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Comparative Study on the Thin-layer Chromatographic Pattern of Methyl Mycolates of *M. leprae* and Related Species[1]

Arun K. Datta, Vishwa Mohan Katoch, Vishnu Dutt Sharma, and Kiran Katoch[2]

Serology ([23]), biochemical tests ([17]), and chemical analysis of lipids present in the mycobacterial cell wall ([5]) have proved to be powerful tools for the classification and identification of mycobacteria. These tests have also been applied for the taxonomic identification of *Mycobacterium leprae* ([9, 16, 29]). But most of these tests are generally based on the biochemical properties of cultures growing *in vitro* ([17]). Therefore, identification of *M. leprae* is still a formidable problem, more than 100 years after its discovery by Armauer Hansen, because *M. leprae* cannot as yet be cultivated in any artificial media. Different identification tests for *M. leprae* have been described from time to time depending on its immunological and biochemical characteristics in *in vivo* conditions ([26]). But none of these tests are solely reliable for its taxonomic identification since differences may exist in immunological and biochemical characteristics between *in vivo*- and *in vitro*-grown mycobacteria ([1, 24]). However, the mycolic acids' pattern seems to be a stable character, as has been found in the case of *M. microti* ([6]). The thin-layer chromatographic (TLC) pattern of *M. microti*-derived methyl mycolates does not show any difference between its *in vivo* and *in vitro* growth. Therefore, detailed analysis of *M. leprae*-derived methyl mycolates offers promise as a useful taxonomic marker.

The object of this study is to evaluate the thin-layer chromatographic technique using methyl mycolates for the differential identification of *M. leprae*. Classes of mycolic acids obtained from *M. leprae* isolated from infected tissues have been compared with those from several strains cultivated *in vitro* from leprosy biopsies ([3, 22, 27]). Comparisons have also been made with the other cultivable mycobacteria described as related to *M. leprae* ([13, 19, 21]).

MATERIALS AND METHODS

Mycobacterial strains. The cultivable strains studied are listed in Table 1. All of these strains are kept in our Mycobacterial Typing Center and are maintained on Löwenstein-Jensen (LJ) medium.

Skin biopsy material from five, highly bacillary-positive lepromatous leprosy patients was studied. Normal skin tissue was also collected as a control.

The bacterial masses of cultivable mycobacteria were collected in the mid-logarithmic phase of their growth in LJ media. They were washed with distilled water and freeze dried.

Skin biopsy materials were chopped into small pieces, homogenized by grinding in normal saline, washed with saline, and then processed for lipid extraction.

Lipid extraction. Mycobacterial cells were extracted with chloroform-methanol (2:1) for 24 hr at room temperature using Folch's method ([15]). Tissue samples were extracted using Bligh and Dyer's monophasic system ([4]). The residue was then extracted with chloroform-methanol (2:1) at room temperature for 48 hr. These extracts were pooled, evaporated to dryness under N_2, and kept at $-20°C$ for further use.

The material remaining after chloroform-methanol extraction from both the cells and the tissue materials was saponified by 0.5% (w/v) KOH in methanol for 4 days at 37°C

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[2] A. K. Datta, M.Sc., Assistant Research Officer; V. M. Katoch, M.D., Senior Research Officer; V. D. Sharma, M.Sc., Research Assistant, Department of Microbiology; K. Katoch, M.D., Senior Research Officer, Department of Medicine, Central JALMA Institute for Leprosy (I.C.M.R.), Taj Ganj, P.O. Box 31, Agra 282001, India.

TABLE 1. *Mycobacterial strains used in this study.*[a]

Species	Lab. no.	Strain no.	Source	Year isolated	Place	Collected from
M. vaccae[b]	J2 J13 J28	R-877R 426 R-859				Dr. J. Stanford, U.K.
ICRC strain	J11	1203	Skin lesion, patient	1961	Bombay, India	Dr. J. Stanford, U.K.
"*Mycobacterium w*"	J14 J16	1300R 1300S	Sputum, patient	1978	Madras, India	Prof. G. P. Talwar, India
Skinsnes' strains	N29 N30	MO-75 HI-75	Skin biopsy, patient	1975	Hawaii, U.S.A.	Dr. O. K. Skinsnes, U.S.A.
"*M. lufu*"	N34	464	Soil	1980	Lake "Lufu," Central Africa	Dr. F. Portaels, Belgium

[a] Strains were characterized using standard biochemical tests ([17, 30]).
[b] Type strain.

