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Differentiation of *Mycobacterium genavense* and *Mycobacterium simiae* by Automated Mycolic Acid Analysis with High-Performance Liquid Chromatography

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***Mycobacterium genavense*, a fastidious opportunist in patients with AIDS, cannot be identified by conventional biochemical methods. Computerized mycolic acid analysis by high-performance liquid chromatography offers an alternative that distinguishes the mycolic acid profile of *M. genavense* from those of all other organisms in the database developed at the Centers for Disease Control and Prevention.**

The first case of infection with *Mycobacterium genavense* was reported by Hirschel et al. (6) in 1990. Since then more than 60 cases of *M. genavense* infection have been reported in both adult and pediatric patients with AIDS in Europe, North America, and Australia (10–13). Invasive infection with *M. genavense* was considered the sole or contributing cause of death for most of these patients. Because of its fastidious growth, extended incubation times in primary broth cultures (median, 7 weeks) (16) and subsequent subculture to supplemented solid medium (median, 7 weeks) (4) are required for the detection of *M. genavense*. To date, presumptive identification of *M. genavense* has been based on its slow growth in BACTEC AFB broth and failure to grow on Lowenstein-Jensen slants or Middlebrook 7H11 plates unless the latter are supplemented with mycobactin J (MJ) (4) or human blood (9).

At present the definitive identification of *M. genavense* depends on sequencing of hypervariable regions of the 16S rRNA gene (1). Mycolic acid analysis by the high-performance liquid chromatography (HPLC) method developed at the Centers for Disease Control and Prevention (CDC) for mycobacterial identification (3) potentially offers a more cost-efficient and technically simpler means of identifying mycobacterial species. While less cell mass is needed for the sequencing method, the final identification by any method requires sufficient colony maturity to ascertain that the culture is in fact pure. In a recent evaluation of HPLC for routinely identifying more than 1,100 clinical isolates, Thibert and LaPierre (14) found that noncomputerized analysis of the mycolic acid profiles obtained by HPLC identified more than 96% of isolates, including a number of rare mycobacterial species. CDC researchers, using Pirouette pattern recognition software (InfoMetrix, Woodinville, Wash.), found computerized HPLC identification of mycobacteria to be highly reliable, with an overall accuracy of ≥97% (5).

Because *M. genavense* was not recognized as a new species until relatively recently (2), its mycolic acid profile was not available for inclusion in the CDC database for the HPLC software. Therefore, the HPLC system misinterprets *M. genavense*'s three clusters of mycolic acid peaks as those of *Mycobacterium simiae*, the only species in the database with a trimodal mycolic acid profile (TMAP). In the present study we developed and evaluated a computer-generated model for the identification of *M. genavense* and *M. simiae* isolates. Our model demonstrates that automated HPLC mycolic acid analysis can reliably differentiate *M. genavense* from *M. simiae* isolates gathered from collections across the United States.

The methods for the identification of the *M. genavense* and *M. simiae* species in the present study included determination of growth patterns, conventional biochemical characteristics, whole-cell fatty acid profiles in the Microbial Identification System (Microbial ID, Inc., Newark, Del.), and mycolic acid profiles by HPLC and sequencing of hypervariable regions of the 16S rRNA gene (4, 8). The mycolic acid profiles of isolates from eight patients at the Harborview Medical Center (HMC) were chosen to calculate the average profile for *M. genavense*. Results of mycolic acid analyses for eight representative *M. simiae* isolates (including the type strain, ATCC 25275) were used to calculate the average profile for this species. The 16 isolates were chosen through the use of cluster analysis performed with the Pirouette software. These same isolates and the model-building portion of the Pirouette software were then used to build a two-class model based on multivariate analysis analogous to the CDC model for mycobacterial identification by HPLC (5).

