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Comprehensive Approach to Identification of Serovars of *Mycobacterium avium* Complex

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Serotyping of nontuberculous mycobacteria, especially those of the *Mycobacterium avium* complex, provides important epidemiological information, particularly in tracing origins of infections. Seroagglutination with whole cells and polyclonal rabbit antibodies was the original way of identifying serovars and is still commonly used. The discovery of the glycolipid nature of the typing antigens allows differentiation of serovars on the basis of thin-layer chromatography of whole antigens and gas chromatography-mass spectrometry of the characteristic sugars of the oligosaccharide haptens of these antigens. In particular, the generation of monoclonal antibodies to the glycolipid antigens allows facile differentiation of serovars through enzyme-linked immunosorbent assay. All of these protocols were applied in developing a comprehensive approach to the typing of members of the *M. avium* complex.

In 1926, J. Furth (15) first reported on the ability of the "avian tubercle bacillus" to produce an aqueous suspension that was much more homogeneous than those obtained with the tubercle bacilli. In 1965 and then in 1980, W. B. Schaefer (29, 30) described protocols for the generation of rabbit antisera and their application in specific, sensitive agglutination-absorption reactions to the identification and classification of nontuberculous mycobacteria. When they were combined with results of animal virulence studies (21, 29, 30), these developments led to the recognition of serovars 1 to 3 of *Mycobacterium avium*, 17 serovars (serovars 12 to 28) of *M. intracellulare*, and 8 intermediate serovars (serovars 4 to 11). This classification was again altered on the basis of genomic analyses (1), and currently, with some ongoing debate as to which serovars are of the *M. avium* or the *M. intracellulare* species (1, 25, 27, 34), the 28 well-documented serovars are termed the *M. avium* complex.

The seroagglutination protocol has been instrumental in the assembly of reference strains (29, 33), in the study of the geographical distribution of serovars in humans with underlying AIDS (18, 32, 36) and in animals (16, 21, 32), and in tracking the sources of such isolates (14).

The serovar-specific typing antigens of the *M. avium* complex have been identified (3, 4) as glycolipids, specifically glycopeptidolipids (GPLs), composed of an invariant monoglycosylated lipopeptide core to which a variable oligosaccharide hapten is glycosidically linked. These have been termed polar GPLs to distinguish them from the singly glycosylated, apolar GPLs which are nonspecific, being present in all serovars. The structures of the oligosaccharide haptens from the polar GPLs of most of the more commonly encountered serovars have been elucidated (4, 22). This fundamental knowledge has allowed the development of simple chemical tools for the identification of *M. avium* complex isolates, notably thin-layer chromatography (TLC) of the alkali-stable, serovar-specific polar GPLs (5–7, 32). The facile resolution by gas chromatography (GC) of the characteristic sugars that make up the haptenic oligosaccharide of the specific GPLs (2, 5, 6, 8–11, 23, 24) has provided yet another powerful tool for the identification and classification of mycobacteria. Enzyme-linked immunosorbent assay (ELISA) based on the GPL antigens and the original type-specific rabbit antisera has proved beneficial in this respect (35), and more recently, the generation of murine monoclonal antibodies (MAbs) to specific epitopes within the GPLs of individual serovars (26) has increased the ease and specificity of identifications. These protocols were applied as part of a comprehensive strategy for serovar identification of the *M. avium* complex.

MATERIALS AND METHODS

Mycobacterial strains. The majority of the clinical isolates used in this study were from patients with AIDS (32). Isolates from the well-characterized Serotyping Culture Collection (33) were also used.

Growth of mycobacteria. Cultures were grown for 2 to 3 weeks at 37°C on three plastic petri dishes (100 by 15 mm) containing 7H11 agar (12, 33). To obtain enough material for the different procedures, bacilli from two plates were scraped from the surface with sterile, deionized water and were transferred aseptically to sterile screw-cap glass culture tubes (20 by 120 mm). Cells were autoclaved at 121°C for 60 min, centrifuged for 5 min at 2,400 × *g*, and lyophilized prior to the extractions. Bacilli from the third plate were collected in phenol–phosphate-buffered saline and were transferred to sterile screw-cap glass culture tubes (20 by 125 mm) for seroagglutination.

Seroagglutination. Suspensions of cells in phenol–phosphate-buffered saline were allowed to stand at room temperature for 3 weeks. Agglutinations were performed as described previously (17, 29, 33) by using well-characterized and, at times, cross-absorbed rabbit antisera.

