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Identification of *Mycobacterium avium* Complex Strains and Some Similar Species by High-Performance Liquid Chromatography

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Strains of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium xenopi*, and *Mycobacterium gordonae* were identified by high-performance liquid chromatography (HPLC) analysis of mycolic acids as bromophenacyl esters. HPLC criteria were used to develop a flow chart identification scheme, which was evaluated in our laboratory with a set of 234 strains representing five species and a hitherto undescribed species. Correct identifications of *M. gordonae* and *M. xenopi* were easily made. Flow chart differentiation of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* was done with 97.9, 97.5, and 89.2% accuracies, respectively. Independent evaluation of the flow chart at a separate laboratory demonstrated an overall identification accuracy of 97% for *M. avium* complex. Strains that have been described biochemically as being intermediate between *M. avium-M. intracellulare* and *M. scrofulaceum* were identified as one or the other of these known species. Strains which were negative with the species-specific radioactive probe for *M. avium* complex but which were positive with the nonradioactive SNAP X probe were usually identified as *M. intracellulare* and *M. scrofulaceum* but rarely as *M. avium*.

A close phylogenetic relationship existing within a group of slowly growing organisms has been described by such terms as *Mycobacterium avium* complex (MAC), *M. avium-M. intracellulare* (MAI), *M. avium-M. intracellulare*, and *M. scrofulaceum* (MAIS), and strains biochemically intermediate between *M. avium-M. intracellulare-M. scrofulaceum* (MAIS-intermediate). Confusion continues to surround the proper identification of these species. The traditional assignment of *M. avium* to serotypes 1 to 3 and *M. intracellulare* to serotypes 4 to 28 (16) was revised on the basis of DNA-DNA hybridization (2). Recent studies with species-specific genetic probes suggested that *M. avium* was represented by serotypes 1 to 6, 8 to 11, and 21, while serotypes 7, 12 to 20, and 25 were *M. intracellulare*. Serotypes 41 to 43 have been recognized as *M. scrofulaceum*. Other serotypes were not clearly identifiable and are still under study (15). Biochemical tests normally used for *Mycobacterium* species identification will not distinguish *M. avium* and *M. intracellulare* (2, 10); specific identification of these species has been indicated by DNA-DNA homology studies (1, 2). Moreover, DNA-RNA hybridization tests with commercially available single-stranded radioactively labeled DNA probes (Gen-Probe, Inc., San Diego, Calif.) have provided a precise 2-h test to distinguish *M. avium* from *M. intracellulare*. Positive test results with probes parallel the results of DNA-DNA homology studies (6). Chemical studies with thin-layer chromatography have shown that *M. avium*, *M. intracellulare*, *M. scrofulaceum*, and *M. xenopi* share the same structural classes of mycolic acids, the α-, keto-, and wax ester groups (11).

We previously used high-performance liquid chromatography (HPLC) to describe chromatographic patterns for *M. avium* and *M. intracellulare*. Patterns were similar, with minor differences in the heights and areas of comparable peaks (5). Differences, both quantitative and qualitative, in fatty acid peak size have been successfully used for bacterial identifications with gas-liquid chromatography (20). Differences in peak heights worked well for HPLC patterns with the closely related species *M. nonchromonogenicum* and *M. terrae* (14), and it was reasonable to apply the method to this cluster of similar species. The purpose of this investigation was to develop an HPLC decision scheme for MAIS species identification that would be useful in a clinical laboratory. We examined *M. xenopi* and some strains of *M. gordonae* which had been found to produce HPLC patterns with peaks in common with MAC and *M. scrofulaceum*. These *M. gordonae* strains were unusual since it had been established that they contain α-, keto-, and methoxy mycolic acids and produced a single clustered mycolic acid pattern (5, 11). It was also important to distinguish *M. avium* and *M. intracellulare* from the drug-susceptible *M. xenopi*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Culture collection strains served as standards for the test and were from the Trudeau Mycobacterial Culture Collection, formerly at the National Jewish Hospital and Research Center, Denver, Colo., maintained at the American Type Culture Collection, Rockville, Md., or the Mycobacteriology Laboratory, Centers for Disease Control (CDC), Atlanta, Ga. Some of the culture collection strains had been used previously as standards (5). Conventional methods were used to identify strains to the species level (10). In addition, *M. avium*, *M. intracellulare*, and *M. gordonae* strains were confirmed with species-specific DNA-RNA hybridization probes (Gen-Probe). A series of 12 biochemically similar *M. xenopi*-like strains were submitted by W. Gross, Veterans' Administration Medical Center, West Haven, Conn. These represented

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a group of clinically important but biochemically unidentified strains. Mycobacteria were harvested from Lowenstein-Jensen (L-J) slants that were grown at 35°C for 21 days.

