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Routine Application of High-Performance Liquid Chromatography for Identification of Mycobacteria

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Mycolic acid analysis by high-performance liquid chromatography (HPLC) was introduced in our laboratory as the routine technique for identifying all clinical isolates of mycobacteria referred to us. HPLC identified 96.1% of the 1,103 strains analyzed, whereas the biochemical procedures and/or the commercial DNA probes identified 98.3% of strains, for an overall agreement of 94.4%. Compared with the probes, there was 100% specificity and 98.9% sensitivity for *Mycobacterium tuberculosis* identification. HPLC allowed early detection and identification of the rare mycobacterial species *M. haemophilum*, *M. malmoense*, *M. shimoidei*, and *M. fallax* as well as uncharacteristic strains of *M. simiae*. After 18 months of routine use, HPLC proved to be reliable, easy to perform, rapid, and less costly than other identification methods.

It has been demonstrated that reverse-phase high-performance liquid chromatography (HPLC) of the high-molecular-weight mycolic acids of the cell wall can provide a species-specific, rapid, and reproducible method for mycobacterial species identification (2–6, 12–14, 18). We previously studied (unpublished data) 474 strains using as "gold standards" the isotopic probes (Gen-Probe, Inc., San Diego, Calif.) for *Mycobacterium tuberculosis* complex (MTBC) and *M. avium* complex (MAC) and standard biochemical procedures (15) for the other mycobacterial species. In that study, HPLC identified 95.3% of the strains, with 100% specificity and sensitivity for MTBC. It also proved to be more sensitive than the isotopic probes in use at that time for MAC, identifying 66.6% (26 of 39) of the strains missed by the probes as well as 98% of the probe-positive MAC isolates. The results of that previous study (unpublished data) and those done at other laboratories (2, 14, 16) led us to use mycolic acid analysis by HPLC as the main technique for identifying all clinical isolates referred to the Québec Public Health Laboratory (Laboratoire de Santé Publique du Québec [LSPQ]). In this report we present our results and observations after using HPLC as an identification method on a routine basis over an 18-month period.

(The results of the present study were presented in part at the 1992 General Meeting of the American Society of Microbiology, New Orleans, La., 26 to 30 May 1992.)

MATERIALS AND METHODS

Bacterial strains. A total of 1,103 clinical isolates, including the 474 isolates from the previous study (unpublished data), was analyzed by HPLC. These isolates represented 17 mycobacterial species and complexes. All isolates were also analyzed by standard biochemical procedures (15) and/or by isotopic DNA probes for MTBC and MAC. A small number of MAC strains (n = 19) was analyzed with the nonisotopic chemiluminescent AccuProbe (Gen-Probe, Inc.). A higher proportion of probe-negative MAC, *M. bovis*, and *M. bovis* BCG isolates than are normally encountered in the mycobacterial population in our laboratory was used; some of these strains were selected for our previous study.

Mycolic acid preparation. Cells were harvested directly from the original cultures submitted to our laboratory or from a subculture on appropriate media. For some isolates, the subculture was made from Middlebrook 7H9 broth kept at −20 or at −70°C. The mycolic acids were prepared as described previously (2). In brief, cells were saponified in methanolic potassium hydroxide, and the mycolic acids were extracted into chloroform and were then derivatized to UV-absorbing esters with *p*-bromophenacyl bromide.

HPLC. Chromatographic separation was performed with a modular system composed of a Waters automated gradient controller (model 680; Millipore Corporation, Milford, Mass.) with model 501 and 510 pumps, a 5-μl injector loop (model 7125; Rheodyne Inc., Cotati, Calif.), a diode array detector (model 1040A; Hewlett-Packard Co., Avondale, Pa.) set at 254 nm, a Hewlett-Packard integrator (model 3392A), and a Hewlett-Packard desktop computer (HP 85) for data processing. During the course of the study, the modular system was modified in part by replacing the Rheodyne injector with a Waters model 715 Ultra Wisp sample processor and the Hewlett-Packard integrator with a Nelson analytical data system consisting of a model 950 Intelligent Interface and PC Integrator Software (model 2100, version 5.0; Perkin-Elmer Nelson Systems Inc., Cupertino, Calif.) for data collection and analysis as well as the plotting of the chromatograms. Mycolic acid esters were separated with a reverse-phase C_{18} ultrasphere XL analytical cartridge column (4.6 mm by 7 cm; particle size, 3 μm; Beckman Instruments, Inc., San Ramon, Calif.) with a methanol-methylene chloride gradient elution. Solvent gradient conditions and flow rate were those described previously (2).