Extraction and TLC of lipids. Lyophilized cells were extracted with chloroform-methanol (2:1; vol/vol) as described previously (33). Prior to TLC and other manipulations, samples of lipid were treated with alkali to destroy nonspecific lipids (33).

TLC was performed as described previously (33), with the

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following modifications. Strips (6 to 7 by 20 cm) were cut from aluminum-backed thin-layer sheets of kieselgel 60 (0.2 mm; E. Merck, Darmstadt, Federal Republic of Germany), and lipids (10 to 100 μg) from strains were applied to duplicate strips. One primary developing solvent was chloroform-methanol-water (65:25:4) (7). The reference GPLs used in this system were from serovars 8, 10, and 22. Other primary solvents were chloroform-methanol-water (30:8:1 or 30:10:1), in which case the reference GPLs were from serovars 1, 12, and 7; because of variations in R_f values arising from fluctuations in temperature and humidity, it was important to include the GPLs from known serovars. Developed plates were dried and lightly sprayed with 10% H_2SO_4 in ethanol and were heated at 110°C in an oven. The minimal amount of GPLs required for detection was 1 μg; however, 5 μg was usually applied.

ELISA. Use of alkali-treated lipid preparations reduced the problem of the interaction of nonspecific lipids with the polyclonal rabbit antisera (35). The alkali-treated lipids from isolates were dissolved in absolute ethanol (50 μg/ml) through pan sonication in a Bransonic Ultrasonic Cleaner 1200 for about 5 min. Aliquots (50 μl; 2.5 μg) were applied to round-bottom wells of 96-well polystyrene microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) as described previously (35). Rabbit antiserum to the known serovars (29, 33), diluted in phosphate-buffered saline–Tween, was added (50 μl per well). Cross-reactivity, as noted by Yanagihara et al. (35), was reduced by using cross-absorbed antisera. The color absorption was read at dual wavelengths of 490 and 540 nm.

ELISA with whole cells and murine MAbs. The advantage of using MAbs was that whole cells could be used in ELISA. Cells, which were taken from the original 20- by 120-mm tubes after autoclaving, were diluted (50 μg/ml) in carbonate-bicarbonate buffer (pH 9.6) and coated in 50-μl aliquots onto 96-well polystyrene microtiter plates (Dynatech). Plates were incubated overnight in a humid chamber at 37°C. MAbs prepared to the 10 most common serovars (26), diluted in phosphate-buffered saline–Tween, were added to the plates (50 μl per well), which were incubated for 1 h at 37°C. The ELISA was completed as described above.

GC analysis. GPLs in the alkali-treated lipid extracts were partially purified prior to GC analysis. SEP-PAK silica gel cartridges (Waters Associates, Bellefonte, Pa.) were fitted with 5-ml disposable syringes, which acted as reservoirs for the eluting solvents. Twelve samples at a time could be processed with the vacuum manifold (Supelco, Bellefonte, Pa.). The SEP-PAK cartridges were equilibrated with chloroform (5 ml) followed by the application of the alkali-treated extracts dissolved in 0.5 ml of chloroform. The nonspecific apolar GPLs were removed with 5 ml of chloroform. Gradients of increasing concentrations of methanol in chloroform removed the remaining polar GPLs according to their polarities. For instance, 10 to 15% methanol eluted GPLs with R_f values of between 0.3 and 0.6; 25 to 30% methanol removed those with R_f values of between 0.25 and 0.01. A portion of the partially purified GPL (10 to 200 μg) was hydrolyzed with 2 M trifluoroacetic acid at 121°C for 2 h. Acid was evaporated under a stream of air, and the residue was reduced with 250 μl of a solution (10 mg/ml) of sodium borodeuteride in 1 M ammonium hydroxide–absolute ethanol (1:1) (22). A drop of glacial acetic acid was added to neutralize the borate, which was removed by coevaporation with 10% acetic acid in methanol and, finally, by coevaporation with methanol. The residue was dissolved in dry pyridine (100 μl) followed by the addition of distilled acetic anhydride (100 μl) and overnight incubation (16 h). Chloroform (300 μl) was added to the mixture, which was washed twice with water. The alditol acetate-containing chloroform phase was dried prior to GC analysis.

