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Investigation of "atypical" mycobacteria

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INVESTIGATION OF " ATYPICAL " MYCOBACTERIA ISOLATION, CULTURAL, CULTURAL AND BIOCHEMICAL STUDIES

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บทคัดย่อ

Atypical Mycobacteria เป็นเชื้อ Acid—fast จัดอยู่ในพวกเดียวกับเชื้อวัณโรค ทำให้เกิดโรคผิวหนังลักษณะเป็นตุ่มหรือแผลเรื้อรังในคนและสัตว์ อาจทำให้เกิดโรคคล้ายวัณโรคในคน เชื้อนี้เข้าใจกันว่ามีบทบาทใน " ปฏิกริยาทับซนิด " (cross reaction) กับทูเบอร์คิวของวัวและไก่ จึงได้มีผู้ค้นคว้าหาแหล่งที่มาของเชื้อนี้ ส่วนที่ว่าเชื้อเหล่านี้จะมีบทบาทดังกล่าวมาแล้วหรือไม่นั้น ก็ยังไม่มีการสรุปยืนยัน ผู้เขียนได้พบว่า เชื้อนี้พบได้ทั่วไปในดินและน้ำของนครโคเปนเฮเกน เดนมาร์ค ส่วนใหญ่เป็นพวก " เจริญเร็ว " (Runyon' s group IV) แบ่งออกได้เป็นสามพวกย่อยโดยอาศัยลักษณะการให้สีและอิทธิพลของแสงสว่างที่มีต่อเชื้อ ผู้เขียนได้ทำการศึกษาเชื้อนี้อย่างละเอียดทางรูปร่าง การเจริญเติบโต และคุณสมบัติทางชีวเคมีบางอย่าง

The terms "atypical mycobacteria" and their synonyms, such as "unclassified mycobacteria", "anonymous mycobacteria", "paratubercle bacilli", are confused by many authors. These terms refer to mycobacteria which differ from M. tuberculosis, but were found in pulmonary disease in man. Youmans (1963) preferred the term "atypical" which he thought was meaningful, whereas the others were meaningless. Kovacs (1966) agreed with Runyon that these mycobacteria should be called "unclassified mycobacteria". After the study of the properties of these mycobacteria, any of these terms can be used, but in my work I prefer "atypical mycobacteria".

The number of reports on the isolation of atypical acid-fast organisms from pulmonary disease with symptoms and roentgenographic findings resembling those of tuberculosis have increased (Beck 1959, Engback et al. 1959, 1964, Marks and Trollope 1960, Nassau and Hamilton 1957, etc.). The relationship between the atypical mycobacteria and tubercle bacilli is not understood. Many workers believe that these mycobacteria are the cause of skin lesions in cattle which are sensitive to tuberculin, so that they react as if they were infected with tuberculosis. A lot of cattle are tuberculin-positive, though no tubercular lesions are found on autopsy. This

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phenomenon may be due to the sensitization produced by the ingestion, skin exposure, or entrance through wounds of acid-fast organisms whose habitat may be in soil and water. The reservoir and natural habitat of the anonymous mycobacteria are still uncertain. Dust, water, and soil seem to be the most natural sources of infection. Frey and Hagan (1931) succeeded in isolating acid-fast bacteria from soils in different parts of the U.S.A. Filertsen, Gangasaas, and Hopen (1968) isolated a large number of mycobacteria belonging to Runyon's Group IV from salt water of the harbour of Bergen and several swimming pools in Western Norway. They concluded that swimming pool infection with atypical mycobacteria may be important for the non-specific tuberculin reactions.