The new model was evaluated with 58 additional mycobacterial isolates that had been identified by CDC's mycobacterial library as *M. simiae* on the basis of their TMAPs. These included 19 *M. simiae* isolates, 15 *M. genavense* isolates referred to our laboratory for identification, and a unique collection of 24 non-*M. genavense*/non-*M. simiae* (NG/NS) isolates with TMAPs, most of which were provided by colleagues from other institutions (Table 1). These 24 NG/NS isolates were initially identified by the HPLC method as *M. simiae*; however, whole-cell fatty acid patterns in Microbial Identification System studies did not confirm these identifications. A number of tests

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TABLE 1. Sources of mycobacterial isolates with TMAPs

Species	Isolate no.	Source[a]
M. genavense	358	Hirschel
	360, 1012, 1014, 1025, 1274, 1275, 1276, 1305, 1565	HMC
	392	Ohman
	393, 596, 1150	DeGirolami
	429	Fierer
	786, 1151	Besser-Wiek
	787	Plorde
	788, 1149	Morrissey
	789, 791, 792	Gilkison
M. simiae	359	Hirschel
	M25, 1083	HMC
	M39	ATCC 25275
	M46, M47, M49	Ferguson
	M54	ATCC 25273
	M55, M58, M65, M67, M70, M71	Kilburn
	M86, M87, M88, M89	Tsang
	M101, M102, M103, M105, M108, M111, M112, M117, M122	Cage
NG/NS	422, 531, 642, 648, 713, 898, 1019	HMC
	M66, M68, M69, M72, M73, M74, M75	Kilburn
	M100, M104, M113, M114, M119, M120, M121	Cage
	M126, M127, M128, M134	Böttger

[a] Source abbreviations: Hirschel, B. Hirschel, Hospital Cantonal Universitaire, Geneva, Switzerland; HMC, Harborview Medical Center, Seattle, Wash.; Ohman, C. Ohman, Lutheran General Hospital, Park Ridge, Ill.; DeGirolami, P. DeGirolami, New England Deaconess Hospital, Boston, Mass.; Fierer, J. Fierer, Veterans Administration Hospital of San Diego, San Diego, Calif.; Besser-Wiek, J. Besser-Wiek, Minnesota Department of Health, Minneapolis; Plorde, J. J. Plorde, Seattle Veterans Administration Medical Center, Seattle, Wash.; Morrissey, A. Morrissey, University Hospitals of Cleveland, Cleveland, Ohio; Gilkison, V. Gilkison, Emmanuel Hospital, Portland, Oreg.; ATCC, American Type Culture Collection; Ferguson, R. Ferguson, New Mexico Department of Health, Alburquerque; Kilburn, J. O. Kilburn, Centers for Disease Control, Atlanta, Ga.; Tsang, A. Tsang, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.; Cage, G. Cage, Arizona Department of Health Services, Phoenix; Böttger, E. Böttger, Institut für Medizinische Mikrobiologie, Hannover, Germany.

were used to rule out *M. genavense* and *M. simiae* as appropriate identifications. None of the NG/NS organisms were MJ dependent, and the results of neither PCR studies nor conventional biochemical tests fit the features of known *M. genavense* and *M. simiae* strains.

The *M. genavense* isolates were grown on Middlebrook 7H11 plates supplemented with MJ and were harvested at 8 weeks of growth; one strain, however, was harvested at 20 weeks. All non-*M. genavense* isolates were harvested from Middlebrook 7H11 plates at 3 weeks. Subcultures of all organisms were incubated at 37°C in an aerobic atmosphere with 5% CO_2, and harvested cells were stored at −70°C until analysis, when they were thawed at room temperature.

