A Method of Classifying Solid Leprosy Bacilli Based on the Number of Band Structures on Them[1]

S. Izumi, M. Nishiura, M. Sugimori, S. Okada and H. Takizawa[2]

Mycobacterium leprae and other mycobacteria have peculiar band structures visible on the surface of the bacillary cell body by electron microscopy (Fig. 1 a & b). These were first noticed by Okada in 1958 ([3]). Structural details of these bands have been described ([2]). In the present study, a method of recording the number and distribution pattern of band structures is presented.

MATERIALS AND METHODS

Preparation of the specimen for the counting of band structures. A small tissue fragment taken directly from a leproma is macerated with two surgical knives in a drop of saline on a Maximow slide. After adding a few drops of saline, the suspension is stored overnight in a refrigerator in order to remove soluble tissue components from the surface of the bacillus. The supernatant of the suspension is then carefully removed with an injection syringe. After adding a few drops of fresh saline, the suspension of leprosy bacilli is sucked in and out of a tuberculin syringe several times so as to make a fairly even suspension.

Four hundred mesh copper grids for electron microscopy, coated with collodion film, are supported over the edge of a desk by means of sharp forceps. A small drop of the bacillary suspension is placed on each grid. When the grids have dried, they are washed in distilled water to remove the salt crystals. Later the grids with bacilli are shadow-cast with platinum-palladium, and examined with an electron microscope. As the counting of the band structures is directly carried out on the fluorescent screen of the electron microscope, it is necessary to examine the bacilli at the accelerating voltage of about 80,000.

Method of counting band structures. The size of each hole of the 400-mesh copper grid being about 30 μ x 30 μ, it is easy to examine all bacilli in a hole of the grid, and by shifting the holes one by one on the fluorescent screen, all bacilli in all holes of the grid can be counted without repetition.

Solid bacilli are classified into the following different types based on the number of band structures on them.

(a) Solid bacilli with no band ($B = 0$)
(b) Solid bacilli with one band ($B = 1$)
(c) Solid bacilli with two bands ($B = 2$)
(d) Solid bacilli with three or more bands ($B \geq 3$)

A special recording form is used on which 50 bacilli are classified and recorded in one line of the form. By repeating the counting and recording of five groups of 50 bacilli, 250 bacilli from each clinical case of lepromatous leprosy are examined.

The Band Index is intended to indicate the average number of band structures per single solid bacillus and is defined as follows:

$$\text{Band Index} = \frac{\text{Total number of band structures}}{\text{Total number of solid bacilli examined}}$$

Band structures have been found on leprosy bacilli in all active lepromatous lesions from the 25 cases thus far studied. The word "active" here means that the lesion contains a large number of solid bacilli. Classification of the band structures of solid leprosy bacilli has been carried out in 14 cases of lepromatous leprosy. Count-

1 Received for publication 23 September 1969.
2 S. Izumi, M.D., Leprosy Research Laboratory, Kyoto University and the Department of Chemotherapy, Komyo-en Leprosarium, Okayama-prefecture, Japan; M. Nishiura, M.D., M. Sugimori, Phar. B, S. Okada, M.D. and H. Takizawa, M.D. Leprosy Research Laboratory Kyoto University School of Medicine, Sakyo-ku, Kyoto, Japan.