Alditol acetates were routinely resolved on a Varian model 3700 gas chromatograph with fused-silica capillary columns (30 m by 0.25 mm) of SP-2340 (13, 22, 24) by using a temperature program consisting of 190°C for 6 min and 2°C/min up to 235°C. Alditol acetates were also analyzed on a gas chromatograph (5710; Hewlett-Packard, Avondale, Pa.) equipped with a Durabond-1 fused-silica column (22, 24); this analysis consisted of an initial temperature of 160°C for 2 min followed by analysis at 4°C/min to a final temperature of 240°C for 16 min. The carrier gas was helium. The retention times of each encountered sugar derivative was programmed into a Hewlett-Packard 3396A Integrator which was connected to the Hewlett-Packard gas chromatograph so that the R_t of alditol acetates from the test organisms could be compared with those from well-characterized serovars.

RESULTS

Application of TLC to identification of isolates. The total alkali-treated lipid extracts from reference strains of each serovar were applied to a TLC plate and chromatographed in chloroform-methanol-water (30:10:1) (Fig. 1). This solvent was chosen because it can best differentiate the polar GPLs of all serovars with their widely varying mobilities. The system also demonstrated which GPLs have similar R_f values and which could be readily distinguished one from another. Serovars 3, 9, 10, 11, 25, 26, and 27 contained the most polar of the polar GPLs; serovar 2 contained the least polar; the GPLs from serovars 1, 4, 6, 14, and 20 had similar, if not identical, R_f values, as did serovars 3 and 9, 10 and 11, 8 and 19, 12 and 16, and 23 and 24. TLC alone could not distinguish between certain serovars. For instance, isolate 5-16 in lane 3 of Fig. 2 could well be serovar 1, 4, 6, 14, or 20. Nevertheless, TLC does allow some tentative identifications and reduces the possibilities for others.

Some isolates from patients with AIDS were shown to agglutinate equally well with several antisera. Among the more commonly observed reactions were those against antisera to serovars 4 and 8, serovars 1 and 8, and serovar 4 and *M. xenopi*. This phenomenon of dual serovars/spp. within one isolate was corroborated by the use of ELISA and the appropriate MAbs or polyclonal antibodies and by isolating single colonies of individual serovars. TLC also proved particularly adept at confirming this feature. For instance, when the GPLs from several clinical isolates that displayed agglutination reactions indicative of a mixture of serovars 1 and 8 were applied to a TLC plate, the GPLs of both serovars were readily observed (Fig. 3). It should be emphasized that independent, chemical evidence for the presence of *M. xenopi* in mixed isolates is not provided in this work.

TLC also proved effective in exploring the relationships among *M. avium* complex isolates obtained from a group of infected animals. Armadillos had been experimentally infected with *M. leprae* (31), but on harvesting of their tissues, cultivable organisms of the *M. avium* complex were found. TLC demonstrated that all of the isolates had the same patterns, suggesting a point source of contamination of the experimentally infected armadillos, perhaps contamination of the *M. leprae* inoculum. More extensive chemical analysis of the isolate and reaction with the appropriate MAb established the identification as that of serovar 14.