Randomly selected CDC strains and culture collection strains were used in a training set to define the standard parameters for the construction of a flow chart decision scheme based on HPLC criteria. Included in this standard training set were *M. avium* TMC 701, TMC 706, TMC 715, TMC 716, TMC 1464, and ATCC 25291 and 26 *M. avium* CDC strains; *M. intracellulare* TMC 1411, TMC 1469, TMC 1473, TMC 1476, and ATCC 13950 and 27 *M. intracellulare* CDC strains; *M. scrofulaceum* TMC 1302, TMC 1303, TMC 1305 to 1307, TMC 1309, TMC 1312, TMC 1314, TMC 1316, TMC 1320, TMC 1321, and TMC 1323 and 21 *M. scrofulaceum* CDC strains; *M. gordonae* TMC 1326 and 5 *M. gordonae* CDC strains; and *M. xenopi* TMC 1470, TMC 1482, ATCC 19970, and 9 *M. xenopi* CDC strains.

An evaluation set to test the decision scheme was composed of different isolates of biochemically well-characterized strains that included *M. avium* TMC 1461, TMC 1462, and TMC 1479 and 140 *M. avium* CDC strains; *M. intracellulare* TMC 1419 and 39 *M. intracellulare* CDC strains; 28 *M. scrofulaceum* CDC strains; 13 *M. xenopi* CDC strains; and 10 *M. gordonae* CDC strains.

Mycolic acid sample preparation. Whole cells were removed from L-J slants by being washed with 2.3 ml of methanolic saponification reagent (25% potassium hydroxide in 50% methanol) or, if growth was eugonic, with 10-μl plastic loops. Cells were thoroughly mixed in a final volume of 2 ml of saponification reagent in glass tubes (13 by 100 mm), capped tightly, and autoclaved for 1 h at 121°C. Free mycolic acids were extracted and derivatized to UV-absorbing bromophenacyl esters as described previously (4). Briefly, mycolic acids were acidified with 1.5 ml of a 50% solution of concentrated HCl and water and extracted from the mixture with 2 ml of chloroform. The chloroform layer was dried under air at 85°C, and 2 mg of potassium bicarbonate was added. This preparation was resuspended in 1.0 ml of chloroform, and 50 μl of *p*-bromophenacyl-8 reagent (Pierce Chemical Co., Rockford, Ill.) was added. Derivatization was completed in 20 min at 85°C. Tubes were cooled, the mixture was acidified with 0.5 ml of a solution of HCl and water (1:1 [vol/vol]), and 0.5 ml of methanol was added. After the solution had been thoroughly mixed, the bottom chloroform layer was removed and evaporated to dryness. The samples were resuspended in 100 μl of chloroform for injection.

HPLC. Liquid chromatography conditions for using an HPLC model System Gold (Beckman Instruments, Inc., Berkeley, Calif.) with methanol-methylene chloride gradient elution have been described previously (4). Briefly, mycolic acids were separated with a Beckman C-18 reverse-phase ultrasphere-XL cartridge column. The UV-absorbing bromophenacyl mycolic acid esters were detected with a Beckman model 166 UV detector set at 260 nm. The column was equilibrated with 98% methanol–2% methylene chloride. For 1 min, the solvent conditions were changed to 80% methanol–20% methylene chloride. After 1 min, the solvent was changed linearly for 9 min to 35% methanol–65% methylene chloride with a flow rate of 2.5 ml/min. After the 9-min gradient run, the column was reequilibrated for 0.5 min in 98% methanol–2% methylene chloride and held at this concentration for 1.5 min.

Location of chromatographic peaks. A high-molecular-weight standard (Ribi ImmunoChem Research, Inc., Hamilton, Mont.), used to determine relative retention time (RRT) for each peak has been described previously (4). Automatic identification of peaks was controlled with peak identification tables by the HPLC software. An arbitrary system of labeling was used to designate the peaks.

Flow chart design. A training set of 115 strains including *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. xenopi*, and *M. gordonae* strains described above were analyzed by HPLC. Chromatographic patterns for each strain were examined for differences in the heights for pairs of peaks. A statistical software system (SAS Institute Inc., Cary, N.C.) was used for chromatographic ratio data analysis with a system 310 personal computer (Dell Computer Corp., Austin, Tex.). Peak height ratios were determined for pairs of peaks by dividing the peak height value of the peak that eluted first by the peak height value of the peak that eluted next in sequence for all peaks (4). HPLC patterns were grouped according to species, and calculated values for each ratio were combined, sorted in numerical order, and examined for their ability to discriminate species. Specific ratios were selected and combined with RRT determinations to develop a 12-step flow chart identification scheme.