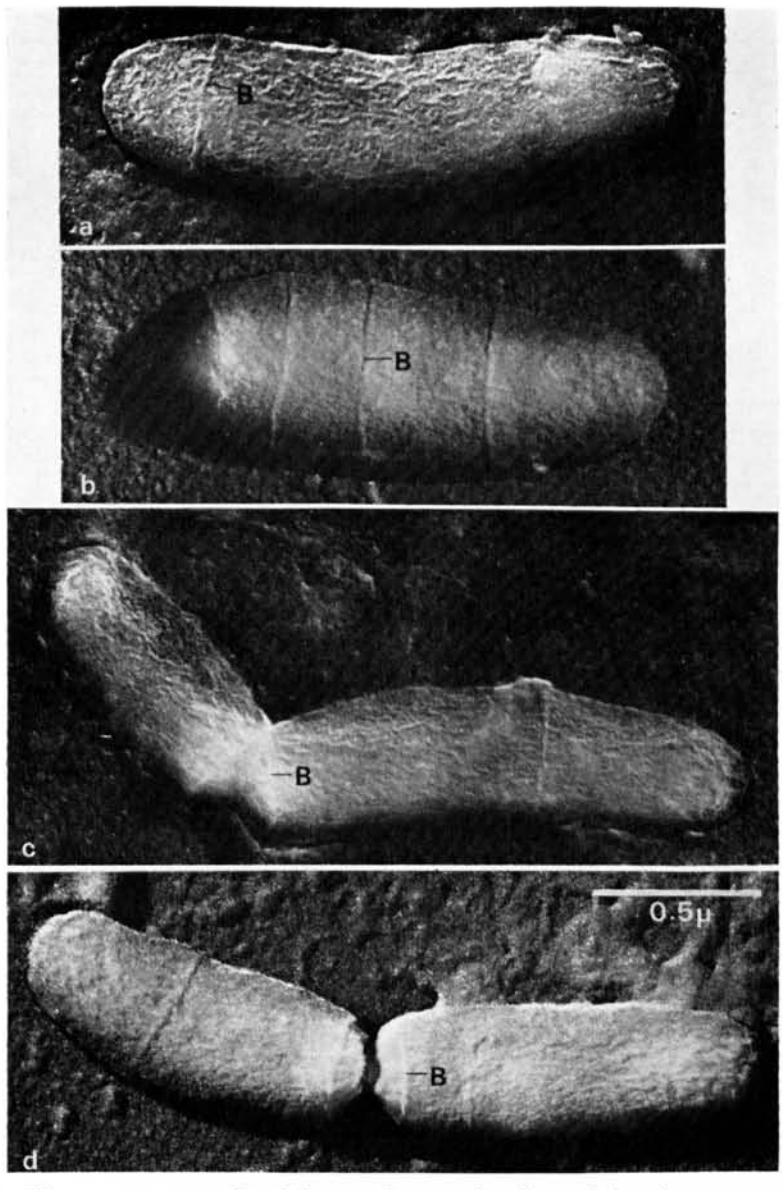

FIG. 1. Electron micrographs of human leprosy bacilli with band structures (**B**) (a) Bacillus with one band; (b) bacillus with four bands; (c) and (d) bacillus during cell division. Newly formed band structures are noted on both sides of strictures. Magnification: 60,000X.

ing of other mycobacteria was also done on H37Rv, H37Ra, BCG, *M. avium* (Jucho strain), *M. bovis* (MR strain) and *M. lepraemurium* (Hawaiian strain).

RESULTS

Band structure counts. Table 1 shows the percentage of each band-type (B = 0, B = 1, B = 2, and B ≧ 3) of solid leprosy bacilli examined in 14 lepromatous patients. Band indices are also shown in the same table.

The results of similar counting of band structures of mycobacteria other than human leprosy bacillus are shown in Table 2.

As recorded in Table 1, there are various patterns of band distribution. Among the various band types, B=0 and B=1 usually

TABLE 1. *Band types and Band Index of leprosy bacilli.*

Case No.	B = 0 %	B = 1 %	B = 2 %	B ≧ 3 %	Band Index
1	47.2	31.6	15.2	6.0	0.82
2	28.8	58.4	11.6	1.2	0.85
3	33.5	43.8	18.1	4.6	0.95
4	38.0	38.0	16.4	7.6	0.94
5	30.0	48.4	16.8	4.8	0.97
6	38.4	38.0	19.6	4.0	0.90
7	29.6	37.6	24.8	8.0	1.13
8	39.2	41.2	15.6	4.0	0.85
9	26.0	52.0	16.4	5.6	1.03
10	34.8	43.6	17.6	4.0	0.91
11	19.6	36.4	25.6	18.4	1.51
12	43.6	38.4	15.2	2.8	0.77
13	25.2	39.2	25.2	10.4	1.26
14	32.0	48.4	15.8	4.4	0.92

TABLE 2. *Band types and Band Index of various mycobacteria.*

	B = 0 %	B = 1 %	B = 2 %	B ≧ 3 %	Band Index
H37Rv	78.0	21.6	0.4	0.0	0.22
H37Ra	66.8	28.4	3.6	1.2	0.39
BCG	67.2	31.2	1.2	0.4	0.35
M. bovis (MR)	83.6	16.4	0.0	0.0	0.16
M. avium (Jucho)	56.0	31.6	10.4	2.0	0.58
M. smegmatis	56.0	43.6	0.4	0.0	0.44
M. lepraemurium (Hawaii)	7.6	59.6	25.6	7.2	1.33

