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Whole-Cell Protein Electrophoresis for Typing *Mycobacterium tuberculosis*

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A method of discriminating between strains of *Mycobacterium tuberculosis* by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins combined with a sensitive silver stain is described. Thirty-five isolates of *M. tuberculosis* and five isolates from other species of *Mycobacterium* were examined, including serial isolates from the same patients and isolates from a small cluster of hospital cases. Different species of *Mycobacterium* were clearly distinguished, and within the species *M. tuberculosis*, different fingerprints were obtained, allowing discrimination of some strains from different patients. The reproducibility and discrimination of the technique are discussed.

Investigation of the epidemiology of *Mycobacterium tuberculosis* in relation to recurrences in individual patients and the association of cases within clusters has suffered from the lack of a generally accepted typing scheme. Phage typing has been attempted but is slow and cumbersome, and only a few different types can be recognized (1, 3). More recently, molecular methods have been applied to produce DNA fingerprints (4, 9). However, DNA sample preparation is complex, especially when carried out under containment conditions. Preparation of whole-cell proteins is much simpler and has been used to type other bacterial species successfully (7, 10).

Protein fingerprints stained with Coomassie blue have been used to differentiate between strains of different mycobacterial species (2). In this previous study, "minor differences in the relative amount of protein in some bands" were observed (2). Silver staining is up to a hundred times more sensitive than Coomassie blue staining and thus might intensify the differences between strains within a single species. If such differences were reproducible, silver staining might be a useful method of typing *M. tuberculosis*. We have therefore examined the whole-cell protein fingerprints of wild isolates of *M. tuberculosis* visualized with silver.

MATERIALS AND METHODS

Strains. Thirty-five isolates of *M. tuberculosis* were examined. These included 22 kindly supplied by the Mycobacterial Reference Laboratory, South African Institute for Medical Research, Johannesburg, South Africa; 13 from patients at Hammersmith Hospital; and strain H37RV. Two isolates of *Mycobacterium kansasii* from the same patient, single isolates of *Mycobacterium chelonae* and *Mycobacterium fortuitum* from patients, and *Mycobacterium avium* NCTC 8559 were also included. All wild isolates were identified to species level by standard methods. United Kingdom isolates were submitted to the Public Health Laboratory, Dulwich, London, for confirmation of identity.

Isolates were stored on Lowenstein-Jensen slopes at room temperature for up to 1 month or at −20°C for longer periods.

Sample preparation. Isolates were subcultured on Lowenstein-Jensen slopes at 37°C for 3 to 4 weeks until good visible growth was seen. Only fresh subcultures were used for typing. If a culture was adherent or difficult to emulsify, an additional slope was used. Growth was removed from the entire slope, with care taken not to include any agar; suspended in 2 ml of distilled water; and sonicated for two periods of 10 min each in a sealed bijou with a 300-W, 20-kHz ultrasonicator (Vibra-cell, Danbury, Conn.) set at 2-s cycle, 2-s rest. Sodium dodecyl sulfate was added to a final concentration of 2% (wt/vol), and the soluble proteins were dissociated by boiling for 2 min. After the cooled suspension was centrifuged, the supernatant was collected and adjusted such that a 1-in-10 dilution had an optical density of 0.115 ± 0.035 when the protein content was assayed spectrophotometrically at 280 nm. Samples were then diluted 1 in 2 in electrophoresis sample buffer (5) and stored for up to 8 weeks at −20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (5), with 3%

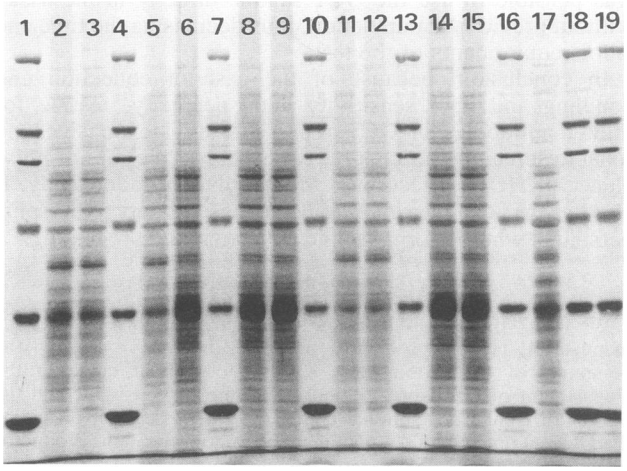

FIG. 1. Lanes 1, 4, 7, 10, 13, 16, 18, and 19 are molecular weight marker tracks with bands of 200,000, 116,250, 97,400, 66,200, 45,000 and 29,000. Lanes 2 and 11, 3 and 12, and 5 represent isolates 040, 048, and 049, respectively, from patient 12. Lanes 6 and 14, 8 and 15, and 9 represent isolates 047, 046, and 045, respectively, from patient 15. Lane 17 is the stain control.