HPLC pattern identification. The peaks were identified by their relative retention times, which were determined with a high-molecular-weight internal standard (Ribi Immunochem Research Inc., Hamilton, Mont.). For analysis of the chromatographic patterns, we used two flow charts requiring the calculation of peak height ratios: one for species presenting a single cluster of peaks (2) and a second one for species presenting two clusters of peaks (8). Those species that presented a single cluster of peaks were *M. tuberculosis-M. bovis*, *M. bovis* BCG, *M. marinum*, *M. gordonae*, *M. asiaticum*, *M. szulgai*, *M. gastri*, and *M. kansasii*. Those that presented two clusters of peaks were *M. xenopi*, *M. xenopi*-like (8, 22), *M. gordonae*, *M. avium* and *M. intrac-*

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TABLE 1. Comparison of identification by probes and/or biochemical tests and HPLC

Species	No. of strains	No. (%) positive by:	
		Probe or biochemical method	HPLC
MTBC	297	297[a] (100)	292[b] (98.3)
MAC			
Probe-positive	227	227 (100)	224 (98.7)
Probe-negative	54	54 (100)	26 (48.1)
M. gordonae	121	121 (100)	120 (99.2)
M. fortuitum	102	102 (100)	101 (99)
M. chelonae	101	101 (100)	101 (100)
M. xenopi	41	41 (100)	41 (100)
M. kansasii	35	35 (100)	32[c] (91.4)
M. terrae complex	31	31 (100)	31 (100)
M. simiae	25	6 (24)	25[d] (100)
M. haemophilum	16[e]	16 (100)	16 (100)
M. szulgai	14	14 (100)	13[c] (92.8)
M. marinum	11	11 (100)	11 (100)
M. gastri	11	11 (100)	11 (100)
M. chelonae-like organism	6	6 (100)	5 (83.3)
M. malmoense	5	5 (100)	5 (100)
M. shimoidei	5[f]	5 (100)	5 (100)
M. fallax	1	1 (100)	1 (100)
Total[g]	1,103	1,084 (98.3)	1,060 (96.1)

[a] Includes 5 *M. bovis* and 20 *M. bovis* BCG isolates.

[b] The chart misidentified three strains of *M. tuberculosis* as BCG ($n = 2$) and *M. gastri* and two strains of BCG as *M. gastri* and separated 18 of 20 BCG strains from *M. tuberculosis* and *M. bovis* strains.

[c] The chart misidentified three strains of *M. kansasii* as *M. szulgai* ($n = 2$) and *M. gastri* ($n = 1$) and one strain of *M. szulgai* as *M. kansasii*.

[d] *M. simiae* chromatogram confirmed by the Centers for Disease Control and Prevention (16).

[e] Two isolates were sent from the southeastern part of the United States for susceptibility testing.

[f] Four isolates were kindly provided by the Public Health Laboratory for Northern Alberta.

[g] Overall agreement, 94.4%.

ellulare (for *M. avium* and *M. intracellulare*, we used a simplified version of the chart which did not differentiate between *M. avium* and *M. intracellulare* [8]), and *M. scrofulaceum*. For those strains that did not fall within the two flow charts, identification was based on visual observation of the species-specific patterns (16).

Isolate identification. Final identification of the mycobacterial isolates relied upon HPLC patterns as well as on the results obtained by conventional procedures and/or those obtained with the commercial DNA probes.

RESULTS

Identification. Identification results are presented in Table 1. As with the probe, HPLC could not separate *M. tuberculosis* from *M. bovis* but did allow for the differentiation of the BCG strains. The pattern shared by *M. tuberculosis* and *M. bovis* was characteristic, thereby virtually eliminating the risk of a false-positive identification. The use of the probe for MTBC was discontinued in favor of HPLC after testing 297 strains.

More than 97% of the *M. gordonae* strains from Québec present a typical pattern with a single cluster of peaks (2, 3, 9); no other species produced this pattern. The very small number of *M. gordonae* strains that produced an HPLC pattern with two groups of peaks (8, 9) was always singled out by the flow chart. These strains were culturally and biochemically identical to the single-cluster *M. gordonae* strains. Only one strain of *M. gordonae* identified biochemically could not be identified by HPLC. Considering the results for 121 *M. gordonae* strains, HPLC was substituted for biochemical tests for *M. gordonae* identification.