TABLE 3. *Repeated count of Band Index in five groups of 50 human leprosy bacilli in each case.*

Case No.	Group 1	Group 2	Group 3	Group 4	Group 5	Average
1	0.90	1.10	0.50	0.80	0.82	0.82
2	1.02	0.72	0.78	0.84	0.90	0.85
3	0.94	0.98	0.96	0.88	1.00	0.95
4	0.60	0.90	0.98	1.16	1.06	0.94
5	0.78	1.02	0.76	1.28	1.02	0.97
6	1.00	0.94	0.82	0.90	0.86	0.90
7	1.06	1.06	1.12	1.16	1.24	1.13
8	0.86	0.90	0.70	0.78	1.00	0.85
9	1.22	1.24	1.10	0.90	0.68	1.03
10	0.86	1.14	0.76	0.74	1.06	0.91
11	1.78	1.44	1.42	1.42	1.48	1.51
12	0.64	0.78	0.80	0.74	0.90	0.77
13	1.26	1.22	1.30	1.28	1.26	1.26
14	0.96	0.92	1.08	0.72	0.94	0.92

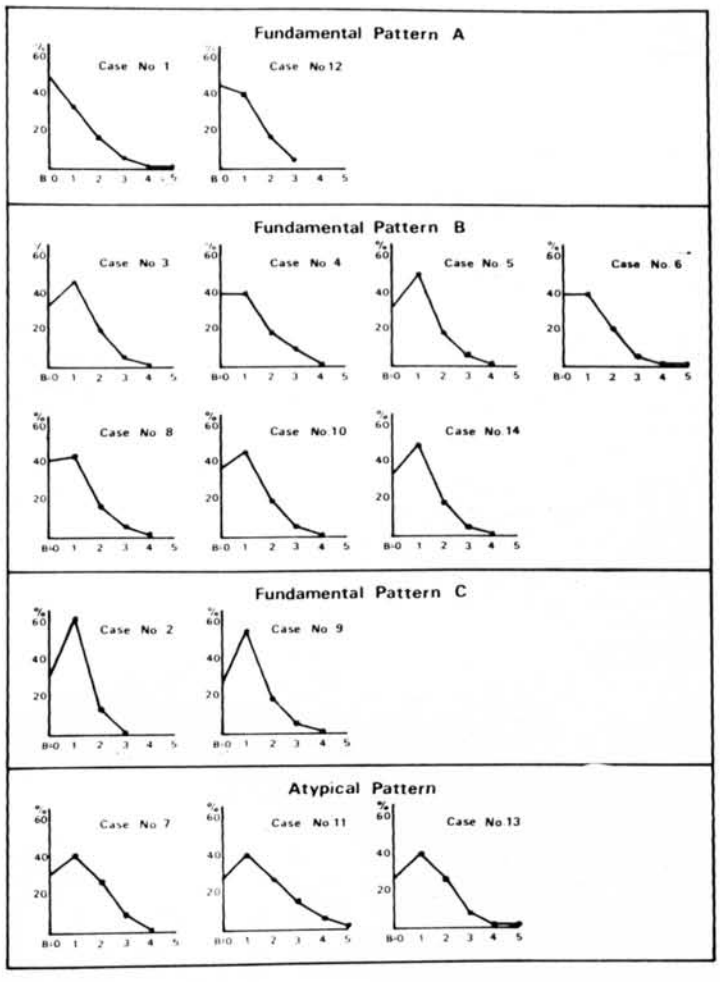

FIG. 2. Four patterns of band distribution of human leprosy bacilli.

represent the largest percentage. Therefore the various band distribution patterns can be grouped into three fundamental and one atypical pattern:

Fundamental pattern A: The number of B = 0 is larger than that of B = 1.

Fundamental pattern B: The number of B = 0 is almost equal to that of B = 1.

Fundamental pattern C: The number of B = 0 is smaller than that of B = 1.

Atypical pattern: This pattern is distinguished from the other patterns by having a higher percentage of bacilli of the B = 2 and B = 3 types.

Figure 2 shows the result of the grouping of each case based on detailed statistical calculation. It is still difficult to correlate these patterns directly with the clinical activity of the lesions. However (Fig. 3), the band distribution of all the cultivable mycobacteria examined shows a steep fundamental pattern A which is very different from the patterns of human leprosy bacilli. Only *M. lepraemurium* (Hawaiian strain) showed a fundamental pattern C with high Band Index.