([10]). The pellets were washed twice with methanol and then extracted with ether. These ether-soluble lipids, known as bound lipids, contain methyl mycolates. Methyl mycolates of *M. leprae* (isolated from armadillo-infected tissue) were obtained from Dr. P. Draper of the Medical Research Council, U.K., who also supplied methyl mycolates of *M. tuberculosis* kindly prepared by Dr. N. Polgar.

Thin-layer chromatography (TLC). Precoated, high-performance TLC plates (5 × 5 cm) of silica gel $60F_{254}$ (E. Merck, Germany) were used. Samples were applied, and the plates were developed first with petroleum ether (b.p. 60–80°C)-diethyl ether (9: 1, v/v) five times and then once with methanol ([13]).

For two-dimensional TLC, the system followed was that of Minnikin, *et al.* ([18]). The plates were developed three times with petroleum ether (b.p. 60–80°C)-acetone (95: 5) in one direction, and then once with toluene-acetone (97:3) in the second direction. Spots were visualized either by charring the chromatograms at 120°C for 8–10 min after spraying with 0.5% $K_2Cr_2O_7$ in 10% H_2SO_4 ([6]) or by charring the chromatograms at 120°C for 20–30 min after spraying with a 1% ethanolic solution of molybdophosphoric acid.

RESULTS

The results showed that the TLC pattern of the methyl mycolates extracted from infected human tissue material is the same as that of *M. leprae*-derived methyl mycolates (armadillo origin) kindly sent by Dr. P. Draper (Fig. 1A). The normal tissue extract, however, did not show any presence of methyl mycolates. Chromatogram 1A shows the appearance of two spots of methyl mycolates derived from leprosy-infected tissue. By comparison with the standard methyl mycolates from *M. leprae* of armadillo origin as well as from *M. tuberculosis*, these spots were characterized as α- and keto-derivatives of methyl mycolates. Thus the present results corroborate earlier findings ([8, 10, 14, 28, 33]). A spot "F" appeared in the case of the leprosy-infected tissues which was not found in normal tissue. This appeared to be a higher fatty acid methyl ester and has not been reported earlier.

Two-dimensional TLC of *M. leprae*-derived methyl mycolates also showed two distinct spots for α- and keto-derivatives (Fig. 2). Some additional spots of unknown structure, however, appeared in the case of infected tissue-derived materials.

"*M. lufu,*" Skinsnes' strains, and "*Mycobacterium w*" showed the same TLC patterns (unidimensional) containing keto- and dicarboxy derivatives of methyl mycolates (Fig. 1B). *M. vaccae* showed one extra spot which co-chromatographed with the methoxy derivative of *M. tuberculosis*-derived methyl mycolates. The strain ICRC also showed a relatively faint spot which co-chromatographed with the methoxy deriv-