HPLC identified 250 isolates as MAC and 31 isolates as *M. scrofulaceum* by using the current flow chart (8). These 281 isolates were identified as MAC by relying on conventional procedures and/or probes and on HPLC. Of the 31 strains identified as *M. scrofulaceum* by HPLC, 28 (90.3%) were negative for MAC by the probe method. Twenty-one of these 28 strains had biochemical reactions typical of those of MAC strains, 5 had a positive urease test, and 2 had a positive urease test and a semiquantitative catalase test result of >45 mm. These two strains were reported as MAC because they were not pigmented and one of the isolates had an HPLC pattern that keyed to MAC by using a previous flow chart (16). Twenty-six other strains not recognized by the probes were identified as MAC by HPLC and biochemical features. On the basis of the results for these 281 strains, HPLC replaced the probes for the identification of MAC strains, and the nonchromogenic strains identified as *M. scrofulaceum* by the flow chart are reported as MAC.

With regard to the rapidly growing mycobacteria, we looked at strains from the *M. fortuitum* complex since they represent approximately 95% of all rapid growers submitted to LSPQ. Of the 102 strains reported as *M. fortuitum*, one was missed by HPLC because of an unusual chromatographic pattern. No distinction between the three biovars of the species (*M. fortuitum* biovar *fortuitum*, *M. fortuitum* biovar *peregrinum*, *M. fortuitum* 3rd biovariant) could be made by HPLC. However, HPLC could separate *M. chelonae* from *M. chelonae*-like organisms as well as differentiate the two *M. chelonae* subspecies *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus*) with a discriminant power of 95.3%. One strain reported as an *M. chelonae*-like organism on the basis of the carbon source test (20) was identified by HPLC as *M. chelonae* subsp. *abscessus*.

Final identification of 25 strains as *M. simiae* was based on HPLC results. Only seven strains could be detected biochemically. Seventeen other strains did not present cultural or biochemical features typical of those of *M. simiae* such as photochromogenicity and/or positive niacine and/or urease and catalase (>45 mm) activities (15). One was a fastidious strain sent to LSPQ in BACTEC 12B broth. It grew poorly and exclusively on Middlebrook 7H10 agar. Attempts to grow the strain on other solid media with or without an added iron source or mycobactin J at 30 and 36°C failed. No further analysis could be performed.

HPLC allowed for the correct and easier identification of all strains of the rare species *M. haemophilum*, *M. shimoidei*, *M. malmoense*, and *M. fallax*.

Technician time and costs. The average time required by a technician to analyze one strain was 41 min for the conventional procedures, 20 min for HPLC, and 19 min for the isotopic probe method. The costs of reagents and supplies for HPLC proved to be less than those for the other techniques, with an average cost for HPLC of $3.17 per sample, compared with $7.91 for conventional testing and $29.37 for the isotopic probes (all monetary units are in Canadian dollars). Since the probes were used only for the identification of MTBC and MAC, we calculated the average time and cost for the two complexes.

DISCUSSION

The purpose of the present study was to ascertain whether HPLC could advantageously be substituted for the time-consuming study of mycobacterial biochemical features as well as for the expensive commercially available genetic probes in an extent 4/level III laboratory. After 18 months of routine use of HPLC and an analysis of 1,103 mycobacterial isolates, we concluded that HPLC is a reliable, rapid, simple, less costly, and specific identification method.

HPLC identified 96.1% of all strains studied, whereas the biochemical procedures and/or commercial DNA probes identified 98.3%, for an overall agreement of 94.4%. Compared with the MTBC probes, there was 100% specificity and 98.9% sensitivity for *M. tuberculosis*. HPLC proved to be more sensitive than probes for the identification of MAC organisms, identifying 48.1% of the strains missed by the probes and 98.7% of the probe-positive MAC isolates. Other laboratories reported overall agreements with biochemical methods for mycobacterial identification of 94.1% (14), 99.1% (2), and more recently, 95.4% (13). A sensitivity of less than 100% for *M. tuberculosis* identification was acceptable to us, because the identification relies on the cultural characteristics of the strains tested as well as on the pattern and the flow chart. For the three *M. tuberculosis* strains missed by the chart, the cultural characteristics were clearly those of *M. tuberculosis*, and the chromatograms, although atypical, were also suggestive of *M. tuberculosis*. Final identification of these strains required further testing, in this case, by the DNA probe method.

HPLC can be applied to all mycobacterial species. Our experience confirmed the usefulness of mycolic acid analysis for the identification of *M. simiae*, *M. malmoense*, and *M. shimoidei*, organisms that may be confused with MAC (18), as well as for the easier detection and identification of other rare species. This was particularly advantageous in the case of the *M. malmoense* isolates that grew poorly and some of the *M. haemophilum* isolates that were sent to LSPQ without any pertinent information.