Flow chart evaluation. Strains used to evaluate the accuracy of the flow chart were either Gen-Probe-positive *M. avium*, *M. intracellulare*, or *M. gordonae*. Strains of *M. scrofulaceum* and *M. xenopi* were identified by conventional biochemical methods. Mycolic acid peaks were located by RRT, and ratios were calculated by dividing peak heights values as described above. Peak height ratio values for the evaluation strains were compared with the standard peak height ratio values of the flow chart in a sequential manner, from steps at the top of the chart to the steps below. Strains in the evaluation set were run as unknowns, and the final identification was deferred to the HPLC flow chart criteria alone.

Mycobacteria not clearly distinguishable. After the flow chart was evaluated with well-characterized strains, we examined groups of mycobacteria that did not exactly fit the biochemical description of *M. avium*, *M. intracellulare*, or *M. scrofulaceum*. One group consisted of 31 strains that had failed to react to the species-specific radioactive probes (Gen-Probe) for *M. avium* or *M. intracellulare* and had been identified biochemically as MAIS-intermediate. Discrepant reactions occurred with scotochromogenicity, semiquantitative catalase (bubbles >45 mm in height), or urease compared with authentic MAC and *M. scrofulaceum* reactions. *M. avium* and *M. intracellulare* are usually negative in these tests, although some have been recognized to be pigmented. *M. scrofulaceum* has been traditionally considered positive for the three tests. These aberrant strains have been reported previously as MAIS-intermediate strains and their descriptions have always disagreed with one or two of the biochemical test results described above for the known species (9, 12).

Another group of 15 strains, identified as MAC because of positive reaction with the SNAP X probe (Syngene Inc., San Diego, Calif.), a nonradioactive oligonucleotide probe, was also studied. Biochemically, these strains were confirmed as MAC but had failed to react with the radioactive probe (Gen-Probe) for *M. avium* or *M. intracellulare* (17, 18).

Strains examined by the LSPQ. The flow chart was sent to the Public Health Laboratory of Quebec (Laboratoire de Santé Publique du Québec [LSPQ]) for evaluation and final adjustment of peak height values with different HPLC equipment and clinical strains. The LSPQ compared HPLC results with biochemical and genetic probe test results for 302 isolates. Included were 146 strains of *M. avium*, 62 strains of

M. intracellulare identified with species-specific radioactive probes (Gen-Probe), and 19 MAC strains identified to the complex level with the AccuProbe (Gen-Probe), a chemiluminescent probe. Although the detection system used in the two Gen-Probe kits is different, the results with the AccuProbe and the radioactive probe are identical for the species in the MAC, since at the molecular level their detection abilities are the same. Also included were 54 biochemically identified MAC strains that had not reacted with the radioactive probes. Additionally, the flow chart was tested with 17 strains of *M. xenopi* and 4 strains of *M. gordonae* producing an HPLC pattern with two groups of peaks. Most isolates were either tested directly from the original L-J slant sent to the LSPQ or from a 2- to 3-week-old subculture on Middlebrook 7H10 broth. For the older isolates, the subculture on 7H10 was made from the Middlebrook 7H9 broth kept at −20°C for less than a year. The LSPQ used a Waters automated gradient controller (model 680; Millipore Corporation, Milford, Mass.) with model 501 and 510 pumps. Samples were injected onto the column through a Rheodyne 5-μl injector loop (model 7125; Rheodyne Inc., Cotati, Calif.). A diode array detector (model 1040A; Hewlett-Packard, Waldbronn, Germany) set at 254 nm was used to detect the bromophenacyl esters of the mycolic acids. Chromatographic analysis was performed with a Hewlett-Packard integrator (model 339A). Mycolic acid sample preparation, HPLC solvent gradient conditions, flow rate, and column components were identical to those described for the CDC analysis. Because the clinical treatment of MAC infection does not differ for different species, the LSPQ used a modified version of the decision chart that did not differentiate between *M. avium* or *M. intracellulare* and ended after six steps.