The procedure for extracting mycolic acids from whole cells was based on the work of Butler et al. (3). Briefly, harvested cells were saponified in 25% potassium hydroxide; this was followed by chloroform extraction of the mycolic acids in an acidic environment. Potassium bicarbonate provided the catalyst for derivatization of the mycolic acids by crown ethers into their UV-detectable bromo-phenacyl esters. The samples were clarified in acidic methanol, and an internal standard was added before injection on the high-performance liquid chromatograph. The HPLC hardware consisted of a Beckman Gold System that included a manual injector (or a 507 autosampler), a 126 pump, a 166 detector, and an ultrasphere column (4.5 mm by 7.5 cm with 3-μm particles). Peak integration and identification were performed by the Beckman System Gold software by using an International Business Machines PS/2 model 56SX computer in accordance with the parameters forwarded to HMC by CDC.

The Beckman System Gold software analyzed each extraction for the relative retention time (RRT) of each mycolic acid peak and the proportion or percentage of the total height that each peak represented (percent peak height [PPH]). When the chromatograms exhibited a total area of less than 10, the analysis was repeated with fresh subcultures. Figure 1 shows representative chromatograms for *M. simiae* ATCC 25725 and *M. genavense* ATCC 51233, with the peaks named according to the CDC scheme (5). For each of the eight *M. simiae* strains and eight *M. genavense* strains used in the model, Table 2 presents a summary of the average RRT and average PPH for each peak in CDC's identification table. Table 2 also contains the standard deviation for the PPH and the *P* values for the two statistical analyses performed on the data, the Wilcoxon rank sum test and the two-sample Student *t* test for independent samples with unequal variances.

Our model correctly named all of the challenge isolates of *M. genavense* ($n = 15$) and *M. simiae* ($n = 19$). The model contained only a two-class database; therefore, as expected, the model could not correctly identify the 24 NG/NS isolates included in the study. Of these, 19 were consistently named *M. simiae*, 1 isolate was repeatedly named *M. genavense*, and 4 isolates had different identifications in repeat runs.

Upon visual inspection, the TMAPs of *M. simiae* and *M. genavense* resemble each other (Fig. 1) but can be distinguished

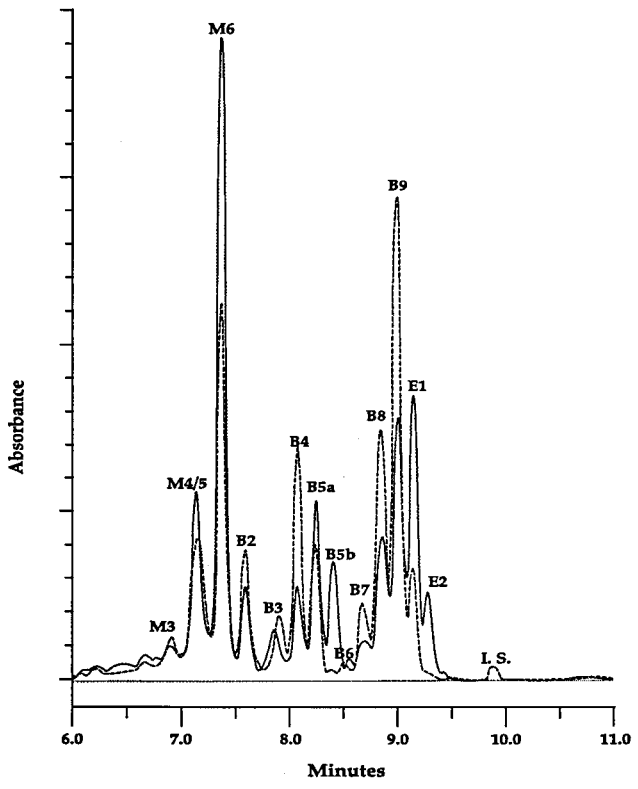

FIG. 1. Mycolic acid profiles from HPLC analyses of *M. simiae* ATCC 25725 (solid line) and *M. genavense* ATCC 51233 (broken line).