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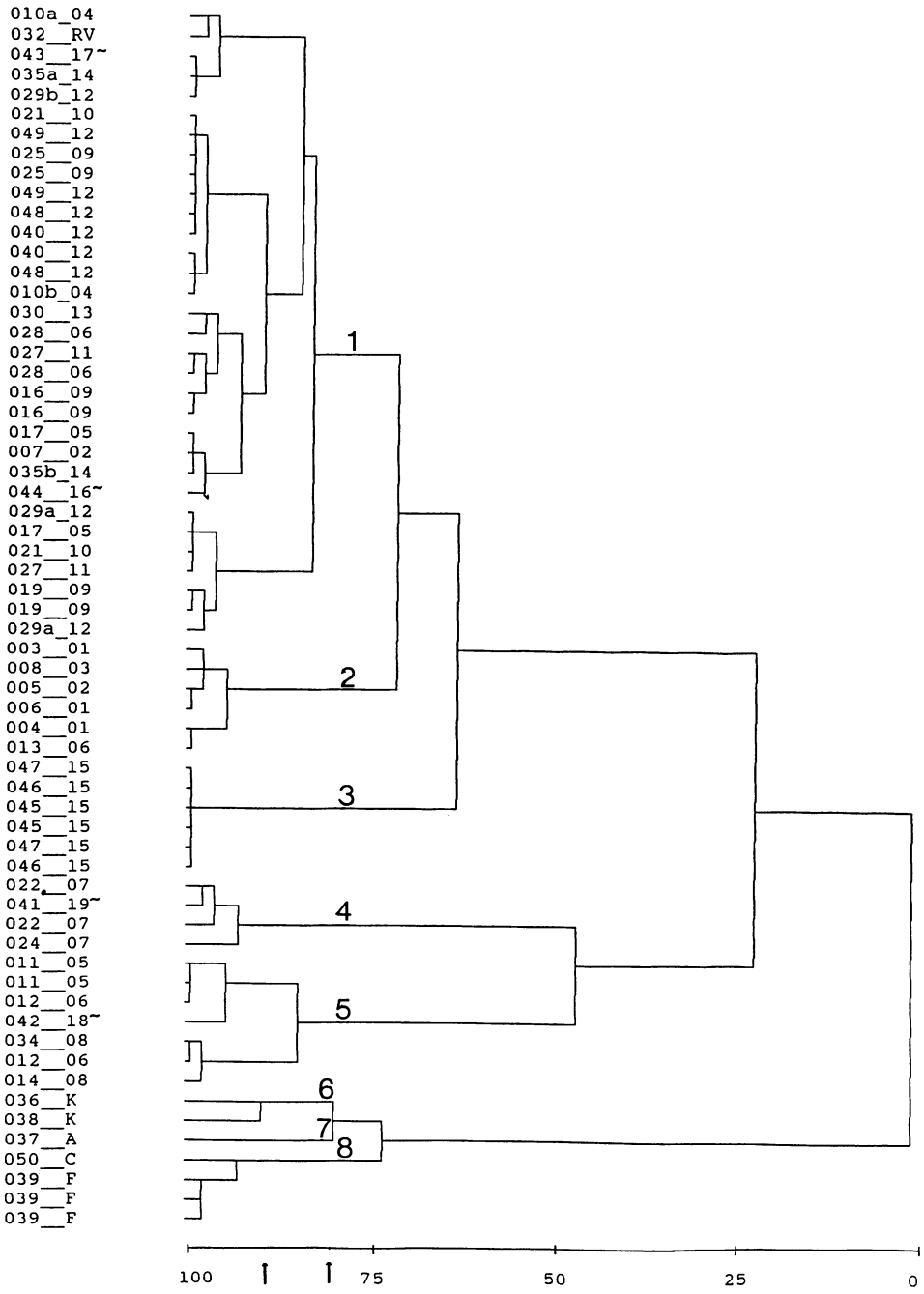

FIG. 2. Cluster analysis of 40 isolates of mycobacteria. Horizontal axis is hierarchical level of percent similarity. Isolate designations at the left include the strain number; a or b, to indicate different subcultures of the same isolate; and the patient number for isolates of *M. tuberculosis*. Isolates marked with a tilde (~) are from a cluster of cases (see text). Arrows indicate 90% (left) and 83% (right) hierarchical levels (see text for details). Abbreviations: K, *M. kansasii*; A, *M. avium*; C, *M. chelonae*; F, *M. fortuitum*; RV, *M. tuberculosis* H37RV.