Application of ELISA for identification of isolates. Previ-

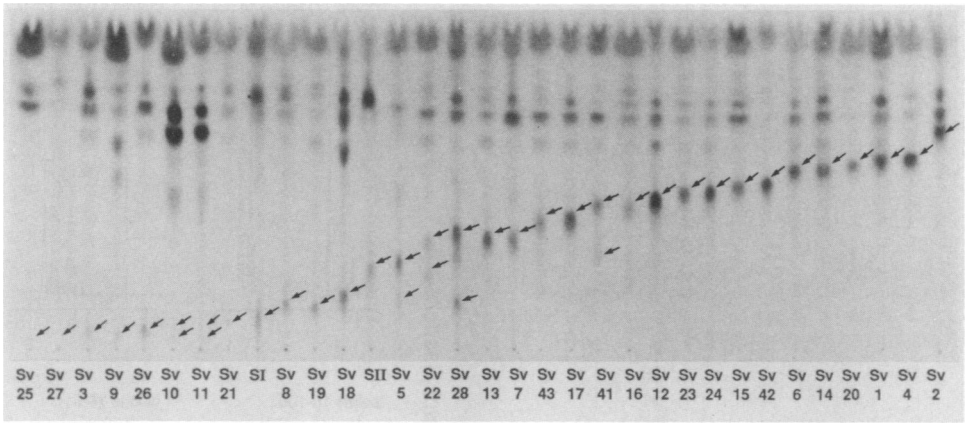


FIG. 1. TLC of the lipids from *M. avium* complex serovars (Sv) 1 to 28, *M. scrofulaceum* serovars 41 to 43, and *M. simiae* I (SI) and *M. simiae* II (SII) in order of the polarities of the seroreactive GPLs (arrows). Serovars 25, 27, 26, 3, and 9 (far left) have the most polar GPLs, whereas serovar 2 (far right) has the most apolar GPL. The developing solvent system consisted of $CHCl_3$-CH_3OH-H_2O (30:10:1).

ously, we described the application of ELISA to the partially purified GPLs from the entire range of serovars with the purpose of resolving discrepancies in identifications (35). Rabbit antisera raised against whole bacteria were used. Now, however, with the availability of MAbs that selectively recognize individual GPLs (26), more specific and more sensitive reactions are feasible. For instance, when the GPLs from 47 random clinical isolates of the *M. avium* complex were tested, GPLs from 9 of them reacted with MAb 33B8 to serovar 4 and subsequently yielded TLC and GC patterns characteristic of the GPL of serovar 4 (data not shown). Of even more practical benefit was the fact that these MAbs were also shown to be capable of differentiating whole bacteria. For example, when the whole cells of reference strains of *M. avium* complex serovars 1 to 28 and *M. scrofulaceum* serovars 41 to 43 were applied to a 96-well plate, MAb 33B8 reacted only with the reference strains of serovar 4 (Fig. 4).

ELISA with the appropriate MAbs (26) was also able to distinguish between serovars 8 and 21, which differed only by the presence or absence of a 3-methoxyl group on the terminal pyruvyl-glucose of the GPL (5, 26); this differentiation was not possible by use of polyclonal antisera or TLC. ELISA, in conjunction with the corresponding MAbs, was also able to distinguish between other serovars with similar R_f values on TLC, such as serovars 1, 4, 6, 14, and 20. One important finding was that whole cells of *M. avium* complex serovar 1 react readily with the corresponding MAb (26), whereas the serovar 1-specific GPL does not. Apparently, extraction and alkali treatment destroyed the antigenicity of the specific GPL from serovar 1, probably through de-*O*-acetylation of the terminal sugar (26).

Application of GC to identification of isolates. Because each *M. avium* complex serovar contains a unique oligosaccha-

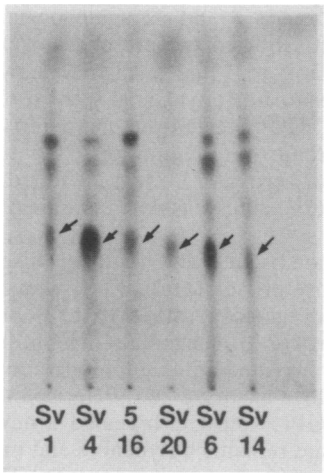


FIG. 2. TLC of GPLs with similar mobilities. *M. avium* complex serovars (Sv) 1 (strain 11907-300), 4 (TMC 1463), 20 (TMC 1419), 6 (34540 Wales), and 14 (TMC 1403) are shown. Isolate 5-16 was obtained from a patient. The arrows indicate the GPLs. The developing solvent system was $CHCl_3$-CH_3OH-H_2O (30:8:1).

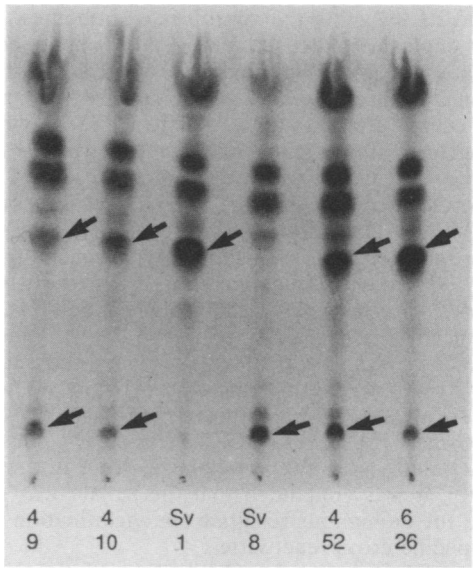
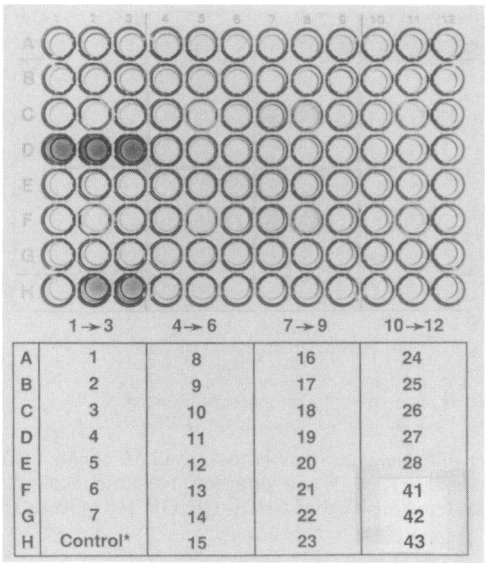