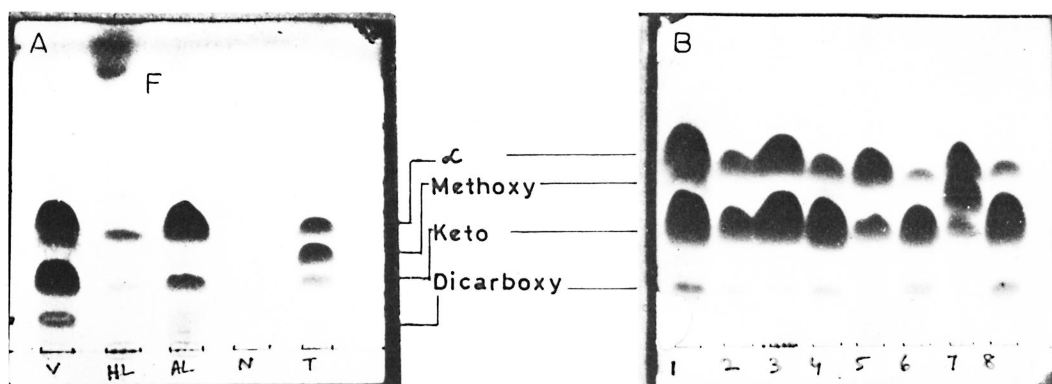

FIG. 1. Thin-layer chromatography of methyl mycolates run 5 times in petroleum ether (b.p. 60–80°C)-ether (9:1) and once in methanol. Plates were sprayed with 0.5% $K_2Cr_2O_7$ in 10% H_2SO_4 and then charred at 120°C for 8–10 min. In chromatogram A, the samples were from *M. vaccae* (v), leprosy-infected human tissue (HL), *M. leprae* (armadillo origin, AL), normal skin tissue (N), and *M. tuberculosis* (T). In chromatogram B, the samples were from *M. vaccae* (1), "*Mycobacterium w*," 1300 R (2) and 1300 S (3), ICRC strains (4), *M. leprae* (armadillo origin, 5), Skinsnes' strain MO-75 (6), *M. tuberculosis* (7), and "*M. lufu*" (8). F = non-hydroxylated fatty acid methyl ester.

ative. The two-dimensional system, however, clearly distinguished between *M. vaccae* and the ICRC strain (Fig. 3). "*M. lufu*" and the ICRC strain showed a similar pattern when the two-dimensional TLC system was followed.

DISCUSSION

One of the most characteristic features of mycobacteria is the presence of mycolic acids in their cell wall structure. These mycolic acids show a specific TLC pattern for individual species, and this can be used for the taxonomical identification of various cultivable mycobacteria ([18]). The presence of α- and keto-derivatives of methyl mycolates in *M. leprae*, therefore, appears to serve as a useful taxonomical marker for it.

The presence of α- and keto-methyl mycolates in *M. leprae* was previously shown by other workers using material derived from both human sources ([8, 14, 33]) and from infected armadillos ([10, 28, 32]). Previously, Draper, *et al.* ([11]) reported two-dimensional TLC patterns of methyl mycolates from *M. leprae* (armadillo origin). The present study is the first report showing the same from human sources.

In this study, it was found that leprosy patients' tissue-derived *M. leprae* contained α- and keto-mycolates in the proportion of about 70:30. Asselineau, *et al.* ([2]), however, found evidence of the presence of methoxy mycolates in armadillo-derived *M. leprae* along with the α- and keto-mycolates by mass spectrometry. We, however, could not detect methoxy mycolate in *M. leprae* using high-performance TLC plates. This might be due to their presence in very small amounts and could also be due to the fact that the reagents used might not be sensitive enough to detect such a small quantity of methoxy mycolates.

Several reports have appeared about alleged *in vitro* cultures of *M. leprae* ([7]). Therefore, it was of interest to analyze the mycolates of mycobacteria which have been cultured from leprosy-infected tissue biopsies. The Skinsnes' strains MO-75 and HI-75 ([27]) and the ICRC bacillus ([3]) were isolated from leprosy-infected biopsy materials. "*Mycobacterium w*" was collected from sputum ([22]). In the present study, all of these isolates showed patterns identical to those of the *M. avium-intracellulare-scrofulaceum* (MAIS) group. Young ([32]) also carried out somewhat similar studies with other isolates from leprosy lesions (three FMR isolates, ICRC strain C44, and Kato's strain A5) and showed their TLC patterns to be identical to those of the MAIS group ([32]) but different from that of *M. leprae* obtained from human tissues ([33]). Among the mycobacterial species included in the present study, "*M. lufu*" has been found to bear a close similarity to *M. leprae* in some re-