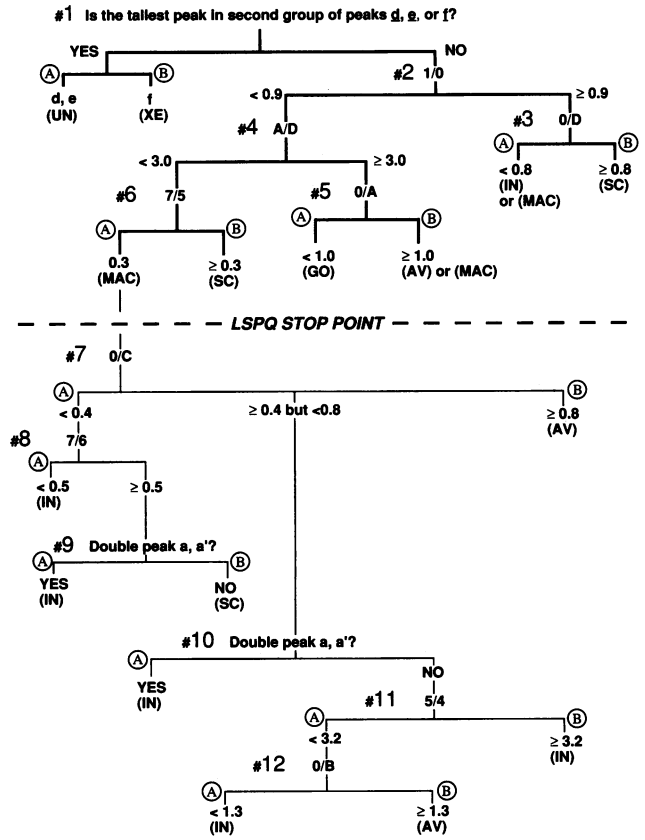

FIG. 1. Flow chart decision scheme for *M. avium* (AV), *M. intracellulare* (IN), MAC, *M. scrofulaceum* (SC), *M. xenopi* (XE), and *M. gordonae* (GO). Bold lines indicate the modified version of the chart used by the LSPQ. See text for RRT and peak height ratio calculation. Unidentified strains (UN) which resembled *M. xenopi* by HPLC may represent a new species. DNA examination and 16S RNA sequencing are currently being conducted.

RESULTS

CDC results. Nine peak ratios and the RRT criteria for specific peaks were incorporated into an identification scheme with 12 steps (Fig. 1). All strains examined in the evaluation set produced RRTs that were identical to the RRTs of the training set (Table 1). Final identifications of well-characterized strains in the evaluation set with the flow chart decision scheme are shown in Table 2.

We had previously shown that *M. gordonae* strains produced a single group of mycolic acid peaks but later noted that, additionally, some demonstrated a multiple group of peaks similar to those of *M. avium* (5). Only those *M. gordonae* strains that produced multiple clustered peak groups having a slight resemblance to the HPLC pattern of MAC or *M. scrofulaceum* were examined.

M. xenopi strains produced similar HPLC patterns containing mycolic acid peaks comparable to those of MAC and *M. scrofulaceum* (Fig. 2). However, examination of *M. xenopi* strains showed that they contained additional peaks that eluted later than peak e, which was usually found as the last peak for all MAC and *M. scrofulaceum* strains (Table 1).

The *M. xenopi*-like strains examined gave an HPLC pattern that had the same RRT as the *M. xenopi* profile (Fig. 2). Unlike *M. xenopi*, these strains were resistant to additional drugs and did not grow consistently at 45°C. However, all the true *M. xenopi* strains consistently had peak f as their major peak, and the *M. xenopi*-like strains had either peak d or peak e as the major peak in the second cluster of peaks.

Representative chromatographic patterns for *M. avium*, *M. intracellulare*, and *M. scrofulaceum* consisted of two groups of peaks (Fig. 2; Table 1). The first group of seven peaks emerged from the column quickly between 5.4 to 6.7 min and was followed by a time interval of 1.0 min when no peaks emerged. The last group of eight or nine peaks emerged between 7.7 and 9.2 min. Reproducibility of the patterns was demonstrated by testing multiple strains (Table 1).

M. avium and *M. intracellulare* strains produced peak 5 as their tallest peak in the first group of peaks for all strains examined. For *M. scrofulaceum*, the tallest peak was peak 5 in 97% of the samples and peak 6 in the remaining 3%. For the second group of peaks, all strains of *M. intracellulare* produced peak c as their tallest peak, but *M. scrofulaceum* strains produced peak c as the tallest peak in 84% of the samples and peak b as the tallest peak in 16%. *M. avium* strains produced peaks 0, c, and d in 46, 48, and 6% of the samples, respectively, as their tallest peaks. Strain diversity resulted in variation in the peak heights and caused a subclustering of the strains by different peak height ratios within a species (7). The different pathways of the flow chart reflected this variation; i.e., the identity of a single species was determined within different decision branches (Fig. 1; Table 2). Neither *M. xenopi*, *M. gordonae*, nor the unidentified strains similar to *M. xenopi* presented this problem.