FIG. 3. TLC of the alkali-treated lipids from *M. avium* complex serovars (Sv) 1 (strain 11907-300) and 8 (strain TMC 1468) positioned between four clinical isolates (isolates 4-9, 4-10, 4-52, 6-26) containing the characteristic GPLs (arrows) of both serovars 1 and 8. The solvent system was $CHCl_3$-CH_3OH-H_2O (65:20:3).



	1→3	4→6	7→9	10→12
A	1	8	16	24
B	2	9	17	25
C	3	10	18	26
D	4	11	19	27
E	5	12	20	28
F	6	13	21	41
G	7	14	22	42
H	Control*	15	23	43

FIG. 4. ELISA demonstrating the reaction of the anti-serovar 4 MAb 33B8 against a collection of whole cells of the *M. avium* complex (serovars 1 to 28) and *M. scrofulaceum* (serovars 41 to 43). Bacterial suspensions were applied in triplicate wells. The control (0.9% NaCl) is shown in well H-1; wells H-2 and H-3 contain a suspension of serovar 4 (isolate TMC 1463).

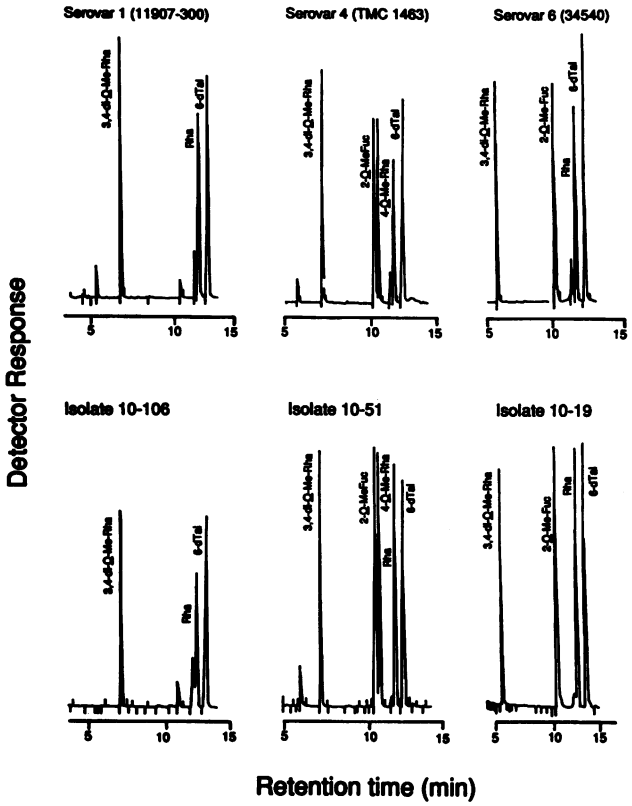


FIG. 5. GC of the alditol acetates derived from the alkali-treated fraction (GPLs) of *M. avium* complex serovars 1, 4, and 6 compared with similarly derived material from isolates 10-106, 10-51, and 10-19, respectively. GC was conducted on an SP-2340 fused-silica column as described in the text. 3,4-di-*O*-Me-Rha, 3,4-di-*O*-methyl-rhamnose; Rha, rhamnose; 6-d Tal, 6-deoxytalose; 2-*O*-Me-Fuc, 2-*O*-methyl-fucose; 4-*O*-Me-Rha, 4-*O*-methyl-rhamnose.

ride unit as part of its specific polar GPL, resolution and identification of component sugars should provide unequivocal means of serovar differentiation. Accordingly, fractions containing the purified specific GPLs from reference strains and from some clinical isolates were hydrolyzed, and the sugars were converted to the alditol acetates and examined by GC (Fig. 5). Clearly, the sugar compositions of the polar GPLs from isolates 10-106, 10-51, and 10-19 matched those of the reference strains of serovars 1, 4, and 6, respectively. The identities of these isolates were further corroborated by TLC and ELISA with the appropriate antibodies.