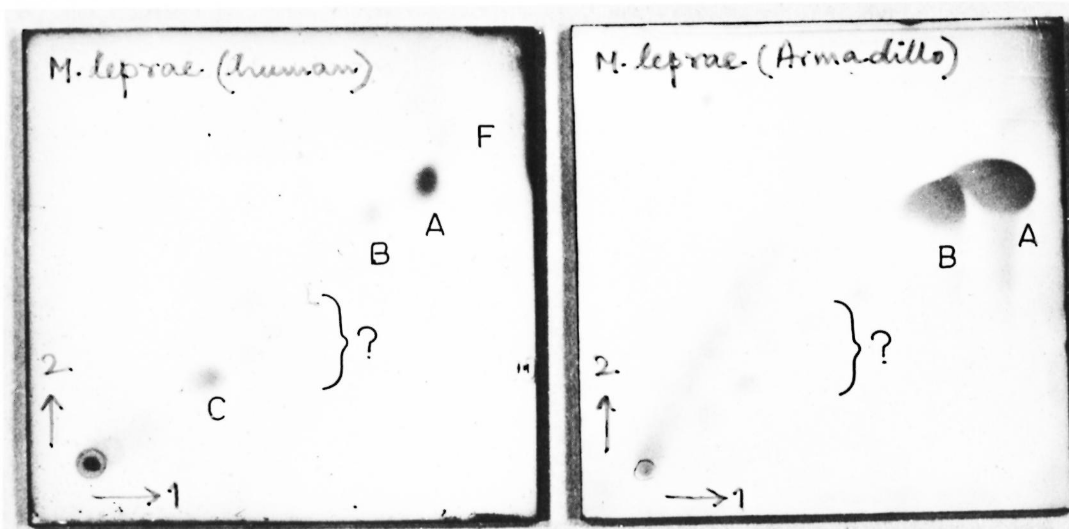

FIG. 2. Two-dimensional thin-layer chromatography of methyl mycolates run 3 times in petroleum ether (b.p. 60–80°C)-acetone (95:5) in the first direction and then once in toluene-acetone (97:3) in the second direction. Plates were sprayed with a 1% ethanolic solution of molybdophosphoric acid and then charred at 120°C for 20–30 min. In the chromatograms, samples from human infected tissue and the standard (*M. leprae*, armadillo origin) sent by Dr. P. Draper were used. F = non-hydroxylated fatty acid methyl esters; A = α-mycolate; B = keto-mycolate; C = unknown long-chain polar components; ? = unidentified minor components.

spects ([25]). *M. vaccae* has also been shown to have some biochemical and antigenic characteristics similar to those of *M. leprae* ([12, 13, 19, 20, 29, 31]). But none of the organisms showed a TLC pattern comparable to that of *M. leprae* (Table 2). Thus, it could be possible that the TLC pattern of methyl mycolates could serve as a marker for taxonomical identification. However, a detailed analysis should be done using all of the available mycobacterial species before considering this pattern as *M. leprae* specific.

Studies on methyl mycolates have been reported by other workers. In the present study, high-performance TLC plates have been used because of their very high effi-

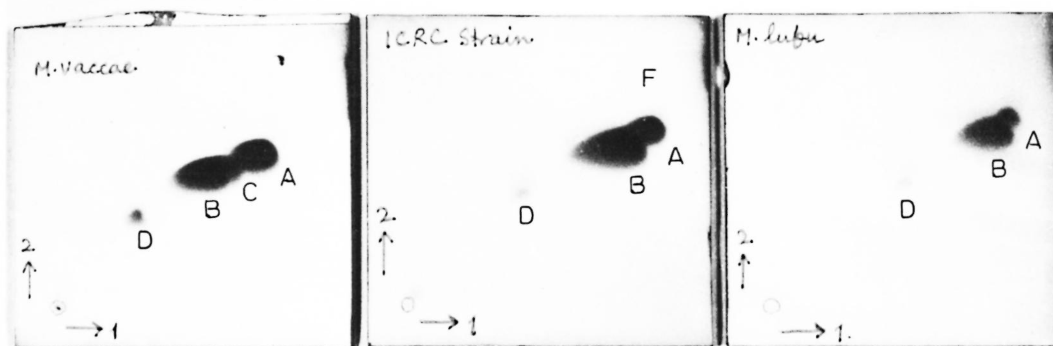

FIG. 3. Two-dimensional thin-layer chromatography of methyl mycolates run 3 times in petroleum ether (b.p. 60–80°C)-acetone (95:5) in the first direction and then once in toluene-acetone (97:3) in the second direction. Plates were sprayed with a 1% ethanolic solution of molybdophosphoric acid and then charred at 120°C for 20–30 min. In the chromatograms, samples were from *M. vaccae* J2, ICRC strain J11, and "*M. lufu*" N34. F = non-hydroxylated fatty acid methyl ester; A = α-mycolate; B = keto-mycolate; C = methoxy mycolate; D = dicarboxy mycolate.