Reproducibility of the *M. avium* and *M. intracellulare*

TABLE 1. Reproducibility relationship of characteristic bromophenacyl mycolic acid ester peaks for mycobacteria

Peak no.[a]	RRT (±SD) of peaks for				
	M. avium (*n* = 33)[b]	*M. intracellulare* (*n* = 43)	*M. scrofulaceum* (*n* = 32)	*M. gordonae* (*n* = 16)	*M. xenopi* (*n* = 25)[c]
10	—[d]	—	—	4.99 (0.01)	—
9	—	—	—	4.73 (0.01)	—
8	—	—	—	4.64 (0.02)	—
7	4.45 (0.03)	4.44 (0.02)	4.46 (0.01)	4.43 (0.02)	—
7′	4.32 (0.03)	4.32 (0.02)	4.33 (0.02)	4.31 (0.02)	—
6	4.14 (0.02)	4.13 (0.02)	4.14 (0.02)	4.11 (0.01)	4.17 (0.01)
6′	4.05 (0.02)	4.02 (0.03)	4.05 (0.01)	3.97 (0.01)	—
5	3.85 (0.02)	3.84 (0.02)	3.84 (0.01)	3.88 (0.01)	
4	3.59 (0.02)	3.58 (0.02)	3.59 (0.01)	3.58 (0.01)	3.61 (0.01)
3	3.35 (0.02)	3.35 (0.01)	3.36 (0.01)	—	3.35 (0.01)
3′	—	—	—	—	3.10 (0.01)
2	2.19 (0.04)	2.20 (0.01)	2.18 (0.02)	2.17 (0.03)	2.14 (0.005)
1	2.00 (0.02)	2.00 (0.01)	2.00 (0.009)	1.97 (0.01)	1.93 (0.005)
0	1.82 (0.02)	1.82 (0.01)	1.81 (0.01)	1.77 (0.008)	—
a	1.65 (0.01)	1.62 (0.02)	1.62 (0.03)	—	—
a′	—	1.52 (0.02)	1.57 (0.02)	1.58 (0.008)	1.49 (0.006)
b	1.40 (0.01)	1.36 (0.02)	1.39 (0.01)	1.39 (0.005)	1.32 (0.007)
c	1.22 (0.01)	1.21 (0.007)	1.21 (0.009)	1.21 (0.006)	—
d	1.05 (0.01)	1.04 (0.007)	1.04 (0.008)	1.04 (0.008)	—
e	0.89 (0.01)	0.89 (0.01)	0.88 (0.01)	—	0.88 (0.006)
f	—	—	—	—	0.73 (0.005)
g	—	—	—	—	0.58 (0.005)
h	—	—	—	—	0.45 (0.01)

[a] See Fig. 2 for the locations of peaks in the chromatograms.
[b] *n*, number of strains tested.
[c] The unidentified *M. xenopi*-like strains had exactly the same RRTs as *M. xenopi*.
[d] —, no peak.

HPLC patterns was shown previously with different conditions of analysis (5). A common pattern was produced by *M. scrofulaceum*, with a similar number of peaks (Fig. 2; Table 1). *M. avium* normally had 15 common peaks, whereas *M. scrofulaceum* and *M. intracellulare* demonstrated 16 peaks. Double peaks (a and a′) were produced by 54% of the *M. intracellulare* strains and 16% of the *M. scrofulaceum* strains. *M. avium* strains never produced these double peaks.

M. avium evaluation strains were correctly identified at the flow chart decision steps 5B, 7B, and 12B, for 16.7, 64, and 16.7% of the strains, respectively. Three strains of *M. avium* were misidentified as *M. intracellulare*, one at step 8A and two at step 12A (Fig. 1; Table 2). Accurate identification of *M. avium* was made for 140 of 143 (97.9%) of the strains examined.

Identification of the *M. intracellulare* species was correct for 39 of 40 (97.5%) of the strains examined. Accurate identification of *M. intracellulare* was made at steps 3A, 8A, 9A, 11B, and 12B. One *M. intracellulare* strain was misidentified as *M. avium* at step 12B (Table 2). This misidentified strain was highly pigmented but was shown to hybridize 27.7% with the radioactive probe for *M. intracellulare*.

M. scrofulaceum was accurately identified at decision steps 3B, 6B, and 9B for 53.5, 21.4, and 14.3% of the strains examined, respectively. Misidentifications of *M. scrofulaceum* strains occurred 3.6% of the time at decision steps 8A, 11B, and 12B (Table 2).