(wt/vol) acrylamide in the stacking gel and 10% acrylamide in the resolving gel. Electrophoresis was performed in a protean dual-vertical-slab-gel apparatus (Bio-Rad Laboratories) at a constant current of 10 mA for the stacking gel and 15 mA for the resolving gel. The tank cooling water was recirculated at 18 ± 1°C. Gels were 16 cm in the vertical dimension and were run until the dye front had travelled to 1 cm from the bottom of the gel. Molecular weight marker proteins of 200,000, 116,250, 97,400, 66,200, 45,000 (all BioRad high-molecular-weight standards), and 29,000 (Sigma) were placed at the edges and in every third track along the gel. Nineteen tracks were run on each gel; a 20-well comb was used.

Silver staining was performed by the method of Patel et al. (8), with some minor modifications. In brief, gels were fixed overnight in 50% methanol, washed for 30 min in water, and soaked in silver diamine solution (21 ml of 0.36% [wt/vol] NaOH, 1.4 ml of 35% [wt/vol] NH_4OH, and 4 ml of 20%

[wt/vol] silver nitrate made up to 100 ml with water) for 15 min with constant shaking. Gels were then placed in reducing solution (2.5 ml of 1% citric acid and 0.26 ml of 36% [wt/vol] formaldehyde made up to 500 ml with water) until protein bands appeared. The reaction was stopped with 5% (wt/wt) acetic acid, and the gel was destained in Farmer's solution (0.6% sodium thiosulfate, 0.3% sodium carbonate, 0.1% potassium ferricyanide) overnight, washed in water until all the yellow Farmer's solution was eluted, fixed three times for 1 h each time in 50% methanol, and restained with silver as before. The intensity of silver staining was controlled by placing gels in reducing solution for 6 to 8 min and monitoring band development with a bacterial protein preparation of known pattern in each gel.

Gels were digitized on the day of staining. The detailed method of analysis will be published elsewhere (6). In brief, gels in a plastic bioassay dish (Nunc) were placed on a high-intensity light box (Wardray) and digitized with a charge-coupled-device camera (model 90; Datacopy Corp.) with a 55-mm Micro-Nikkor lens and Hoya HA50 infrared filter giving 1,728 by 2,240 picture elements on a 256-point grey scale. Recording was controlled by an IBM PC/AT-compatible microcomputer running MS-DOS and using WIPS software supplied with the camera. All further image processing used customized software. Gels were viewed in detail, and the unwanted margins were deleted with programs compiled by the Microsoft C compiler, version 5.1. They were then transferred to the Xenix partition of the hard disk. Tracks were located automatically, and a one-dimensional density plot was produced by taking an average across the track at each point along its axis. The plot lengths were normalized by using a linear fit on segments of $\log_e$ of the molecular weight, and background density variation was corrected by a fast Fourier routine to apply a high-pass step filter. Density plots were then compared by using Fourier routines to calculate the correlation between pairs of plots and to derive a similarity coefficient. Cluster analysis of the similarity matrix produced was by a Euclidean distance agglomerative algorithm.

RESULTS AND DISCUSSION

We used Lowenstein-Jensen slopes rather than liquid media because Lowenstein-Jensen is the medium normally used for primary isolation. Sufficient growth of a pure culture permits fingerprinting within 24 h, and it avoids the difficulties of obtaining sufficient growth in liquid media.

All isolates had a 30- to 40-band protein fingerprint. Reproducible differences between isolates occurred in the relative densities and shapes of bands as well as in band positions (Fig. 1).

Similarity coefficients from known replicate samples should approach 100%. A histogram of 57 similarity coefficients for replicate tracks of the same isolate on the same gel was a beta distribution of mean 95% (standard deviation, ±4%), and one of 82 coefficients between gels had a similar distribution of mean 82% (standard deviation, 6%). Similarity coefficients for pairs of tracks within a gel were therefore multiplied by 0.9 before a cluster analysis was performed to allow for the greater between-gel variation. Values of 0.8 to 1 were inspected, and as we have found for gram-negative bacteria (unpublished data), 0.9 clustered the maximum number of known replicates.

