (R_x) values provided a means of standardizing the technique between laboratories.

MATERIALS AND METHODS

Sources of mycobacterial strains. *M. avium* serovars 2, 5, 7, 10, 12, and 13 from an authenticated collection (19), *Mycobacterium paratuberculosis* Holland-1 and -2, *M. paratuberculosis* BB410 (from a UCLA isolate), and *M. paratuberculosis* Linda were generously provided by R. Chiodini (Mycobacteriology Unit, Department of Medicine, Rhode Island Hospital, Brown University, Providence, R.I.). *M. avium* serovars 1 (strain 4002), 2 (4511), 4 (22), 8 (138), and 10 (2199), *Mycobacterium phlei* (3198), *Mycobacterium flavenscens* (33001 Tsukamura strain), *M. scrofulaceum* (W262), *Mycobacterium kansasii*, *Mycobacterium smegmatis* (NVSL strain), and four strains of *M. bovis* were provided by J. Jarnagin (U.S. Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, Ames, Iowa). Clinical isolates of *M. gordonae* were provided by T. Cleary (Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Fla.).

Growth and harvesting of mycobacteria. Isolates were grown on Middlebrook 7H10 agar plates containing 100 ml of Middlebrook oleic acid-albumin-dextrose-catalase enrichment medium per ml at a pH of 7.9 for 21 days at 37°C. Samples of *M. paratuberculosis* required 10 to 12 weeks in culture under similar parameters. For *M. avium* and *M. paratuberculosis* cultures, 2 mg of mycobactin J (Allied Monitor, Fayette, Mo.) per liter was also added.

MSPD and TLC. MSPD was performed as previously described (9), with the following modifications. A sample consisting of 60 mg of mycobacteria was scraped from the plates and blended with 400 mg of C_{18} reverse-phase HPLC packing resin (Bakerbond, catalog no. 702500; J. T. Baker Co.) in a 2-oz. (ca. 60-g), clear-glass mortar and pestle. The blended material was transferred to a 10-ml syringe column where it was eluted with 3 ml each of five solvents (hexane, methylene chloride, acetonitrile, methanol, and ultrapure water) in sequential order. An additional 7 ml of 100% methanol was mixed with the water-derived fraction to enhance evaporation. All five fractions from each bacterial sample were then evaporated to dryness under nitrogen gas at 40°C. Once dried, samples were resolubilized in 60 μl of chloroform-methanol-water (5:4:1) and vortexed briefly. TLC was performed by using prescribed methods, with the following modifications (17). Five-microliter portions of each of the five MSPD fractions from each organism were spotted on separate lanes of a silica gel TLC plate (Baker S125-PA-19C, catalog no. 7009-04). This was repeated three times for a total of 15 μl of each fraction per lane. The plates were allowed to dry at room temperature and then were developed in a TLC chamber containing 100 ml of mobile phase consisting of chloroform-methanol-water (5:4:1). Plates were removed when the mobile phase has risen approximately 10 cm. After they were dried in a chemical-fume hood, the plates were sprayed with 10% H_2SO_4, air-dried, heated at 110 to 120°C for 15 to 20 min, and photographed.

The R_x value was determined for each band by dividing the distance from the origin of each band by the distance from the origin of a common reference band in lane 4 (16). For standardization, band measurements were taken from the leading edge to prevent problems associated with thick bands. For crescent- or arrow-shaped bands, measurements were taken at the apex of the arc.

RESULTS

TLC following fractionation by the MSPD technique provided uniquely different chemotype profiles. All species of mycobacteria tested thus far had different chromatograms and could be easily distinguished from one another. These included *M. smegmatis*, *M. phlei*, *M. kansasii*, *M. flavescens*, 11 isolates of *M. gordonae*, two strains of *M. bovis*, four strains of *M. paratuberculosis*, and 10 serovars of the *M. avium* complex, including 1 isolate of *M. scrofulaceum*. Representative plates of some of these organisms are presented in Fig. 1. There was an observable difference in the chemotype profiles of the various *M. avium* serovars, although many bands were shared between serotypes. These differences also held true for different strains of other species, including *M. bovis* and *M. paratuberculosis*.