Pigmentation in the dark, semiquantitative catalase, and urease reactions were used to group 31 strains of mycobacteria that were a "best fit" biochemically for the MAIS-intermediate group. These strains had biochemical reactions for these tests different from the expected results for MAC and *M. scrofulaceum*. HPLC examination identified the mycobacteria as 19 strains of *M. scrofulaceum*, 10 strains of *M. intracellulare*, and 2 strains of *M. avium* (Table 3).

Fifteen strains reported as MAC because of a positive reaction to the SNAP X probe (Syngene) were tested with

TABLE 2. Distribution and identification of mycobacteria in the CDC evaluation sets by the flow chart decision scheme compared with conventional biochemical identification

Mycobacterium species (*n*)[a]	No. of strains correctly identified/no. incorrectly identified at HPLC decision step:											
	1B	3B	5A	5B	6B	7A	8A	9B	10A	11B	12A	12B
M. avium (143)	0/0	0/0	0/0	24/0	0/0	92/0	0/1	0/0	0/0	0/0	0/2	24/0
M. intracellulare (40)	0/0	0/0	0/0	0/0	0/0	0/0	25/0	0/0	10/0	3/0	1/0	0/1
M. scrofulaceum (28)	0/0	15/0	0/0	0/0	6/0	0/0	0/1	4/0	0/0	0/1	0/0	0/1
M. gordonae (10)	0/0	0/0	10/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
M. xenopi (13)	13/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

[a] *n*, number of strains correctly identified by standard methods.