The costs of reagents and supplies were 60% less than those for the conventional methods and 89% less than those for isotopic probes. Compared with standard biochemical procedures, HPLC, like the probes, not only reduced the hands-on time per strain by more than 50% but also shortened the identification time, which can take up to 4 to 6 weeks. Because a greater biomass was necessary for mycolic acid analysis than for the probe technique, the subculturing time, if required, was included. Since HPLC is much faster than conventional methods, a culture could be processed within 3 h if necessary.

As expected (10, 12), *M. bovis* and *M. tuberculosis*, which share the same types of mycolic acids, were indistinguishable by HPLC analysis, whereas the BCG strain, with mycolates different from those of *M. bovis* and *M. tuberculosis*, could be recognized. The probes do not separate the species belonging to MTBC. *M. bovis*, with its dysgonic growth of small, translucid, and colorless colonies on egg medium, can be morphologically differentiated from *M. tuberculosis*, while the BCG strain, with its more rapid eugonic growth of rough buff-colored colonies, would be mistaken for *M. tuberculosis*. For two of our BCG isolates, the flow chart did not confirm the visual observation of the pattern suggestive of BCG. The strains resembled the Russian BCG, which produces an HPLC pattern different in appearance from those of the other BCG strains (12) and would be associated with *M. gastri* or *M. kansasii* by the flow chart. As already reported (2), when growth conditions or the ages of the referred cultures are not available, we have found that for cultures resulting in an HPLC identification of *M. asiaticum* and *M. szulgai* (and, in our case, to a lesser extent, *M. kansasii*, *M. gastri*, and BCG), a fresh subculture must be analyzed to obtain a correct identification. The one strain of *M. gordonae* missed by the flow chart was sent to the Centers for Disease Control and Prevention. The DNA probe was found to be positive for *M. gordonae*, and the HPLC pattern had been seen previously but is not typical of *M. gordonae* (11). With the chart, it keyed to *M. szulgai*, although it did not compare with other *M. szulgai* profiles. With the flow chart, similar profiles could also be confused with *M. kansasii*. The probe method or selected cultural and biochemical tests such as pigment production, Tween hydrolysis, nitrate reduction, and urease must be performed for final identification of strains with unusual patterns.

M. avium, *M. intracellulare*, and *M. scrofulaceum* are often referred to as the MAIS complex, even though *M. scrofulaceum* is clearly distinct in key cultural and biochemical features as well as in semantide-based studies (21). A closeness is seen, with similar HPLC patterns shared only by these three species (8), making it difficult for the flow chart to always correctly distinguish between these species. The identification of 11% of the MAC isolates as *M. scrofulaceum* by HPLC, 90.3% of which were also not recognized by the probes, occurred for strains that, with the exception of one, were nonpigmented or that had a pale yellow pigment along with typical biochemical features of MAC in most cases. *M. scrofulaceum* is rarely encountered in Québec (<0.5% of all isolates in the past 10 years). Strains identified as *M. scrofulaceum* at LSPQ are usually part of the College of American Pathologists' surveys, and HPLC always accurately identifies these scotochromogenic strains.

Until the introduction of HPLC, *M. simiae* was considered an uncommon species in Québec. Precise biochemical identification is known to be problematic (15), and it has been reported that about 10% of strains identified as MAC by conventional procedures are in fact *M. simiae* according to their mycolate composition (18). Our results showed that, because of atypical cultural and/or biochemical features, 17 of the 25 *M. simiae* strains identified by HPLC could have been reported as MAC without the mycolic acid analysis. With the knowledge of the HPLC results, the photochromogenicity of the 17 strains was retested, and 9 of the 17 strains were then considered to be weakly photochromogenic. Visually, it is easy to separate *M. simiae* from the MAIS complex by HPLC, with its three distinct clusters of peaks (6). Recently, better growth on Middlebrook 7H10 agar and urease activity were obtained with the fastidious strain of *M. simiae*.

The discrepancy noted with one biochemically identified *M. chelonae*-like organism strain which had a chromatogram characteristic of *M. chelonae* subsp. *abscessus* suggests that HPLC may be more reliable than the carbohydrate utilization test (20) for distinguishing between these rapid growers. *M. chelonae* produces a very different HPLC pattern from those of *M. chelonae*-like organisms (5, 16).