Most clinical isolates can be readily identified by the combination of agglutination, TLC, and ELISA. However, occasionally, discrepancies which require the application of GC occur. For example, isolate 12-55 showed about equal agglutination against antisera to serovars 1, 2, and 8 (Table 1). TLC showed the presence of a GPL consistent with those of serovars 1, 4, and 20, which comigrated; there was no evidence of the presence of GPLs similar to those of serovars 2 or 8. ELISA showed reactivity with the serovar 1 MAb. The question thus arose as to whether a mixture of serovars 1, 2, and 8 existed in the clinical isolate or whether it was simply serovar 1. GC of the alditol acetates resolved the issue; only the sugars characteristic of serovar 1 (3,4-di-*O*-methyl-rhamnose; rhamnose; 6-deoxytalose) were present; the characteristic sugars of serovars 2 (2,3-di-*O*-methyl-fucose) and 8 (3-*O*-methyl-glucose arising from 3-*O*-methyl-4,6-pyruvyl-glucose) were not seen. Consequently, it was concluded that the isolate was serovar 1 and that the ambiguous results from agglutination were due to nonspecific cross-reactivities.

In the case of isolate 1-5 (Table 1), agglutination against antisera to serovars 8 and 42 occurred with equal intensities. TLC demonstrated a characteristic GPL identical to that from serovar 8. ELISA showed equal reactivities with serovar 8 and serovar 21 antisera. GC, however, resolved the confusion in demonstrating the presence of the 3-*O*-methyl-glucose substituent characteristic of serovar 8. In the case of a third isolate (isolate 12-107; Table 1), agglutination against anti-serovars 8 and 42 again occurred with equal intensities. TLC showed the presence of GPLs consistent with those of serovars 1 and 8; ELISA showed reactions of equal intensities to antisera against serovars 1, 8, and 21. On the other hand, GC revealed the characteristic 3-*O*-methyl-glucose of serovar 8. In addition, the monosaccharides characteristic of serovar 1 were also found, leading to the conclusion that the clinical isolate contained both serovars 1 and 8.

A fourth isolate (isolate 12-49; Table 1) displayed reactions identical to those of isolate 12-107. However, GC revealed the presence of glucose rather than 3-*O*-methyl-glucose, leading to a serovar 21 designation. The fifth isolate (isolate 1-58; Table 1) provided an example whereby agglutination, TLC, and ELISA all disagreed. The isolate did contain a characteristic GPL, and GC revealed a new sugar profile. Thus, this isolate remains untypeable and may represent an unclassified *M. avium* complex serovar. The sixth isolate (isolate 1-10; Table 1) provided an example whereby agglutination was negative and yet TLC and GC combined indicated that the isolate was of serovar 42 of the *M. scrofulaceum* complex.

TABLE 1. Results of the application of a variety of approaches to identification of *M. avium* complex isolates with equivocal properties

Isolate no.	Serovar designation according to[a]:				Remarks
	Seroagglutination	TLC	ELISA	GC	
12-55	1, 2, 8	1, 4, 20	1	1	Conclusively serovar 1
1-5	8, 42	8	8, 21	8	Type-specific sugar of serovar 21 is absent; that of serovar 8 is present; conclusively serovar 8
12-107	8, 42	1, 8	1, 8, 21	1, 8	A mixture of serovars 1 and 8
12-49	8, 42	1, 8, 21	1, 8, 21	21	Conclusively serovar 21
1-58	1, *M. xenopi*, *M. simiae* II	2, 17	No reaction	GPL	Unknown
1-10	Untypeable	6, 14, 42	42	42	Conclusively serovar 42

[a] Details of the protocols and reasoning are provided in the text.

