Acta vet. scand. 1983, 24, 225—237.

From the National Veterinary Institute and National Institute of Public Health, Oslo, Norway.

WILD ANIMAL MYCOBACTERIAL ISOLATES

CHARACTERIZATION BY CELLULAR FATTY ACID COMPOSITION AND POLAR LIPID PATTERNS

By

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SAXEGAARD, FINN, OTTO ANDERSEN and ERIK JANTZEN: *Wild animal mycobacterial isolates. — Characterization by cellular fatty acid composition and polar lipid patterns.* Acta vet. scand. 1983, 24, 225—237. — Thirteen strains of mycobacteria isolated from deer and various species of wild birds were analysed by gas chromatography (GC) for cellular fatty acids and by thin-layer chromatography (TLC) for polar lipids. These strains were compared to reference strains of Mycobacterium avium, M. paratuberculosis and M. malmoense. All the examined strains exhibited a generally similar fatty acid pattern characterized by relatively large amounts of hexadecanoate (16:0), octadecenoate (18:1), octadecanoate (18:0) and 10-methyl-octadecanoate (tuberculostearic acid, 10-Me-18:0). Several additional acids were also generally present but in smaller amounts. By means of small but distinct differences in fatty acid composition, the wild animal isolates could be distinguished from both M. paratuberculosis and M. malmoense but not from M. avium.

The TLC polar lipid patterns on the other hand separated the wild animal isolates into 2 distinct groups of complex and simple polar lipid composition which corresponded to the morphologically smooth and rough types, respectively. The complex patterns of the smooth strains were comparable to those of the M. avium serovars whereas both the rough wild animal isolates and all the M. paratuberculosis strains showed a simple pattern of polar lipids.

Both fatty acid profiles and TLC polar lipid patterns support allocation of the wild animal isolates to the MAIS complex. Moreover, the 2 chemical techniques, particularly the GC procedure, are very useful for a more rapid and precise identification of the slow-growing wild animal mycobacterial isolates which have hitherto been characterized on basis of vague criteria.

nontuberculous mycobacteria; wild animals; gas chromatography; thin-layer chromatography.

Mycobacterial infections caused by nontuberculuos mycobacteria frequently occur among wild animals such as roedeer and various species of wild birds (*McDiarmid* 1975). In a systematic study of infected wild animals collected in Norway,

Saxegaard (1981) tentatively characterized most of the etiological agents as Mycobacterium avium-like on the basis of their slow growth rate, nonchromogenicity, and pathogenicity for chickens. The strains did not grow in conventional mycobacterial media, and their extremely slow and sparse growth, even in media containing mycobactin, made it difficult to obtain sufficient cells for biochemical differentiation and for serology according to *Schaefer* (1965). Moreover, owing to the dry and crisp character of the colonies the bacterial suspensions were autoagglutinable and unsuitable for serology.

These problems of unambiguous identification of M. avium-like isolates initiated a search for alternative identification techniques. The use of gas chromatography (GC) of cellular fatty acids of mycobacteria has been successfully applied by several workers (*Thoen et al.* 1971, *Chomarat et al.* 1981, *Tisdall et al.* 1979, 1982, *Andersen et al.* 1982). Several workers have also successfully used thin-layer chromatography (TLC) of polar lipids as an aid in characterization of mycobacterial isolates (*Sehrt et al.* 1976, *Jenkins* 1981, *Brennan et al.* 1978, 1982). We have applied these 2 methods of chemical characterization to the group of M. avium-like strains in order to evaluate their suitability for use in the routine identification of clinical isolates.

Strains belonging to M. paratuberculosis and M. malmoense were also examined due to their assumed close relationship to the M. avium-like isolates.

MATERIALS AND METHODS

Microorganisms

The examined strains and their origins are listed in Table 1. All strains were examined by GC and, apart from the following, also by TLC: M. avium serotype 1 and 2, M. paratuberculosis str. 316F, 1 wood pigeon strain and the 4 strains of M. malmoense.

Three of the wild animal strains were of Danish origin. The remaining strains were isolated at The National Veterinary Institute. As described by *Saxegaard* (1981) the strains from the capercaillie, black grouse and goshawk had been identified biochemically and serologically as M. avium serotype 2. The other strains were characterized as M. avium-like on basis of their growth rate, nonchromogenicity and pathogenicity for chickens which, however, varied considerably.

The reference strains were maintained on Middlebrook's 7H10

Table 1. Origin of strains examined.