As mentioned above, the habitat of the atypical mycobacteria may be soil and water; and the relationship between the different species of mycobacteria has not been adequately studied. The problems always aries in connection with identification of mycobacteria, because it is very difficult to distinguish between two related species. Previously, studies on taxonomic identification of some mycobacteria have been reported by the following workers (Thomson 1932; Gordon and smith 1953, 1955; Bojalil and Cerbon 1961; Cerbon and Bojalil 1961; Bojalil, Cerbon and Trujillo 1962). Gordon and Hagan (1938) studied the saprophytic acid-fast bacteria, which they isolated from soil, plants and animal tissues. They separated them into three groups according to their survival at 60 °C and utilization of carbohydrates. Runyon (1959) grouped the anonymous mycobacteria into 4 groups according to rate of growth and pigmentation, on the bacteriological and clinical evidence the characteristics were regarded as distinct and easy to do.

The purpose of the present investigation was to isolate mycobacteria from water, sludge, soil, and animal faeces and to try to analyze the physiolo gical properties and the relationships of the myco bacteria whose cultural characteristics make them similar to each other.

MATERIALS AND METHODS

A total of 34 samples were collected (Table 1) from different sources: The slyudge-containing samples of 5 litres each were cellected from the top and the bottom of a salmon fish pond, 2 filtrate samples from a swimming pool, 12 sludge samples from sewage plants, 17 soil samples from the "Rϕrrendegaard" farm, and 1 sample of horse faeces, were examined for mycobacterium. Seven strains of Runyon's Gr. IV were obtained from Statens Seruminstitut, Copenhagen, 2 strains were regarded as M. fortuitum, 5 strains were unclassified.

Ths sludge was collected in a sterile Petri dish, and about 1 gm. of sludge was transferred to a sterile test tube. The filtrate samples from the swimming pool were collected in sterile flasks containing 50 ml. of sterile normal saline. After shaking well for 5 min. the saline solution was distributed into centrifuge tubes and centrifuged at 3000 rpm. for 15 min. The supernatant was poured off, and 1 ml was collected from the bottom of the centrifuge tube. Sludeg, soil and horse faeces were collected in sterile flasks, and 1 gm. of each sample was distributed into sterile test tubes. The following method was performed to obtain maximum mycobacterium and avoid contamination: To each test tube containing 1 gm. of sample 5 ml. of 6% H_2SO_4 was added, mixing well. The tubes were allowed to stand at room temperature for 15 min. Tuen the acidic samples were diluted with 10 ml. of sterile normal saline. The mixture was distributed into two centrifuge tubes and centrifuged at 3000 rpm. for 10 minutes. The supernatant was poured off, and 1 ml. of sterile normal saline was added to each centrifuge tube. The contents were mixed well and transferred to sterile test tubes. The contents of each tube were distributed with Pasteur pipettes on Löwenstein-Jensen medium and were allowed to mix with the condensed water in the tubes. Then the tubes were placed in horizontal position for 15 minutes, after which 1 drop was transferred to the other Löwenstein - Jensen medium this procedure is a of dilution. Before incubation the test tubes were sealed with hard paraffin. medium. This

Table 1

Findings and Sources of Mycobacterium

Sources \ Amount of samples	2		2		12		17		1	Strains Nos.
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Neg.	
Water from salmon fish pond	2	-								1 - 6
Filtrate from swimming pool			-	2						-
Sludge (1)					6	6				7 - 17
Soil (2)							10	7		18 - 44
Horse faeces									1	-
State Serum Laboratory (3)										9446 M. fortuitum 615 M. fortuitum ATCC 23005 (unclas) ATCC 23001 (") ATCC 23003 (") ATCC 23012 (") ATCC 23039,(")

(1) The sludge samples were taken from sewage plants.

(2) The soil samples were taken from different sites of the "Rørrendegaard" farm, Tåstrup, Denmark.

(3) Seven strains of Runyon's Gr. IV were supplied by B. Vergmann, State Serum Laboratory, Copenhagen, Denmark. Two strains were classified as Mycobacterium fortuitum, the other 5 strains were unclassified. The sources of the 7 strains were unknown.

The first 10 samples (Table 1) were inoculated in Löwenstein-Jensen media with 0.75% glycerine and were cultivated both at 30 °C and 37 °C. The growth of mycobacteria was not so good, it was especially sparse at 37 °C. After that I decided to inoculate in Löwenstein-Jensen medium with 6% glycerine and incubated only at 30 °C for 30 days. The culture were examined every day during the first week, and every 3 days after that until 30 days. Smears were made of different colonies in the culture, which were stained with the Ziehl-Neelsen method.