TABLE 2. *Thin-layer chromatographic pattern of methyl mycolates: types of mycolates present in the different related specimens.*[a]

Mycobacterial specimens	Derivatives of methyl mycolates			
	α-	methoxy	keto-	dicarboxy
M. leprae				
Human tissue derived	+		+	
Armadillo tissue derived	+		+	
M. tuberculosis	+	+	+	
M. vaccae (3 strains)	+	+	+	+
"*Mycobacterium w*" (2 strains)	+		+	+
Skinsnes' strains (2)	+		+	+
ICRC strain	+	+[b]	+	+
"*M. lufu*"	+		+	+

[a] See Table 1 for strains tested.
[b] A very faint spot appeared.

ciency in separating lipids. Also, this plate requires a very minute quantity of material in comparison to conventional laboratory-prepared TLC plates. Even nanogram quantities of methyl mycolates from tissues with a bacillary load of about 10^6 bacilli would be sufficient for these high-performance TLC plates, while for conventional laboratory-prepared TLC plates 100 to 1000 times this quantity would be required. This is an obvious advantage in dealing with tissue-derived lipids. Another advantage of high-performance TLC plates is the reduced operational time; while conventional laboratory-prepared TLC plates require more than three hours, high-performance TLC plates need only 40 minutes. Further, the spots which appeared in the chromatogram were compact and, therefore, easily identifiable. Finally, these plates are also easy to handle, especially when multiple chromatography is to be performed.

It was shown earlier that the pyridine extractable acid-fastness test is a good identification marker for noncultivable *M. leprae* from infected tissues ([12]). It is therefore possible that the thin-layer chromatographic pattern of methyl mycolates along with the pyridine extractable acid-fastness test may serve as good taxonomical criteria for the identification of *M. leprae*.

SUMMARY

The thin-layer chromatographic (TLC) pattern of methyl mycolates of *Mycobacterium leprae* has been compared with the lipids extracted from *M. leprae*-infected tissue material and also with those of other cultivable mycobacterial species. It has been found that *M. leprae*-derived methyl mycolates give two spots, namely, α- and keto-derivatives, after charring the TLC plates with $K_2Cr_2O_7/H_2SO_4$ spray. Lipids extracted from infected tissue material also show spots with the same Rf values when compared with those of armadillo-derived *M. leprae*. Normal skin tissue extracts, however, do not show any presence of mycolates. This TLC pattern of *M. leprae*-derived methyl mycolates is characteristic and is not shown by any other mycobacterial species isolated from leprosy lesions. The strain and species which have been earlier found to resemble *M. leprae* antigenically or biochemically could readily be distinguished with the help of the TLC pattern of their methyl mycolates.

RESUMEN

Usando la cromatografía en capa fina se comparó el patrón de los metil micolatos del *Mycobacterium leprae* con el de los lípidos extraídos de tejido infectado con el *M. leprae*, y con el de los lípidos de especies micobacterianas cultivables. Se encontró que los metil micolatos derivados del *M. leprae* dan 2 manchas, principalmente de alfa- y ceto-derivados, después de rociar las placas con $K_2Cr_2O_7/H_2SO_4$. Los lípidos extraídos del tejido infectado con *M. leprae* y los lípidos extraídos del *M. leprae* derivado de armadillos, originan manchas con los mismos valores de Rf. Los extractos de piel normal no muestran la presencia de micolatos. El patrón cromatográfico de los metil micolatos derivados del *M. leprae* es característico de este microorganismo y no se encuentra en ninguna otra especie micobacteriana aislada de las lesiones de la lepra. La cepa y especies que antes se han considerado como semejantes al *M. leprae* por criterios antigénicos o bioquímicos, pueden ser fácilmente diferenciables en base al patrón cromatográfico de sus metil micolatos.

RÉSUMÉ

On a comparé les profils chromatographiques en couches minces (TLC) des méthyl-mycolates de *Mycobacterium leprae*, avec ceux des lipides extraits de tissu infecté par *M. leprae*, de même qu'avec ceux provenant d'espèce mycobactérienne cultivables. On a observé qu'après avoir été soumis à l'action de $K_2Cr_2O_7/H_2SO_4$, les méthyl-mycolates dérivés de *M. leprae* pré-

sentaient deux tracés, correspondant à des dérivés alpha-et keto-. Les lipides extraits de tissu infecté présentaient des tracés ayant les même valeurs Rf, lorqu'on les comparait avec ceux obtenus avec des bacilles de la lèpre récoltés chez le tatou. Par contre, des extraits de tissu cutané normal n'ont pas révélé la présence de mycolates. Ce profil chromatographique particulier des méthyl-mycolates de *M. leprae* est caractéristique; aucune autre espèce mycobactérienne isolée dans des lésions de lèpre ne le présente. Les souches et les espèces qui avaient paru jusqu'à présent ressembler à *M. leprae,* tant du point de vue antigénique que sur le plan biochimique, peuvent dès lors être facilement distinguées, grâce au profil chromatographique des méthyl-mycolates.

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