Reference strains investigated

Species	Strain	Origin
M. avium serotype 1	B 87	State Veterinary Serum Laboratory,
serotype 2	6194	Copenhagen, Denmark
serotype 3	6197	
M. malmoense	S 648	Malmø General Hospital,
	S 649	Malmø, Sweden
	S 650	
	S 709	
M. paratuberculosis	2 E	Central Veterinary Laboratory,
	316 F	Weybridge, England
	8 goat isolates	National Veterinary Institute, Oslo, Norway

Wild animal strains investigated

Animal species	No. of strains
Roe-deer (Capreolus capreolus)*	4
Capercaillie (Tetrao urogallus)	1
Black grouse (Lyrurus tetrix)	1
Hazel hen (Tetrastes bonasia)	1
Goshawk (Accipiter gentilis)	1
Sparrow hawk (Accipiter nisus)	1
Wood pigeon (Columba palumbus)**	4

* Two strains were of Danish origin.
** One strain was of Danish origin.

medium (Gibco, Bio-Cult Diagnostics LTD., Paisley, Scotland) without mycobactin or on Dubos' medium with 2 % mycobactin (M. paratuberculosis). The wild animal isolates, most of which required mycobactin, were maintained on 7H10 medium with or without mycobactin. Biomass for GC and TLC was obtained after growth on slants of 7H10 medium. In order to avoid contamination, tubes were preferred to plates. Purity was checked by additional inoculation of blood agar plates. Depending on the growth rate, purity was again checked after 3 to 8 weeks incubation at 37°C. Cultures were harvested in sterile distilled water, followed by heating at 80°C for 30 min, centrifugation

(400 × g, 15 min), and 1 wash in distilled water. Biomass intended for TLC analysis was used without washing.

Chemicals

Solvents of analytical grade were distilled before use. The standards of fatty acid methyl esters were obtained from Applied Science Laboratories Inc., State College, Pennsylvania. Thin-layer plates (20×20 cm 0.2 mm DC Alufolien Kieselgel $60F_{254}$ or 10×10 cm 0.2 mm DC HPTLC Kieselgel $60F_{254}$) were purchased from Merck Inc., Darmstadt, Federal Republic of Germany.

Fatty acid analysis

Dried bacterial cells (1—5 mg) were saponified with 1 ml 0.5 mol/l NaOH in dry methanol at 70°C for 20 min and methyl ester formation was achieved by treatment with 14 % BCl_3 in methanol at 85°C for 30 min as described by *Tisdall et al.* (1979).

Fatty acid methyl esters were analysed on a Hewlett-Packard 5710 chromatograph equipped with a flame ionization detector. A fused-silica capillary column (25 m × 0.2 mm) coated with SP-2100 methyl silicone (Hewlett-Packard Inc.) was used. The column was operated from 140 to 280°C increasing at 8°C/min with a carrier gas (helium) flow rate of 1.5 ml/min, injection port temperature of 250°C and a detector temperature of 300°C. The samples were introduced with the aid of a Hewlett-Packard 18740B-capillary column control in splitless mode. Peak areas and retention times were recorded by a Hewlett-Packard 3390A recorder-integrator.

Fatty acid methyl esters were primarily identified by comparing their retention times with those of the standards. In addition, the identities were confirmed by mass spectrometry using a Hewlett-Packard 5992A gas chromatograph/mass spectrometer computer system equipped with a fused-silica capillary column (25 m × 0.3 mm) coated with SP-2100 (Hewlett-Packard Inc.); ionization conditions were 70 eV at 170°C.

Extraction and deacylation of lipids

The technique of *Brennan et al.* (1978) was used. Dried bacilli (5—10 mg) were extracted by chloroform-methanol (2:1) (32 µl/mg bacteria) in culture tubes with teflon-lined screw caps at 50°C for 18 h. After centrifugation (500 × g, 30 min) the clear supernatant (lipid fraction) was deacylated by adding

an equal volume of NaOH (0.2 mol/l) in methanol followed by incubation at 37°C for 20 min. The reaction was stopped by neutralization by acetic acid and the solvents removed by a stream of N_2. The dry residue was extracted with 0.5 ml chloroform-methanol 2:1 after addition of 100 µl water. The organic phase was dried with N_2 and redissolved in 100 µl chloroform-methanol 2:1 before chromatography.