The largest number of bands were produced in the fourth lane, corresponding to the methanol extract. In contrast, the lane with the least polar fraction (the hexane extract) rarely contained any visible bands. Mycobacterial species containing significant amounts of yellow (carotenoid) pigment, such as strains of *M. scrofulaceum*, *M. smegmatis*, or *M. gordonae*, tended to have prominent bands in lane 2 (methylene chloride extract). A band in the fourth lane with an R_f value of approximately 0.24 was common to every mycobacterial species that we tested. This band was consistently one of the darkest observed for all of the organisms sampled. Because of these characteristics, it was used as the reference for determining R_x values for the other bands. Another band in the fifth lane (water extract), which also had an R_f of 0.24, was also common to all species tested, but its intensity was more variable. This band was useful in determining that the correct band was being utilized for calculating the R_x value. Differences in mycobacterial isolates could be determined by comparing R_x values of each visible band on the TLC plates. The R_x values for two isolates, *M. avium* serovar 12 and *M. scrofulaceum* W262, are given in Table 1. R_x values of other mycobacterial species that have been evaluated can be obtained by request. Bands with approximate R_x values of 0.9 to 1.0 were consistently identified in lane 3 for samples derived from members of the *M. avium* group. A band with an R_x value of approximately 1 was also present in lane 2 for most of the *M. avium*-complex organisms, although this band was not observed for serovar 1 or 12. A similar band was noted in lane 2 of the *M. scrofulaceum* sample. The *M. gordonae* isolates lacked the bands characteristic of the *M. avium* group, but other bands which appear to distinguish this species were consistently present. One or more V-shaped bands with an R_x value of approximately 2.3 to 2.4 in lane 4 and another band with an R_x of 1.1 in lane 4 were consistently present. The crescent- or V-shaped band al-