The colonies consisted of acid-fast rods, which were isolated and subcultured in Löwenstein Jensen - medium. When the colonies were seen, smears were made and stained by the Ziehl-Neelsen method, subcultures were made from acid-fast colonies on 6% glycerol nutrient agar in Petri dish, and they were incubated at30 °C. After the culture showed some colonies, smears were made and stained by the Ziehl-Neelsen method. Colonies of acid-fast bacteria were subcultured on 6% glycerol nutrient broth.

The broth was examined for purity by making films and staind by the Ziehl-Neelsen method. The pure cultures were subcultured to 6% glycorol nutrient agar and kept for the following examination. The impure culture were discarded.

Colonial and Cell Morphology

The cultures grown on glycerol nutrient agar at 30 °C for 7 days were examined for colonial morphology. The same cultures were used to prepare films, which were stained by the Ziehl-Neelsen method and examined for cell morphology. The bacilli were measured with a micrometer.

Rate of Growth

The mycobacteria strains were inoculated on Lowenstein-Jensen medium with 6% glycerol and 6% glycerol nutrient agar, and incubated in 30 °C incubator. The cultures were examined every day. The rate of growth was noted after the first growth has been observed.

Pigmentation

The mycobacteria strains were grown on Löwenstein-Jensen medium in a dark 30 °C incubator. The cultures were examined after 3 days (except strains No. ATCC 23005 and strain No. 17, which were examined after 7 days) for pigmentation. The cultures were then kept in daylight at room temperature (20 °C) for 8 weeks (long exposure). Then the cultures were examined for the pigment of the growth. The pigment was classified as greyish black, yellow, lemon or orange.

Acid Production from Carbohydrates

Inorganic nitrogen agar, a medification of Ayer's Reipp, and Johnson (Gordon and Smith 1953), was inoculated with mycobacteria strains and examined for acid production every day. Incubation at 30° C until 30 days.

Utilization of Benzoate, Citrate, and Succinate

The mycobacteria strains were inoculated on a modification of Koser's citrate agar (Gordon and Smith 1955) which contained 2 gm. of the sedium salt of benzoic, citric, and succinic acid; phenol red was used as indicator. The pink colour of the culture after incubation at 30° C demonstrated the utilization of an organic salt as a carbon source.

Hydrolysis of Starch

Duplicate plates of nutrient agar with 1% potato starch were spot inoculated and incubated at 30 C. The Petri dishes were sealed with adhesive tape before incubation. The hydrolysis was demonstrated by flooding the media with Lugol's iodine (Cowan and Steel 1970), one plate at 7 days and the other at 14 days. The hydrolysis was indicated by a clear zone around and/or underneath the growth. Dark blue colour of the media indicated that starchwas not hydrolyzed.

Hydrolysis of Gelatin

Nutrient agar with 0.5% gelatin was used. The cultures were inoculated across duplicates of the medium. The Petri dishes were sealed with adhesive tape before incubation at 30° C. One plate was examined at 7 days, and the second plate was examined at 14 days for hydrolysis by flooding the media with acid mercuric chloride (Cowan and Steel 1970). A clear zone around and underneath the growth indicated the hydrolysis. The unchanged gelatin medium appeared as opaque precipitate.

Decomposition of Casein

The cultures were spot inoculated on nutrient agar with combined 10% sterile skim milk. The dishes were sealed around the edges with adhesive tape before incubation at 30° C. Observed for the clearing of casein around the growth every week until 30 days.

Decomposition of Tyrosine

The test was carried out as for detection of decomposition of casein, but the skim milk was substituted with 0.5% tyrosine. Observed for the disappearance of the tyrosine for 30 days.