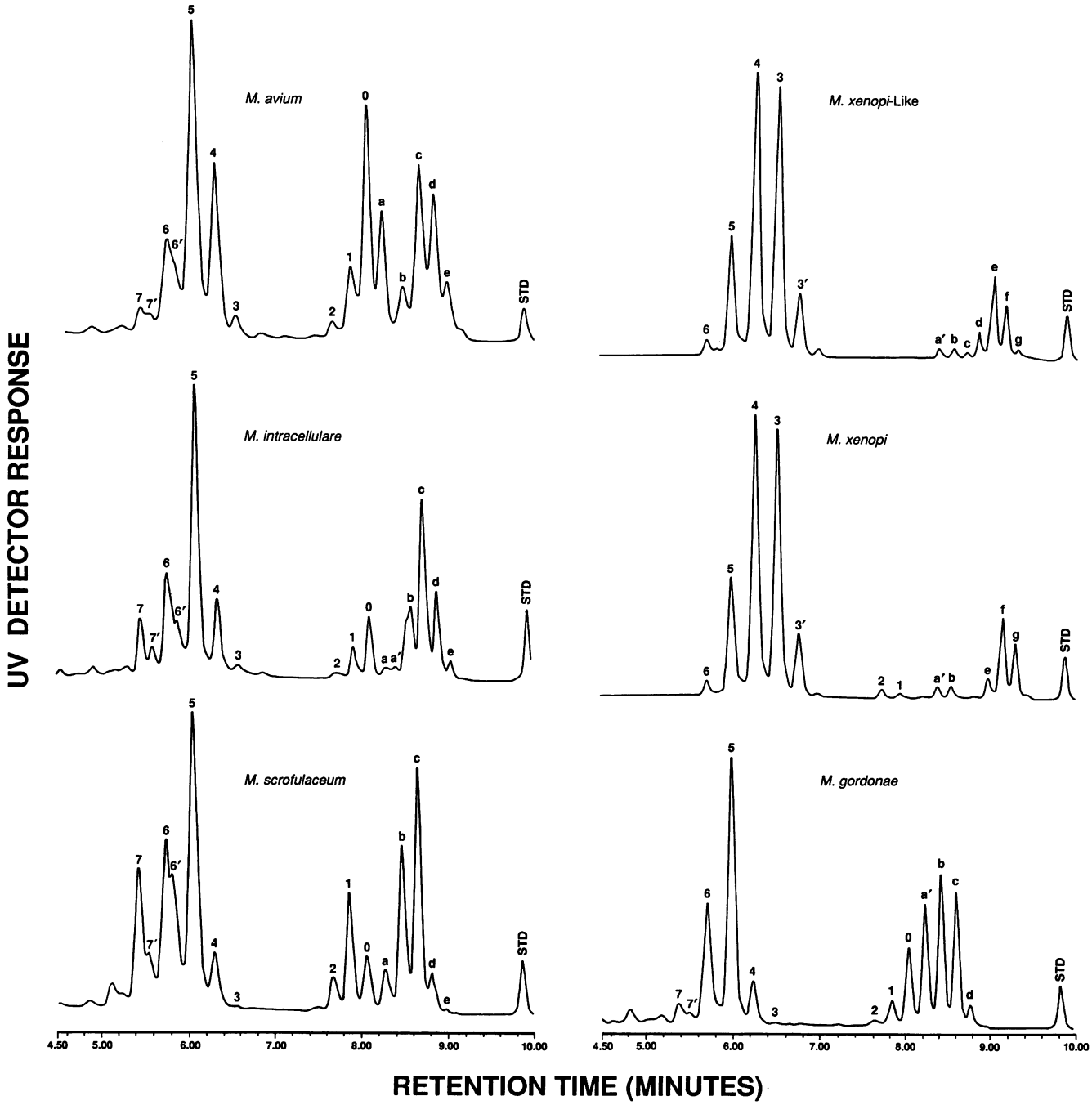

FIG. 2. Representative high-performance liquid chromatograms of bromophenacyl esters of mycolic acids for mycobacteria.

the radioactive probe (Gen-Probe) for *M. avium* and *M. intracellulare* and were negative. HPLC identification resulted in nine strains of *M. intracellulare*, four strains of *M. scrofulaceum*, and two strains of *M. avium* (Table 3). A recent report supporting these results showed that some strains identified as *M. scrofulaceum* had reacted with the SNAP X probe, although others had no reactivity to this probe or to the *M. avium* or *M. intracellulare* probes (21).

Canadian results. The LSPQ accepted, as we did, that a positive reaction to one of the species-specific genetic probes constituted confirmation of the species. For practical purposes, the LSPQ combined the HPLC identification of *M. avium* and *M. intracellulare* species in the designation MAC (Fig. 1). HPLC examination of 208 strains, which were positive for the radioactive probe, showed that two strains were incorrectly identified by HPLC at step 6 as *M. scrofulaceum* because of a peak height ratio for peak 7 divided by peak 5 of ≥0.3. The other 206 strains were identified as MAC.

The LSPQ had also noted that negative results with the radioactive genetic probes did not necessarily rule out species identification as MAC. The LSPQ reexamined 54

TABLE 3. Distribution of unusual mycobacterial strains in the decision scheme

Category (no. of strains)	Decision step no.	Species identified	No. of strains identified
MAIS-intermediate[a] (31)	3A	*M. intracellulare*	2
	3B	*M. scrofulaceum*	9
	6B	*M. scrofulaceum*	10
	8A	*M. intracellulare*	5
	10A	*M. intracellulare*	1
	11B	*M. intracellulare*	2
	12B	*M. avium*	2
MAC (by SNAP X probe) (15)	3A	*M. intracellulare*	1
	6B	*M. scrofulaceum*	4
	8A	*M. intracellulare*	3
	9A	*M. intracellulare*	1
	11B	*M. intracellulare*	4
	12B	*M. avium*	2

[a] See Materials and Methods for explanation.

MAC strains identified by biochemical tests but which were unidentifiable with the species-specific radioactive probe analysis. HPLC identified 26 strains as MAC and 28 strains as *M. scrofulaceum*. Further, HPLC examination of 19 strains that were positive for MAC with the AccuProbe showed 18 strains as MAC and 1 strain as *M. scrofulaceum*.

HPLC pattern analysis of the *M. xenopi* and *M. gordonae* strains using the flow chart gave excellent results. Every one of the 21 strains was correctly identified. All 17 strains of *M. xenopi* produced similar patterns, with peak f as the major peak of the second cluster (Fig. 2). Also, strains of *M. gordonae* isolated in Quebec have been found to produce an HPLC pattern with a single group of peaks for which a flow chart has already been designed (4). For several years, only four strains have demonstrated an HPLC pattern with two groups of peaks, and the MAIS flow chart presented here was able to differentiate them.

DISCUSSION

For this study we selectively used MAIS strains that were identified accurately to the species level because it was important to correctly define the parameters for the HPLC decision scheme. Strain selection was based on results from both biochemical and commercially available genetic probe tests. Strains identified with standard biochemical tests which reacted positively with the radioactive genetic probes for *M. avium*, *M. intracellulare*, and *M. gordonae* were included as authentic strains in the study. Specific genetic probe identification is not available for *M. scrofulaceum*; therefore, identification was done with conventional biochemical tests. We initially used the location of peaks as a criterion for differentiation of species. We determined RRT values relative to a standard to determine the exact location of comparable peaks. The chemical nature of this high-molecular-weight compound caused it to emerge from the column after the mycolic acid peaks, at approximately 10 min. Consequently, earlier emerging peaks had larger RRT values than later emerging peaks. The presence of peaks as reflected by their RRTs was an important criterion for separation of certain species but could not be used alone for identification of the MAIS group.