Band Index. Band Index was determined on the basis of groups of 50 leprosy bacilli. Similar counting was repeated on five such groups of bacilli (total: 250 bacilli) in each case. Table 3 notes that the band indices show fairly uniform values even when these values are based on only 50 bacilli. In order to determine whether or not there are statistically significant differences between the band indices of these 14 cases of lepromatous patients, analysis of variances for one-way layout method were carried

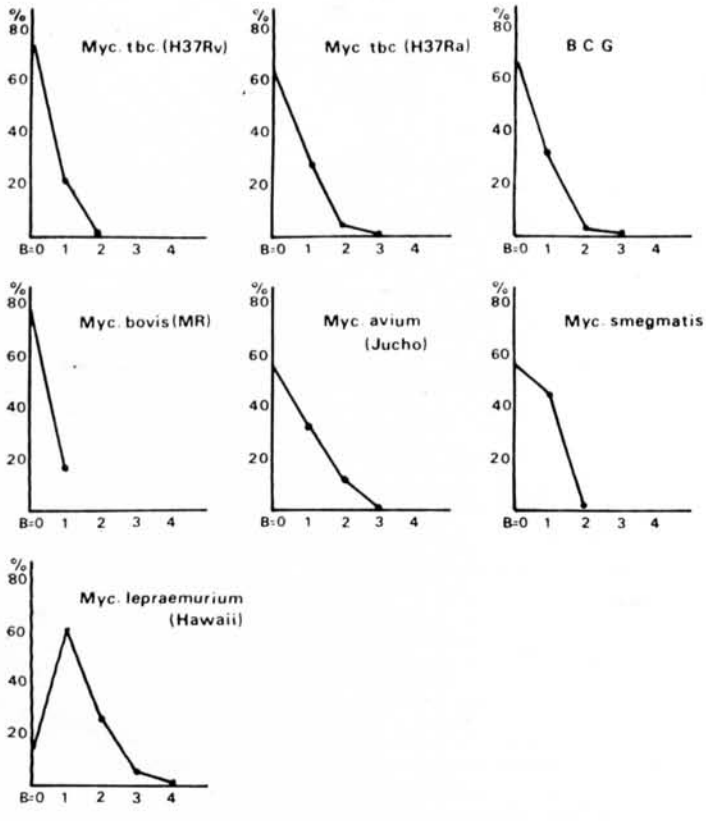

FIG. 3. The band distribution patterns of various mycobacteria other than human leprosy bacillus.

out. The null hypothesis is that "there are no significant differences between band indices of these cases of lepromatous leprosy."

Table 4 presents the results of the calculation as shown by the analysis-of-variance table. As the variance ratio (8.538) is bigger than the acceptance region (2.67), the above null hypothesis is rejected, i.e., "there are statistically significant differences between band indices of these cases of lepromatous leprosy."

Confidence intervals of band indices. The real values of the Band Index of the bacterial population of individual patient is estimated by the possible range of Band Index values based on the actual values of samples. For this estimation Student's t-distribution is used. The results of this estimation of possible ranges (confidence intervals) of Band Index are shown in Figure 4. The band indices of human leprosy bacilli are situated most frequently between 0.7-1.2. Only two patients among 14

TABLE 4. *Analysis-of-variance table.*

Factor	Sum of square	Degree of freedom	Mean square	Variance ratio
Main effect	25,010.3	13	1,923.9	0.000
Error	12,624.0	56	225.4	
Total	37,634.3	69		

Acceptance region: $F_{56}^{12}(0.05) = 2.67$

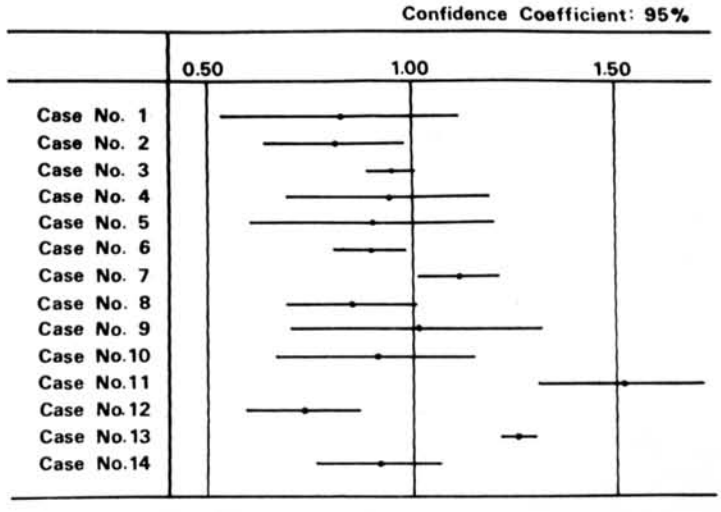

FIG. 4. The confidence intervals of 14 lepromatous patients.

patients showed strong deviation from this general tendency.