Reduction of Nitrate to Nitrite

The cultures were inoculated in tubes of nutrient broth to which 1% KNO_3 had been added. The tubes were incubated in the horizontal position at 30° C. The broth cultures were drawn out with a sterile pipette and tested for nitrite (Cowen and Steel 1970) every day until 14 days.

Niacin Test

The test was done according to the method indicated in the description of Bacto-TB Niacin

Test Strips (Difco Laboratories). But the culture tures on Löwenstein-Jensen medium were 8 weeks old, and the positive control tube was 60 microgram nicotinic acid in sterile normal saline.

Urease

The methods of Singer and Cysner (1952) and Cowan and Steel (1970) were used for the detection of urease production.

Results

Forty-four strains were isolated from different sources. Six strains were isolated from a salmon fish pond, 11 strains from sludge, and 27 strains were isolated from soil. Including 7 strains obtained from the State Serum Laboratory, 51 strains were studied.

Colonial Morphology

The colonial form of all the strains are recorded as follows:

Colonial form	Strains
Rough	615 M. fortuitum, 9446 M. fortuitum, 7, 9, 11, 12, 16, 29, 34.
Smooth	ATCC 23005, ATCC 23001, ATCC 23003, ATCC 23012, ATCC 23039, 1—6, 9, 10, 13-15, 17—28, 30—33, 35—44.

There are four different rough colonies (Figs. 1-4, and two different smooth colonies (Figs. 5-6) (see page 142).

Cell Morphology

The microscopic characteristics of all the strains are recorded as follows:

Table 2

Cell Morphology of Atypical Mycobacteria

Shape of cell	Arrangement	Estimated cell lenghn	Strains
Coccobacillus, or short plump rods	Scattered, single, or clumps	0.8 x 1.5 u	ATCC 23039, 1,2,3,4,5,6,18, 19,21,22,23,24,26,27,28,29, 34,35,37.
Slender, straight and short rods	Scattered, individually	0.6 x 2—3 u	515 M.fortuitum 9446M. fortunitum, ATCC 23003, ATCC 23005, 8, 10, 20,25,30,—33,36,38—44.
Curved, short, irregular rods	Scattered, Chinese letter	0.6 x 2—4 u	11,13,14,15.
Long, curved irregular rods	Scattered, bundles	0.8 x 6—8 u	ATCC 23012, ATCC 23001, 7,9,12,16,17.

Rate of Growth

All strains were rapid growers, showing visible growth within 3 days, except strains ATCC 23005 and No. 17, which did not show visible growth until 6 and 7 days respectively on both Löwenstrin-Jensen medium with 6% glyverol and 6% glycerol nutrient agar.

Pigmentation

Atypical mycobacteria strains are divided into 3 groups (Table 3) according to pigmentation and influence of light (long exposure).

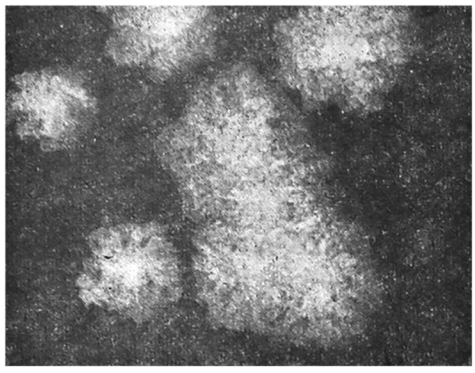

Fig. 1: Mycobacterium, strain 29, rough type of colony from a culture on 6% glycerol nutrient agar, 4 weeks old; X 5.

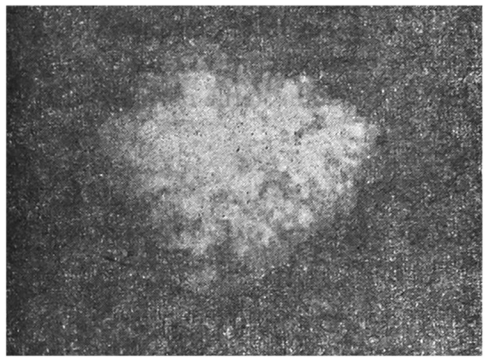

Fig. 2: Mycobacterium, strain 9, rough type of colony from a culture on 6% glycerol nutrient agar, 4 weeks old; X 5.