A cluster analysis of the matrix of similarities is shown in Fig. 2. One method of defining phenons is to estimate the level at which within-cluster variation is at a minimum compared with between-cluster variation. The coefficient of Véron (11) defines five clusters at the 64% level (Fig. 3). However, this results in some visually rather different fingerprints appearing in the same group and is therefore probably not justified.

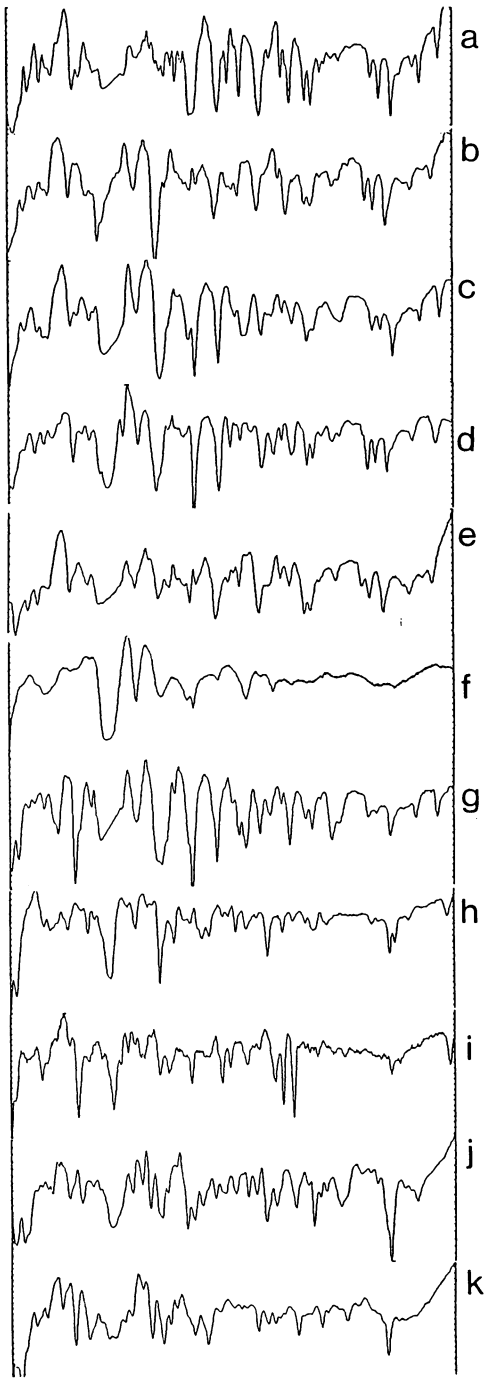

FIG. 3. Representative fingerprints of isolates from each cluster after normalization and application of high-pass step filter. Isolates are from patients 032 (a), 040 (b), 007 (c), 005 (d), 047 (e), 022 (f), 012 (g), 036 (h), 37 (i), 50 (j), and 039 (k). Fingerprints a through g are *M. tuberculosis*, and h through k are *M. kansasii*, *M. avium*, *M. chelonae*, and *M. fortuitum*, respectively.

Another method is to take the hierarchical level at which

all the known replicates cluster appropriately. This is the 83% level, which divides the isolates into eight groups. Isolates from other species are clearly separated from *M. tuberculosis* and, with the exception of *M. chelonae* and *M. fortuitum*, from each other, confirming the ability of protein fingerprinting to distinguish species (6). Five groups of *M. tuberculosis* isolates are delineated; four groups contained isolates from between one and five patients, and one group contained isolates from 12 patients. There was no clear relationship between the geographical origins of isolates and their grouping. Serial isolates from the same patient clustered as expected, with the exception of isolates from African patients 02 and 06.

Most of the known replicate samples cluster at or above the 90% hierarchical level; if all did so, discrimination between strains would be much enhanced. One of the major problems affecting reproducibility is that final stain development is subjective. The charge-coupled-device camera is able to detect many more grey levels than are discerned by the eye, and it would thus be desirable to have stain development controlled digitally.

This technique could be useful for the rapid examination of small clusters of isolates in outbreaks. For instance, isolate 044 is from a nurse, sputum smear positive for acid-fast bacilli, who developed symptoms of pulmonary tuberculosis between 3 and 6 months after nursing two patients (isolates 043 and 041). Both patients also had sputum smear-positive tuberculosis. Isolate 042 was from a human immunodeficiency virus-positive patient who in turn developed new lung lesions and became sputum smear positive for acid-fast bacilli 6 weeks after being in contact with the nurse. Isolates 041 and 042 are clearly distinguished from that of the nurse (044), leaving a possible connection between 043 and 044 only.

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