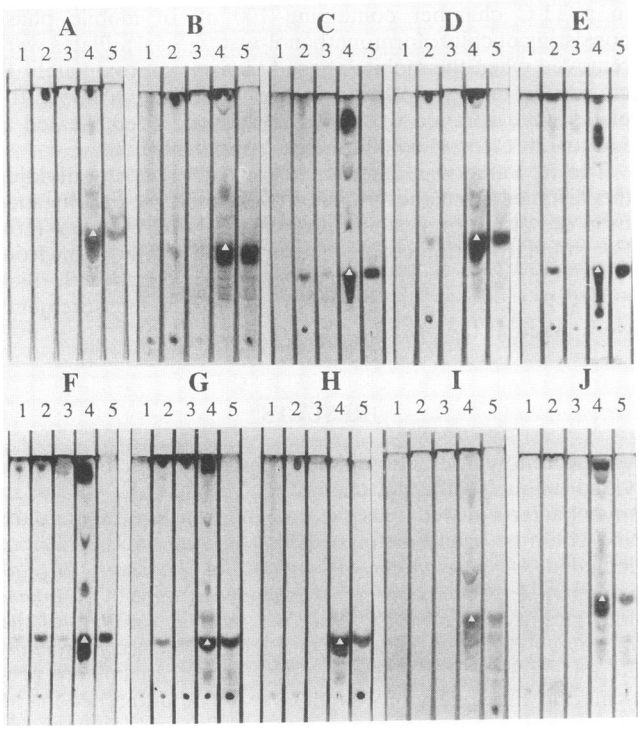

FIG. 1. Chemotypes of various serovars of *M. avium* and other mycobacterial species as determined by the MSPD-TLC method. Fifteen microliters of each MSPD extract was spotted in each of the five lanes. Plates were developed in a chloroform-methanol-water (5:4:1) mobile phase until the mobile phase had risen approximately 10 cm, visualized by spraying with 10% H_2SO_4, and then heated at 120°C for 15 min. (A) *M. avium* serovar 1; (B) *M. avium* serovar 4; (C) *M. avium* serovar 7; (D) *M. avium* serovar 8; (E) *M. avium* serovar 10; (F) *M. avium* serovar 13; (G) *M. scrofulaceum* W262; (H) *M. kansasii* TMC; (I) *M. flavescens* 33001 (Tsukamura strain); (J) *M. gordonae* (isolate 176.233). Lanes: 1, hexane extract; 2, methylene chloride extract; 3, acetonitrile extract; 4, methanol extract; and 5, H_2O extract. Comparison of chromatograms was performed by using band morphology and calculated R_x values compared with those of the reference bands (white triangles).

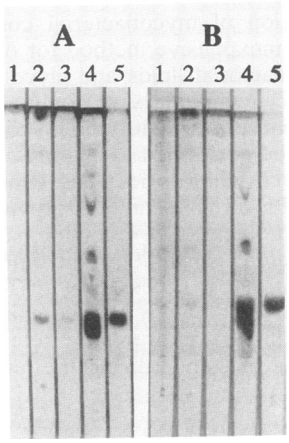

FIG. 2. Chemotypes of two different strains of *M. avium* serovar 2 as determined by the MSPD-TLC method. Fifteen microliters of each MSPD extract was spotted in each of the five lanes. Plates were developed in a chloroform-methanol-water (5:4:1) mobile phase until the mobile phase had risen approximately 10 cm, visualized by spraying with 10% H_2SO_4, and then heated at 120°C for 15 min. (A) *M. avium* serovar 2 (*M. paratuberculosis* 18); (B) *M. avium* serovar 2 (4511). Lanes: 1, hexane extract; 2, methylene chloride extract; 3, acetonitrile extract; 4, methanol extract; and 5, H_2O extract. Note the close similarity of the two chromatograms.

lowed differentiation from other organisms that might also have a band in this region.

Reproducibility was assessed by testing every sample two or more times. Different samples derived from the same culture produced very similar profiles after fractionation by MSPD and subsequent TLC. Isolates from separate sources produced similar chemotype profiles as well, provided that the age of the culture and the type of media were standardized (Fig. 2). These conditions are consistent with the results of previous work with HPLC and TLC which suggested that the standardization of cultures was necessary for concordant results (4).

DISCUSSION

We believe that the MSPD-TLC technique is a promising method for identification and characterization of mycobacteria. Multiple samples can be analyzed by one person in a single day with little expense and with equipment that is available in most clinical laboratories. The small amount of bacterial sample necessary (60 mg [wet weight]) is also less than is necessary with some other methods and allows a shorter culture interval. The technique is reproducible and thus far has been able to differentiate every species and serotype of mycobacteria tested. In addition, different chemotypes of *M. gordonae* and other mycobacterial species that have not been observed previously with other methods have been identified with this method (data not shown). We are currently constructing a data base consisting of the profiles obtained from the organisms that we have tested.

Great variability can arise between laboratories when R_f values are used as the sole quantitative criterion for comparing TLC plates. Factors that can alter R_f values include slight differences in the concentration of the mobile phase, differences in TLC plates, differences in temperature and

TABLE 1. R_x values of bands from mycobacterial chemotype profiles obtained by MSPD-TLC

Organism	R_x value(s) for lane:				
	1	2	3	4	5
M. avium serovar 12	Blank	3.9	1.0, 3.8	0.4, 0.5, 0.7, 1.0, 1.4, 1.8, 3, 3.2, 3.8	0.4, 0.7, 1, 1.5
M. scrofulaceum	Blank	1.0, 1.9, 3.1, 3.6, 3.8, 4	1.0, 4.0	0.5, 0.6, 1.0, 1.4, 1.8, 2.2, 3.0, 3.6, 3.9	0.6, 1.0

saturation of the development chamber, and differences in sample size (17). To allow standardization between laboratories, we have determined R_x values instead of R_f values. The R_x value is derived by comparing bands to an internal standard which is subject to the same developmental factors. Since the bands in lanes 4 and 5 are apparently common to all mycobacteria, an internal control can be established. The relationship between bands should therefore remain constant, even if minor variations are present between laboratories or different plates within the same laboratory. The combination of standardizing materials between laboratories and comparing R_x values instead of R_f values should alleviate many technical difficulties and the need for a template for every species and strain to be tested.