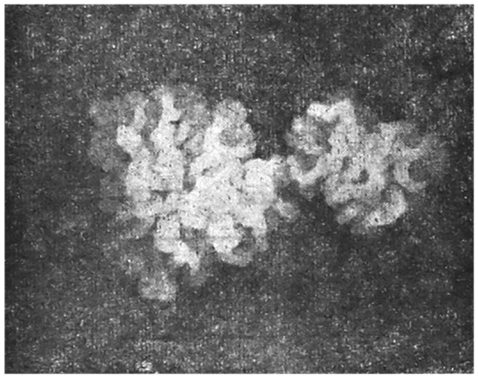

Fig. 3: Mycobacterium, strain 12, rough type of colony from a culture on 6% glycerol nutrient agar, 4 weeks old; X 4.5.

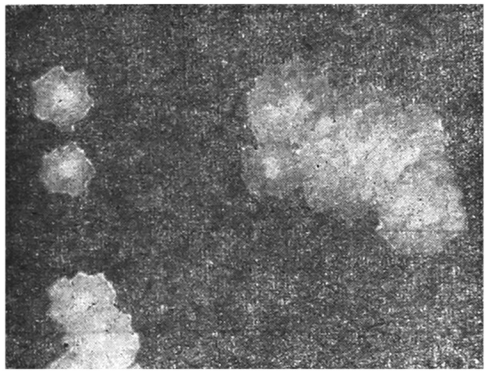

Fig. 4: Mycobacterium, strain 16, rough type of colony from a culutre on 6% glycerol nutrient agar, 4 weeks old; X5.

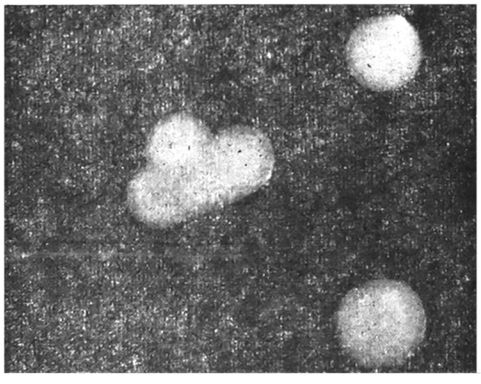

Fig. 5: Mycobacterium, strain 20, smooth type of colony from a culture on 6% glycerol nutrient agar, 4 weeks old, X 5. Note that the colonies are smooth, moist and yellow.

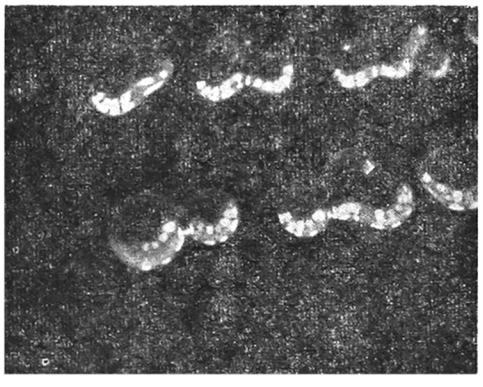

Fig. 6: Mycobacterium, strain 24, smooth type of colony from a culture 6% glycerol nutrient agar, 4 weeks old; X 8. Note the colonies are smooth, moist and heaped up at the centre. They are greyish black.

Table 3.

Grouping of Atypical Mycobacteria According to Pigmentation

Group	Before exposure to light	After long exposure to light	Type of colony	Strains Nos
I	Light yellow, yellow	Deeper yellow, orange	Smooth	ATCC 23009, ATCC 23005 23005, 13,17,20,23,26,28, 28,30,32,35,36,37,39,40.
			Rough	—
II	Cream, buff	Greyish black	Smooth	1,2,3,4,5,6,18,19,21,22,24,27.
			Rough	—
		Yellow	Smooth	25,38,41,42,43,44.
			Rough	—
III	Buff, colourless	—	Smooth	ATCC 23002, ATCC 23003,ATCC 23001, 8, 10, 14,15,31,33.
			Rough	615M. fortuitum, 9446M.fortuitum, 7,9,11, 12,16,29,34.