TABLE 2. Statistical analyses of RRTs and average PPHs for mycolic acid peaks of *M. simiae* and *M. genavense* isolates

Peak name[a]	RRT	*M. simiae*		*M. genavense*		*P* value	
		PPH	SD	PPH	SD	w test[b]	*t* test[c]
A1	0.55	0.51	0.21	0.09	0.09	0.001	≤0.001
A2	0.58	0.68	0.12	0.49	0.27	0.115	0.098
A5	0.59	0.67	0.24	0.38	0.14	0.024	0.011
M1	0.62	0.60	0.16	0.31	0.12	0.003	0.001
M2	0.65	0.56	0.13	0.32	0.19	0.018	0.011
M3	0.68	1.37	0.41	1.46	0.45	0.636	0.661
M4 and/or M5	0.71	8.01	2.63	5.94	0.53	0.388	0.454
M6	0.73	25.51	2.34	18.21	2.07	≤0.001	≤0.001
B1	0.75	3.91	0.61	5.77	0.93	0.002	≤0.001
B2	0.78	0.25	0.46	0.00	0.00	0.218	0.176
B3	0.79	1.77	0.40	2.64	0.73	0.007	0.013
B4	0.81	4.87	0.91	9.24	1.30	≤0.001	≤0.001
B5A	0.83	8.35	1.03	7.70	0.79	0.105	0.185
B5B	0.84	4.56	2.18	0.83	0.55	0.001	0.002
B6	0.85	0.69	0.47	0.65	0.58	0.791	0.886
B7	0.87	1.78	0.52	3.52	0.87	0.001	≤0.001
B8	0.89	6.67	1.82	11.63	2.06	0.002	≤0.001
B9	0.90	11.45	0.69	21.92	2.33	≤0.001	≤0.001
E1	0.92	11.91	2.37	7.79	1.94	0.003	0.002
E2	0.94	4.36	1.66	0.00	0.00	≤0.001	≤0.001

[a] Mycolic acid peaks were identified with the CDC peak ID table (5).
[b] w test, Wilcoxon rank sum test.
[c] *t* test, Student's *t* test for independent samples with unequal variances.

by the fact that *M. genavense* isolates have a relatively large amount of peak B9 and, as noted by Tortoli et al. (15), the absence of a late peak, peak E2. Although visual pattern recognition can be used to distinguish these two species, computerized identification offers many advantages including accuracy, reproducibility, and the option of having the analyses done by technicians, who are less expensive than highly experienced technologists with expertise in mycobacteriology.

When statistical analysis was performed to compare the average PPH for individual peaks from each species, there was a highly significant difference for 11 of the 20 peaks and a significant difference for 3 more peaks. Considering only the peaks beginning with peak M3, 9 of the 15 major peaks exhibit highly significant ($P < 0.005$) difference between *M. simiae* and *M. genavense* isolates. The B3 peaks were significantly different at the 0.05 level.

The earliest attempts to identify isolates of *M. genavense* found that the mycolic acid profile resembled that of *M. simiae* (4, 6). Although reports of *M. simiae* infection in patients with AIDS are very rare (7, 17), it is clinically relevant to accurately distinguish it from *M. genavense* because the latter species is much more susceptible to the antimycobacterial drugs used for the prophylaxis and treatment of mycobacterial infection in patients with AIDS.

Although *M. genavense* and *M. simiae* are closely related phylogenetically (1), our computer model clearly distinguishes these species. However, the existence of at least three recently recognized unnamed taxa with TMAPs that are not in our current model (13, 18) prevents the model from having a high degree of specificity. This possible weakness of HPLC as an identification tool is shared by essentially all identification systems because their databases can include only the taxonomic groups that have been well characterized in the literature. In our laboratory NG/NS organisms were recovered from 5.8% of all patients harboring mycobacteria. At this time, colonial morphologies, biochemical tests, whole-cell fatty acid analyses, and sequencing of the 16S rRNA genes of the NG/NS organisms support their differentiation from *M. genavense* and *M. simiae*. Once these novel organisms have been sorted into new taxonomic groups and their mycolic acid profiles have been added to the database, we believe that HPLC will be a rapid automated method for their identification.

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