In comparison with other commonly used techniques for identification of mycobacteria, the MSPD-TLC technique provides increased specificity and appears to overcome some of the problems associated with other methods. ELISA testing with monoclonal antibodies to cell wall glycolipid antigens is effective in characterizing some mycobacterial species but carries a risk of cross-reactions and inconclusive results. It has recently been combined with other techniques in an attempt to overcome these problems (6, 10, 18, 19). Gas chromatography, as an absolute means of subspecies identification, also has some limitations. It is labor intensive and expensive, and conditions have not been perfected for the release of all sugars of all serovars to allow absolute differentiation (6). Although HPLC has been shown to be effective and reproducible in identifying a number of mycobacterial species, particularly when used in concert with other methods, it does not lend itself to routine, practical use in a clinical situation by most laboratories (3, 4). Only selected reference laboratories are capable of performing the assays, and the equipment necessary to perform HPLC in-house is relatively expensive. In addition, HPLC analysis does not have sufficient sensitivity to differentiate between closely related species, such as *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and *M. bovis*, or between some of the *M. avium* serovars. Genetic techniques for the polymerase chain reaction or restriction fragment analysis cannot distinguish between some closely related mycobacteria and further require specific primers or DNA probes for any species suspected. Like other commonly used techniques, MSPD-TLC may not be able to distinguish all known species of mycobacteria. However, substitution of other visualization agents that allow differentiation of compounds by producing unique color reactions could provide further delineation and increased specificity.

The technique has many potential uses in clinical and research studies. It could be especially applicable in epidemiological studies, in which it is necessary to determine whether two isolates of the same species are related and when direct transmission or a common source of infection is suspected. The technique could also be utilized in determining whether multiple-site infections in AIDS patients are separate or disseminated infections. The MSPD-TLC technique could be utilized safely with *M. tuberculosis* and other highly pathogenic mycobacteria by first irradiating the culture plates. This would eliminate the health risk for laboratory personnel when blending the organisms in a mortar and pestle. Once the majority of mycobacterial species have been evaluated, MSPD-TLC may prove to be a viable method for primary identification of the organisms. Replacement of TLC in current mycobacterial analytic protocols by MSPD-TLC could increase the specificities of these procedures. Examples would include the analysis of mycobactin content in organisms, such as *M. fortuitum*, grown in iron-limited media (1), multiparameter procedures for authenticating collections (18), and the differentiation of new strains from those presently identified (2). The MSPD-TLC technique may also be valuable to researchers studying the pathogenicity of different isolates by comparing chemotype profiles. Potential virulence factors could be identified by the presence of compounds in one isolate that are absent in others. A limiting factor in the widespread clinical applicability of the technique is the necessity for maintaining a level III mycobacterial service laboratory for handling specimens. Irradiating the plates to neutralize the pathogenicity of the organisms may be one way of overcoming this requirement, but the MSPD-TLC technique as we have described it will be more appropriate for state public health, research, and reference laboratories.

The major advantages of MSPD-TLC over current biochemical, immunologic, and genetic techniques are its relative ease of execution and inexpensiveness and the small sample size required. It is our belief that MSPD-TLC, alone or in combination with other techniques, holds great promise in its ability to characterize mycobacterial isolates in a timely, cost-effective manner.

ACKNOWLEDGMENTS

We appreciate the technical assistance of I. Thio, H. Reid, and C. Wang and the editorial assistance of J. M. Kreeger.

This research was supported in part by NIH grant 1P4ORRO4326-01.

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