Similar methods for the estimation of the possible range of band indices were applied to various mycobacteria. Figure 5 shows the results of this estimation, and it is evident that the band indices of cultivable mycobacteria such as H37Rv, H37Ra, BCG, *M. bovis*, *M. avium* and *M. smegmatis* are much lower than those of human leprosy bacilli. A strain of *M. lepraemurium* (Hawaiian strain) has, however, shown exceptionally high values of Band Index.

DISCUSSION

The statistical method used in this study. As described previously, 50 solid leprosy bacilli were recorded as a group, and five such groups (i.e., 250 bacilli) were examined in each case. Thus, five band distribution patterns and five band indices were obtained for each lepromatous patient. These were checked by the chi-square test to determine whether there are statistically significant differences between the five band distribution patterns from each case. When significant differences were found, re-examination of the band structure of this patient was carried out.

Analysis of variance technic for one-way layout method was used to determine whether or not there are statistically significant differences among band indices of these 14 lepromatous patients. In using this statistical method, it is necessary to test uniformity of variances among these 14 clinical cases. This uniformity was confirmed by Hartley's method (1).[3]

The biologic meaning of band structure. In this study, we found that band structures are not characteristic only of human leprosy bacilli, but are commonly seen also on other mycobacterial cell bodies. Twenty-four hour treatment of bacilli with chloroform or petrol ether does not destroy the band structures. It is clear that each species of mycobacteria does not have constant values of Band Index or a definitive pattern of band distribution. But, generally speaking, there are rather striking differences of the band distribution patterns and index values between human leprosy bacilli and other mycobacteria. The present study presents interesting data concerning the

[3] In Hartley's method, the ratio of the largest variance to the smallest variance (F_{max}) is compared with acceptance region ($F_{max}(\alpha)$). $F_{max}(\alpha)$ is computed from

$$F_{max}(\alpha) = \exp \{ w_k(\alpha) \sqrt{[2/\nu - 1]} \},$$

where $w_k(\alpha)$ is the 100α % point of the 'range' w, in independent normal sample of size k, the values of which are already tabulated. 'ν' shows degree of freedom in individual case. As the 'ν' is 4 and w_{14} (0.01) is 5.40, $F_{max}(0.01)$ (i.e. acceptance region) is computed at 82.19. On the other hand, the largest variance in this study is 561.2×10^{-4} from case 9 and the smallest one is 8.8×10^{-4} from case 13. So, F_{max} is computed at 63.8 (561.2/8.8). As the F_{max} is smaller than acceptance region, it is possible to conclude that the variances of the band indices in these 14 cases of lepromatous patients are uniform.

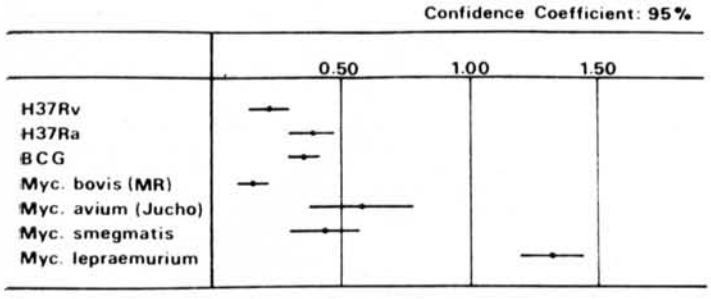

FIG. 5. The confidence intervals of various mycobacteria other than human leprosy bacillus.

band distribution patterns and indices, but the real biologic meaning of band structure is still enigmatic.

While counting band structures, many electron microscopic photos were made of cell division. These suggest some relationships of the band structure to cell division. When cell division occurs at the center of bacterial cell body, band structures are formed on both sides of the stricture (Fig. 1 c & d). However, in case of branching, which often can be seen in the smegma bacillus, the bacilli do not always form band structures. It appears that different types of cell multiplication such as central cell division and branching may introduce different band structure distribution patterns in the various mycobacteria.