Separation of the peaks for these closely related MAIS species required "good" chromatography. Moreover, column deterioration occurred normally with use, resulting in loss of separation of double peaks a and a', leading to the wrong identification. Routine use of *M. intracellulare* ATCC 13950, which demonstrates these peaks, is recommended for checking the condition of the column.

Examination of the mycolic acid bromophenacyl ester peaks for strains of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* resulted in similar chromatographic patterns. Differences in comparable peaks in the patterns with the different strains allowed the computation of peak height ratios. Selection of nine peak height ratios was combined with the presence of peaks a, a', e, and f and was used in a flow chart decision scheme for correct identification of most of the strains tested. Mycobacterial species were identified as belonging to the MAIS group by comparison of RRTs to a standard set of RRTs for characteristic peaks. When RRTs matched the standard set of RRTs and the decision was made to evaluate the strains, the design of the flow chart was such that misidentification occurred for the MAIS group.

M. xenopi and *M. gordonae* were not confused with the MAIS species by HPLC. Although both laboratories noted diversity for HPLC patterns in MAIS species, neither laboratory found pattern diversity with the true *M. xenopi* strains examined. Otherwise, strain variation produced group clustering for each species, which was reflected by different HPLC subgroup decision branches in the flow chart. Strains representative of a single species were identified at different steps in the flow chart.

Differentiation of *M. avium* and *M. intracellulare* can be taxonomically or epidemiologically important, but medical treatment for infections with these species does not differ. Although species-specific genetic probes for *M. avium* or *M. intracellulare* are commercially available, most clinical laboratories identify the strains simply as MAC (8). From the standpoint of everyday laboratory services, it is not practical, either financially or in terms of personnel time, to make the distinction between *M. avium* and *M. intracellulare*. The LSPQ laboratory chose to identify their isolates simply as MAC for these reasons.

The heterogeneity of the class of strains termed MAIS-intermediate has been commonly recognized. Recent studies using serologic methods for identification with alpha antigens for eight of these strains identified as intermediate demonstrated five strains of *M. gordonae*, one each of *M. intracellulare* and *M. avium*, and one unidentifiable strain (19). HPLC identification of strains that were biochemically intermediate showed them to be one of the known MAIS species. Moreover, strains that were negative for the radioactive probes (Gen-Probe) were identified by HPLC as *M. avium*, *M. intracellulare*, *M. scrofulaceum*, or occasionally, other mycobacterial species (3, 17, 18). Strains recognized biochemically as MAC or as MAIS-intermediate which did not react positively with specific radioactive probes or those that reacted with the SNAP X probe were found to be *M. avium*, *M. intracellulare*, or *M. scrofulaceum* by HPLC.

Some strains that we had identified as *M. scrofulaceum* had HPLC patterns with more than three major peak height differences. Examination of these patterns demonstrated a tendency to subdivide these strains into different HPLC groups. Although major height differences were noted, we did not have sufficient evidence to indicate that these strains were new species, subspecies, or biological variations. Recent reports comparing different DNA probe kits showed that strains identified as *M. scrofulaceum* would not react with the genetic probes for *M. avium* and *M. intracellulare*. However, it was shown that some strains identified as *M.*

scrofulaceum reacted with the SNAP X probe. These results supported the HPLC results and showed that all strains that reacted with the SNAP X probe were not either *M. avium* or *M. intracellulare*. These strains pose a taxonomic problem within the *M. scrofulaceum* species and require further work. However, for this analysis, they meet the criteria that we had established for inclusion in the test and were therefore considered to be *M. scrofulaceum*.

HPLC offered several advantages over conventional biochemical and genetic probe identification methods. The HPLC procedure produced simple chromatograms which were easily compared with all other mycobacteria patterns for interpretation and rapid identification. Moreover, computer-controlled pattern recognition procedures for improved interpretation are currently being conducted (13). Because our procedure does not separate the homologous series of mycolic acids, precise chemical identification of the chromatographic components was not possible. However, this was not necessary for identification of the mycobacteria since a species-specific pattern was generated. The autoclave-saponification procedure freed the mycolic acids, killed the mycobacteria, and assured the safety of laboratory personnel working outside the biological safety cabinet. Single samples removed from L-J slants, were saponified, derivatized, and rapidly analyzed by the HPLC in less than 2 h.

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