Thin-layer chromatography

Thin-layer plates were activated at 110°C for 30 min. After cooling in a vacuum desiccator to room temperature the samples (20 µl) were applied. The following 2 developing solvents (*Brennan et al.* 1978) were used: chloroform-methanol-water (60:27:4 v/v) and chloroform-methanol-water (45:5:0.5 v/v).

Developed chromatograms were dried and then sprayed with a solution of 0.1 % orcinol in 40 % H_2SO_4 in ethanol followed by heating at 110°C for 5—10 min.

RESULTS

The fatty acid compositions of the M. avium-like isolates and reference strains of the species M. avium, M. paratuberculosis and M. malmoense are given in Table 2. Examples of chromatographic fatty acid profiles are shown in Fig. 1. All examined strains exhibited a fatty acid composition typical of genus Mycobacterium (*Thoen et al.* 1971, *Tisdall et al.* 1978, 1982, *Chomarat et al.* 1981, *Andersen et al.* 1982). This common fatty acid pattern is characterized by high levels of hexadecanoate (16:0), octadecenoate (18:1), octadecanoate (18:0) and 10-methyl-octadecanoate (10-Me-18:0). Also present, but in lower concentrations, are tetradecanoate (14:0), pentadecanoate (15:0), hexadecenoate (16:1), 10-methyl-hexadecanoate (10-Me-16:0), heptadecanoate (17:0) and the unbranched acids of chain length C_{20} to C_{26}, i.e. pyrolytic products of the long-chain mycolic acids (*Lechevalier et al.* 1977, *Guerrant et al.* 1981).

The standard deviations of the data in Table 2 are unusually large in comparison with previous experience with other bacteria (*Jantzen et al.* 1978). This may be due to the long time interval of the study (about 26 months) and the correspondingly unsystematic variation in experimental conditions. At all events, the M. avium-like strains are indistinguishable from those of the M. avium reference strains. The high degree of similarity be-

Table 2. Cellular fatty acid composition (percentage of total) of the examined mycobacterial species.

Fatty acid[1]	Retention time (min)	M. avium-like (12)[2]	M. avium[3] (3)	M. paratuberculosis[4] (10)	M. malmoense[5] (4)
14:0	*6.73*	**4.0**[6] 2.7—5.5 1.0	**4.7** 3.5—6.2 1.4	**4.2** 0.5—6.4 1.8	**1.2** t—2.5 1.4
15:0	*7.91*	**0.9** 0.3—1.6 0.3	**0.9** 0.5—1.3 0.4	**1.0** 0.4—1.4 0.3	**1.0** t—2.7 1.3
16:1	*8.76*	**5.5** 2.4—8.3 1.7	**6.6** 5.1—8.4 1.9	**7.9** 4.0—17.7 4.5	**8.9** t—13.4 6.0
16:0	*9.17*	**39.1** 23.9—47.8 5.2	**40.0** 37.9—42.1 2.1	**26.6** 20.2—32.2 3.7	**36.2** 27.3—43.7 6.8
10-Me-16:0	*9.61*	**0.9** 0.4—2.0 0.4	**0.7** 0.5—0.8 0.2	**1.7** 0.8—3.0 0.9	t
X_1	*9.96*	**0.3** 0—0.7 0.2	**0.1** 0—0.2 0.1	**1.1** 0.6—1.6 0.4	t
17:0	*10.28*	**0.9** 0.3—2.6 0.7	**0.8** 0.4—1.1 0.4	**1.0** 0.5—1.5 0.4	**1.6** t—3.3 1.6
X_2	*10.80*	t	—	**0.3** 0—0.9 0.4	t
X_3	*11.01*	t	—	**2.5** 0.2—4.5 1.5	t
18:1	*11.14*	**15.4** 12.1—18.3 2.2	**15.7** 14.6—17.3 1.4	**18.0** 11.1—29.7 6.4	**12.5** 2.8—17.6 6.6
18:0	*11.47*	**7.0** 4.0—12.7 2.7	**7.0** 5.3—9.9 2.6	**8.7** 5.2—12.2 2.4	**12.0** 9.3—14.7 2.4
10-Me-18:0	*11.96*	**16.3** 11.9—23.0 3.8	**13.2** 12.1—14.0 1.0	**18.9** 8.4—26.6 5.8	**19.7** 14.9—23.8 4.5
20:0	*13.70*	**2.0** 0.9—3.2 0.7	**1.3** 1.2—1.5 0.2	**1.3** 0.7—2.4 0.5	**2.9** 2.3—3.7 0.6
22:0	*15.78*	**1.0** 0.6—2.0 0.4	**1.1** 0.9—1.3 0.2	**0.5** 0—1.2 0.3	t
24:0	*17.79*	**2.1** 1.1—4.2 0.9	**1.9** 1.7—2.3 0.4	**0.6** 0—1.2 0.4	t
26:0	*20.13*	t	**0.3** 0.1—0.4 0.2	**0.1** 0—0.6 0.2	**0.3** t—1.3 0.4