An analogous structure can be seen on the surface of the yeast cell wall. Using fluorescent staining and electron microscopic technic, Eva Streiblová and co-workers found that formation of this structure has a close relation to cell division (4). However, since yeast cell walls have a very different structure from those of mycobacteria, interpretation of the findings from yeast cannot be applied directly to mycobacteria.

Usual electron microscopic technic such as ultra-thin sectioning or shadow-casting can not demonstrate surface and intracellular structures simultaneously. Some new technic such as freeze-etching may be useful for the further elucidation of structural details. More observations on bacilli at differing cultural stages will be necessary in order to clarify the biologic significance of the band structures.

SUMMARY

Distribution patterns of band structures and the Band Index of leprosy bacilli taken from 14 cases of lepromatous leprosy patients and of other mycobacteria such as H37Rv, H37Ra, BCG, *M. bovis* (MR strain), *M. avium* (Jucho strain), *M. smegmatis* and *M. lepraemurium* (Hawaiian strain) were studied and statistically evaluated.

It was found that the band distribution patterns of human leprosy bacilli can be classified into four different patterns. The band distribution patterns of cultivable mycobacteria were quite different from those of human leprosy bacilli and they belonged mostly to one of the *M. leprae* patterns while the Hawaiian strain of murine leprosy bacilli showed a pattern resembling the third *M. leprae* pattern.

There were statistically significant differences between the band indices of the 14 lepromatous leprosy instances studied. These Band Index values of human leprosy bacilli were much higher than those of cultivable mycobacteria. Generally speaking, the band indices of human leprosy bacilli were situated most frequently between 0.7-1.2. On the other hand the band indices of cultivable mycobacteria had values near 0.3-0.4.

RESUMEN

Los patrones de distribución de las estructuras en banda y el índice de banda de los bacilos de lepra tomados de 14 casos de enfermos con lepra lepromatosa, y de otras micobacterias tales como H37Rv, H37Ra, BCG, *M. bovis* (Cepa MR), *M. avium* (Cepa Jucho), *M. smegmatis* y *M. lepraemurium* (Cepa de Hawaii) se estudiaron y evaluaron estadísticamente.

Se encontró que los patrones de distribución de bandas de los bacilos de lepra humana se podían clasificar en cuatro patrones diferentes. Los patrones de distribución de bandas de las

micobacterias cultivables eran bastante diferentes de los bacilos de lepra humana y correspondían en su mayor parte a uno de los patrones del *M. leprae* mientras que los bacilos de lepra murina de la Cepa de Hawaii mostraban un patrón que se asemejaba al tercer patrón del *M. leprae*.

Habían diferencias estadísticamente significativas entre los índices de banda de las 14 muestras de lepra lepromatosa. Estos valores de índice de banda de los bacilos de lepra humana fueron mucho más altos que los de las microbacterias cultivables. En términos generales los índices de banda de los bacilos de lepra humana estaban situados con mayor frecuencia entre 0.7-1.2. Por otra parte, los índices de banda de las micobacterias cultivables tenían valores cerca de 0.3-0.4.

RÉSUMÉ

Les profils de distribution des structures en bandes, et l'index de bande de bacilles de la lèpre récoltés dans 14 cas de lèpre lépromateuse, et d'autres mycobactéries telles que H37Rv, H37Ra, BCG, *M. bovis* (souche MR), *M. avium* (souche Jucho), *M. smegmatis* et *M. lepraemurium* (souche de Hawaii) ont été étudiés et statistiquement évalués.

On a trouvé que les profils de distribution des bandes dans les bacilles de la lèpre humaine pouvaient être classés en 4 groupes différents. Les profils de distribution des bandes des mycobactéries cultivables étaient tout à fait différents de ceux observés dans les bacilles de la lèpre humaine, et relevaient principalement de l'un des profils de *M. leprae*, alors que les bacilles de la lèpre murine (souche de Hawaii) présentaient un profil ressemblant au troisième profil de *M. leprae*.

On a relevé des différences statistiquement significatives entre les index de bande dans les 14 cas de lèpre lépromateuse qui ont été étudiés. Les valeurs de ces index de bande dans les bacilles de la lèpre humaine étaient beaucoup plus élevés que ceux relevés pour des mycobactéries cultivables. De façon générale, les indices de bande dans les bacilles de la lèpre humaine se situaient plus fréquemment entre 0.7 et 1.2. D'autre part, les indices de bande dans les mycobactéries cultivables présentaient des valeurs proches de 0.3-0.4.

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