HPLC analysis can be applied to the identification of other mycolic acid-containing bacteria (1, 7). On a routine basis, with the same sample preparation method as was used for the mycobacteria, HPLC provided us with an easy method for discriminating between members of the genera *Nocardia*, *Gordona*, *Rhodococcus*, *Corynebacterium*, and *Mycobacterium* as well as between members of the genera *Streptomyces* and *Nocardia*. It has also led to the identification to the

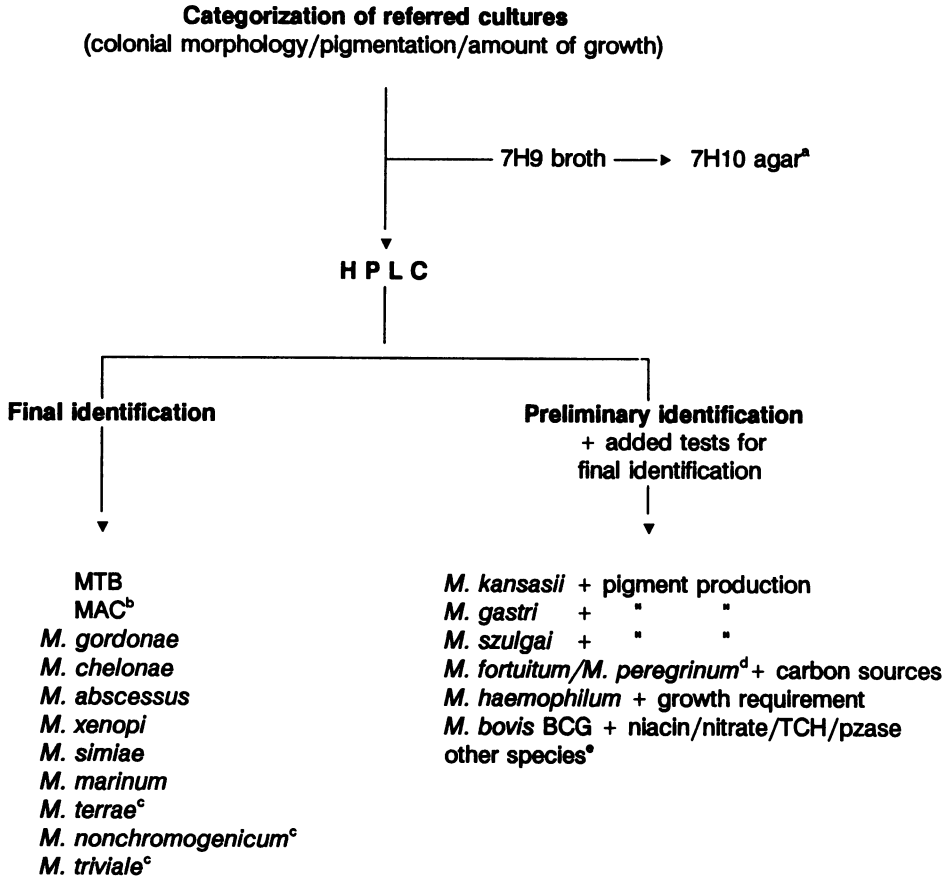

FIG. 1. Scheme for identifying mycobacteria by HPLC. a, For detection of mixed cultures, estimation of growth rate, observation of morphology on agar, and, if necessary, a second HPLC. b, Nonchromogenic isolates identified as *M. scrofulaceum* by HPLC are reported as MAC. c, Although chromatographically similar, *M. terrae* and *M. nonchromogenicum* can be distinguished by HPLC (19). *M. triviale* is chromatographically very different from the other two species of the complex (4, 16). d, Since the majority of *M. smegmatis* isolates produce the same pattern as the *M. fortuitum* and *M. peregrinum* isolates (5), the 3-day arylsulfatase test is performed, but the preliminary report of *M. fortuitum* or *M. peregrinum* is not withheld, since *M. smegmatis* is very rarely encountered in Quebec. e, Appropriate cultural and biochemical tests are performed. TCH, tiophen-2-carboxylic acid hydrazide susceptibility test; pzase, pyrazinamidase test.

species level of isolates of *Gordona sputi*, *Gordona bronchialis*, *Gordona terrae*, *Rhodococcus equi*, and *Tsukamurella paurometabolum*.

Figure 1 describes the simplified routine identification scheme adopted at LSPQ as a result of our experience with HPLC. By using this algorithm, with regard to the recent taxonomic changes upgrading both *M. abscessus* and *M. peregrinum* to species status (17), a rapid final identification can be achieved by HPLC for about 90% of the strains submitted to our laboratory. Less familiar and uncommon species as well as strains that produce atypical patterns are always subjected to additional identification tests.

Overall, compared with biochemical procedures and the probe methods, mycolic acid ester analysis by HPLC is an advantageous species-specific identification method for all mycobacteria.

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