[1] Number before colon denotes number of carbon atoms and number after colon indicates the number of double bonds. 10-Me indicates a methyl branch and its position. 10-Me-18:0 symbolizes tuberculostearic acid and X unidentified compounds.
[2] Number of strains analysed.
[3] M. avium reference strains SVS1, SVS4 and SVS6.
[4] M. paratuberculosis reference strains 316F and 2E and 8 clinical isolates.
[5] M. malmoense reference strains S648, S649, S650 and 709.
[6] First line, mean value; second line, range among strains; third line, standard deviation.
t: Trace value (less than 0.1 %).

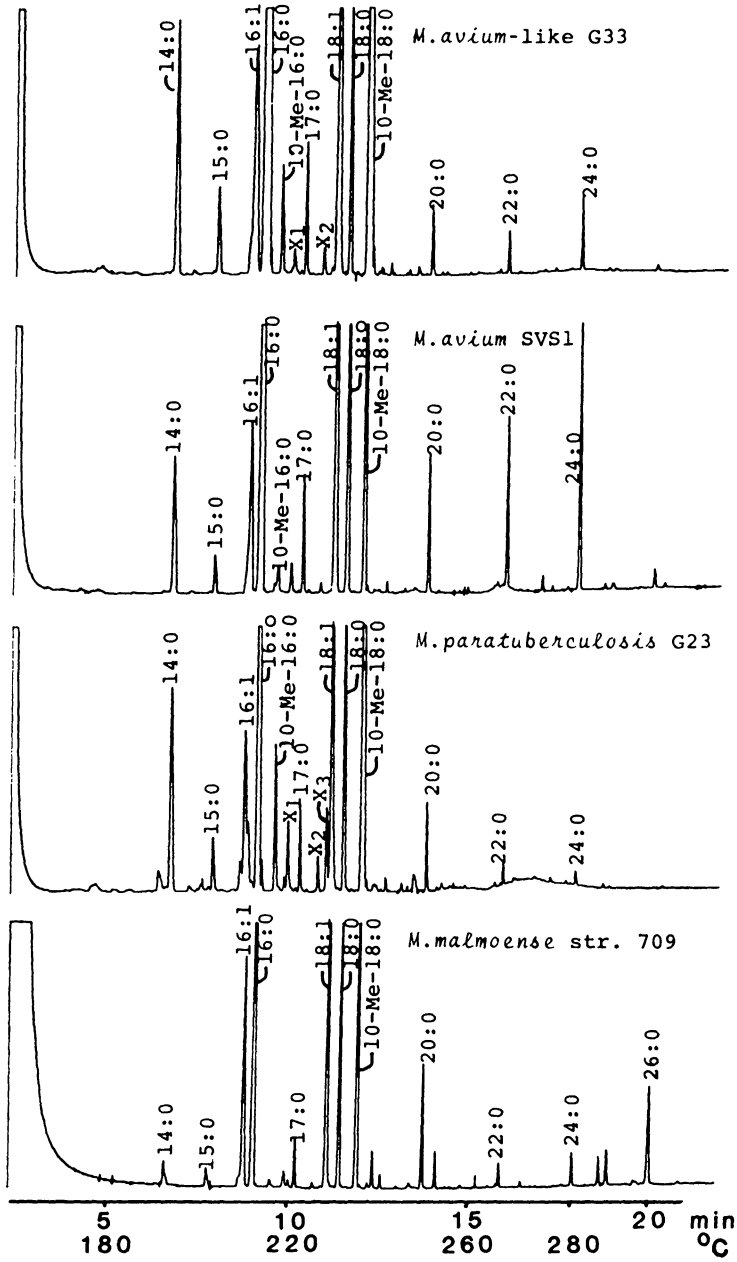

Figure 1. Typical GC fatty acid profiles of the examined mycobacteria. See text for experimental conditions and symbols used.