DISCUSSION

Seroagglutination has been largely responsible for the recognition of the serovars and serotypes of the *M. avium* and related complexes and the designation of these as the *M. avium* serocomplex (30). In some laboratories (16, 28, 34), seroagglutination is still used as the sole tool for serotyping. However, inherent weaknesses caused by a high frequency of cross-reactivity have long been recognized, and there is the occasional presence of nonspecific agglutinating antibodies in sera, leading to false-positive results or multiple reactions (21, 30). Also, agglutinating antibodies cannot distinguish between serovars with similar GPL compositions, such as serovars 8 and 21. There is also the issue of semirough variant strains which autoagglutinate. Accordingly, MAbs and chemical protocols have been applied to the task of achieving accurate identifications.

The form of TLC described here had its antecedents in the early work of Jenkins (19). With modifications (7) arising from structural elucidation of the typing antigens, the GPLs (3, 22), TLC has become an inherent component of serotyping, as long advocated by Jenkins (19) and ourselves (22). In fact, in view of the inherent weaknesses of agglutination, the results of any such study without TLC or an equivalent assay must be questioned. Although it is labor intensive and protracted, TLC in the present context has major advantages, in that results can be obtained for cultures with semirough colonial morphologies. TLC is also particularly adept at demonstrating the presence of more than one serovar in a culture, a situation that now occurs with some frequency. TLC is also effective in demonstrating similarities between a series of isolates, even when their actual identities have not been established. Thus, TLC is a powerful epidemiological tool in indicating the origins of organisms or point sources of contamination.

The inherent weakness of TLC is its inability to distinguish between serovars such as serovars 1, 4, 6, 14, and 20 which have single type-specific GPLs with identical TLC mobilities because of their similar molecular weights or polarities. However, Chatterjee and colleagues (10, 11, 22) have demonstrated that the specific GPL from each of these serovars has its own characteristic sugar composition. Accordingly, this information and the relevant technology were adapted for use in this study. Simple GC of alditol acetates, supported at times by GC-mass spectrometry, allowed unequivocal identification in most cases, aided considerably by computer programming of the relative retention times of the monosaccharide derivatives of each serovar so that those of clinical isolates could be compared and identified.

The form of GC used here is simple yet definitive. SEP-PAK cartridges allowed for the removal of the nonspecific apolar GPLs, the presence of which would interfere with the quantitation of rhamnosyl-, 6-deoxytaloxyl-, and 3-*O*-methyl-6-deoxyhexosyl derivatives, when these occur in polar GPLs such as those of serovars 1, 7, and 19. Yet, GC of the alditol acetates of serovar-specific sugars, at least as an absolute means of serovar identification, presents some problems. First, sample preparation is labor intensive. Second, conditions have not been perfected for the release of all sugars of all serovars, such as those of serovars 14 and 17, such that they can be readily seen by GC.

In light of these strengths and weaknesses of the structural approach to serotyping, there is still a need for strategies based on antigen-antibody recognition. Yanagihara et al. (35) developed an ELISA based on unfractionated GPL-containing fractions and the existing pool of reference antisera. A special advantage of this protocol was the fact that cultures with semirough morphologies, and thus containing a modicum of the surface antigens and which tended to spontaneously agglutinate, were amenable to serotyping. On the other hand, isolates of *M. avium* serovar 1, which was frequently encountered, were not amenable to this form of identification, because, during extraction, as mentioned earlier, the corresponding GPL that was isolated lost its immunoreactivity. Moreover, the protocols are labor intensive and protracted, involving careful quantitation of lipid antigens and titration of sera. In addition, results are confounded by high background ELISA reactions. Thus, murine MAbs, as generated by Rivoire et al. (26) and others (20), which have exquisite specificities for the distal GPL-containing sugars, were a boon to this type of work. These antibodies, which have no appreciable cross-reactivities, have increased the specificity and sensitivity of tests and, above all, have allowed the reaction of whole cells with antibody with no apparent equivocation. However, until a full collection of well-characterized MAbs becomes available, chromatography will also be necessary.

The benefits of serotyping of nontuberculous mycobacteria are seen to have the greatest effect in epidemiological studies. The use of the GPL marker has been very effective in exploring relationships beyond the species level. However, serotyping identification of the *M. avium* complex should no longer be conducted solely by agglutination but, rather, should be conducted by screening the GPL-typing antigens by TLC and by performing ELISA with whole cells and MAbs. If an identification is still not possible, GC that seeks the serovar-specific sugars should be performed. Current goals are to prepare MAbs to the remaining serovars to allow more facile identifications.

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