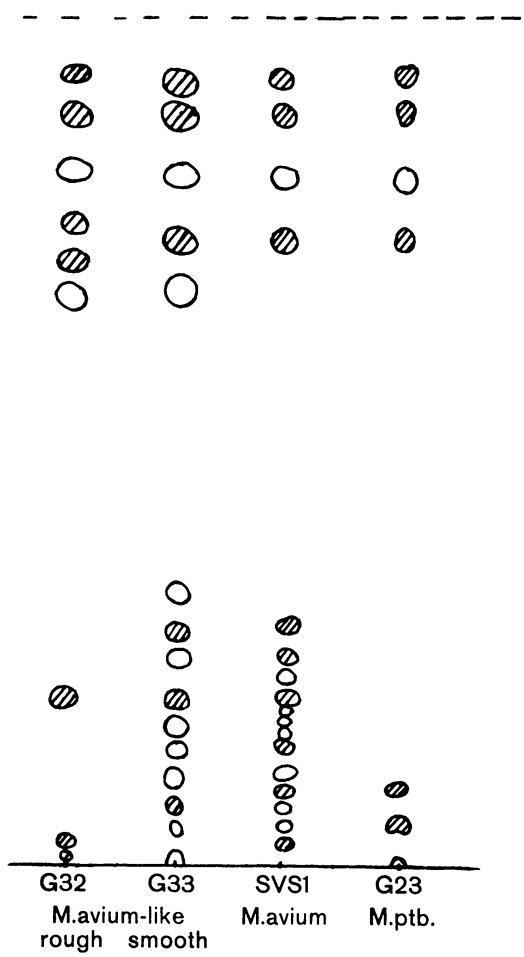

Figure 2. Typical TLC polar lipid patterns of the examined mycobacteria.
Solvent: chloroform-methanol-water 45:5:0.5 (v/v)
Spray reagent: 0.1 % orcinol in 40 % H_2SO_4;
▨: pink; ○: bright yellow or brownish yellow.

tween the examined groups is also evident from this table. However, a few significant differences seem to be useful for group separation. Thus, in comparison with M. avium, including the M. avium-like strains, M. paratuberculosis contained more of 10-Me-16:0 and the 2 unidentified constituents X_1 and X_3 and less of 24:0. The M. malmoense strains contained less of 14:0 and 24:0 but more of 18:0 and 20:0 in comparison with the other groups.

Some typical TLC patterns are schematically presented in Fig. 2. These chromatograms are based on deacylated lipids and orcinol spray as described by *Brennan et al.* (1978, 1982). Such specific patterns can be obtained from 5 to 10 mg cells as starting material. However, employment of the more expensive high performance TLC plates provides well resolved chromatograms from about 2--5 mg cells.

The M. avium-like isolates could be divided into 2 major groups on the basis of their polar lipid patterns. Those of morphologically rough and crisp appearance exhibited only few spots of low mobility whereas those of smooth appearance revealed a complex pattern comparable to those of the M. avium serovars (Fig. 2). The M. paratuberculosis strains were all of a simple TLC pattern which differed from that of the rough M. avium-like strains by the lack of 2 spots, 1 yellow with a relative mobility of 0.7 and 1 pink with a mobility of 0.3.

DISCUSSION

There is disagreement in the literature as to whether mycobacteria isolated from wild animals, mammals or birds, constitute a separate taxon of should be assigned to M. avium or M. paratuberculosis. For instance, in British wildlife, wood pigeons appear to be the most common host (*McDiarmid* 1964) and the strains have accordingly often been called "wood pigeon mycobacteria". These wood pigeon strains, regardless of their origin, resemble M. paratuberculosis in their initial slow, mycobactin dependent growth, their rough colony morphology and their nonpathogenicity for chickens. However, by passaging through chickens they may revert to mycobactin-independent growth of typical smooth chicken pathogenic M. avium colonies (*McDiarmid* 1964, 1975). Therefore, most workers tend to designate "wood pigeon mycobacteria" as M. avium-like. On the other hand, in a recent study of strains isolated from wood pigeons and roe-deer, *Thorel & Desmettre* (1982) concluded, mainly on the basis of cultural and biochemical evidence, that the isolates were more closely related to M. paratuberculosis than to M. avium. Serologically the strains did not agglutinate with any of the 28 examined serovars of the MAIS complex (M. avium -- M. intracellulare — M. scrofulaceum) but behaved also differently from M. paratuberculosis when tested by immunodiffusion in agar.

The fatty acid data of the present investigation add evidence to support the supposition that wood pigeon mycobacteria are members of the MAIS complex, although closely related to M. paratuberculosis and M. malmoense. This applies irrespective of their origin, mammal or avian, and of their colony morphology. Furthermore, fatty acid analysis seems to be potentially useful in assuring more rapid and precise diagnosis of wood pigeon mycobacteria, especially in consideration of the vague criteria hitherto used, namely growth rate, mycobactin dependence, colony morphology and pathogenicity for chickens. For instance, *Matthews et al.* (1981) found that 20 out of 50 deer isolates were nonpathogenic for chickens, and *Matthews & Sargent* (1977) in their study of hare isolates found it necessary to observe cultures and experimental animals for 9 months before a definitive diagnosis was reached. The GC technique is rapid, the answer may be obtained in approximately 1½ h after harvesting, and growth time can be reduced considerably due to the small amount of bacteria required. On the other hand, as previously shown (*Thoen et al.* 1971, *Chomarat et al.* 1981), fatty acid patterns provide no distinction between species or serovars within the MAIS complex.

Four strains of the human pathogen M. malmoense were included in the GC study due to their growth behaviour and morphological properties similar to most of the wild animal mycobacterial isolates. As indicated in Table 2, the fatty acid patterns of these strains showed close resemblance to those of the other groups. However, small distinct quantitative differences (Table 2) distinguished M. malmoense from both the M. avium group and M. paratuberculosis.

TLC analysis of the smooth and serologically typable wild animal isolates gave complex patterns which were comparable to those of the M. avium reference strains. Our limited experience with diagnostic TLC applications did not allow us to allocate any of the 3 smooth wild animal isolates to a particular M. avium serotype, but this small preliminary TLC study has encouraged the more frequent use of TLC in mycobacterial diagnosis due to its very high potential for differentiation within the MAIS complex. However, both a strict standardization of the technique and a large number of reference extracts are required (*Jenkins* 1981, *Brennan et al.* 1978, 1982). Compared to GC, however, the re-

quired amount of cells (2—5 mg) reduces its value as an aid in rapid identification.

In conclusion, the wild animal M. avium-like isolates were indistinguishable from the M. avium reference strains in terms of fatty acid composition and slightly different from both M. paratuberculosis and M. malmoense. The GC analysis provided no differentiation between the M. avium strains. This, on the other hand, is possibly by the TLC technique which may be regarded as a chemical method corresponding to serotyping. Thus, the 2 chromatographic methods are supplementary since the fatty acid data indicate the correct group/complex of an unknown isolate whereas its TLC lipid pattern may provide evidence for allocation of a correct species or subspecies identity.

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SAMMENDRAG

Karakterisering av mykobakterier isolert fra ville dyr ved analyse av fettsyrer og polare lipider.

Tretten ukjente stammer av mykobakterier isolert fra rådyr og ville fugler ble analysert med henblikk på fettsyrer og polare lipider og sammenliknet med referensestammer av Mycobacterium avium, M. paratuberculosis og M. malmoense. Alle undersøkte stammer hadde stort sett sammenliknbare fettsyremønstre karakterisert av relativt store kvanta av heksadekanoat (16:0), oktadekenoat (18:1), oktadekanoat (18:0), og 10 methyl-oktadekanoat (tuberkulostearinsyre, 10-Me-18:0). Andre syrer ble også påvist, men i mindre kvanta. Ved hjelp av små, men distinkte forskjeller i fettsyresammensetning kunne de ukjente mykobakterieisolatene adskilles fra M. paratuberculosis og M. malmoense, men ikke fra M. avium.

Analyse av polare lipider (ved tynnsjiktkromatografi) separerte de ukjente stammer i 2 grupper av henholdsvis enkle og komplekse lipidmønstre som tilsvarte ru og glatt kolonimorfologi. Det komplekse mønsteret fra stammer med glatt kolonimorfologi var av samme type som mønsteret fra M. avium-referensestammene, mens stammene med ru kolonimorfologi og alle M. paratuberculosis-stammene hadde et enklere lipidmønster.

Både analysene av fettsyresammensetning og polare lipider indikerer at mykobakterieisolatene fra rådyr og ville fugler tilhører MAIS-komplekset (M. avium — M. intracellulare — M. scrofulaceum). For øvrig er de 2 kjemiske metodene, særlig fettsyreanalyse, et velegnet hjelpemiddel for en hurtig og mer eksakt identifisering av slike ekstremt sentvoksende mykobakteriestammer.

(Received May 6, 1983).

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