Biochemical, Test

The results of the different biochemical tests of the strains are listed in Table 4. The strains are listed serially according to their ability to ferment hexoses, pentoses, disaccharides and polyalcohols respectively. The hexoses, glucose, mannose, and fructose were widely utilized. Only strains No. 17 was unable to utilize glucose, galactose was utilized by some strains. Seventeen strains produced acid from all the hexoses, glucose, mannose, fructose and galactose. The pentoses were utilized to some extent by the strains. 10 strains utilized rhamnose 18 strains utilized xylose, 27 strains utilized arabinose, and only strain No. 42 utilized arabinose, xylose and rhamnose. No strain was capable of utilizing lactose, while 35 strains produced acid from trehalose, 14 strains utilized maltose, and 12 strains utilized saccharose. Only strain No. 43 produced a slight amount of acid from raffinose. Polyalcohols, especially mannitol and inositol were widely utilized, but only 11 strains utilized sorbitol, none of the strains utilized dulcitol. Strains Nos. 24 and 42 produced slight amounts of acid from salicin.

Modification of Koser's citrate agar which contained different carbon sources, benzoate, citrate and succinate were used for detection of the ability of mycobacteria to use such compounds as carbon source or not. Eleven strains utilized benzoate, and 11 strains utilized citrate, but only 9 strains did not use succinate as carbon source. Strains Nos. 39 and ATCC 23005 were able to utilize benzoate, citrate and succinate as canbon sources.

Explanation to Table 4

a = strains nos. 1, 2, 6
b = strains nos. 3 and 4
c = strauns nos. 7, 11, 34, 9446 M.fortuitum
d = strains nos. 12 and 14.
e = strains nos. 21, 22
f = strains nos. 35 and 37
g = strains ATCC 23012 and ATCC 23003
\+ = the number of days the test is positive
\- = no reaction within 30 days, except the gelatin test and the reduction of nitrate to nitrate which indicated 14 days.
. . = no test,

(1) = average days the test is positive.
(2) = strain No. 37 is negative, the other was not tested.
(3) = strain No. 12 is negative, the other was not tested.
(4) = strains Nos. 7 and 11 are negative, the others were not tested.
(5) = urea solution as described by Cowan and Steel.
(6) = urea solution without glucose, described by Singer and Cysner.
(7) = urea solution with glucose, described by Singer and Cysner.
(8) = slightly positive.

Starch was hydrolyzed by all mycobacteria.

Galatin was hydrolyzed by strains Nos. 1, 2, 3, 4, 5, 6, 24, 30, 21, and 22.

Casein was not decomposed by mycobacteria

Twenty-six strains were tested for the decomposition of tyrosine, none of them decomposed tyrosine.

Forty-eight strains were able to reduce nitrate to nitrite, three strains were negative. The test was carried out up to 14 days, any strain unable to recuce nitrate within 14 days is recorded as negative.

Twenty-one strains were tested for niacin production, all of them were negative.

Urease activity. For detection of urease acitivity of mycobacteria the methods described by Cowan and Steel and Singer and Cysner were used. Although the time required to break down the urea were slightly different, the results indicated that both methods can be used. Strain No. 17 was unable to break down urea from urea solution as described by Cowan and Steel, but it broke down urea from the urea medium described by Singer and Cysner within 30 days. Strain No. 29 could not break down urea solution without glucose, Singer and Cysner urea medium. The other 49 strains broke down urea in the media described by Cowan and Steel and Singer and Cysner.

Discussion

This investigation indicates that the atypical (unclassified, anonymous) mycobacteria are widely distrituted in soil and water. According to available literature the saprophytic mycobacteria have been found not only in their natural habitats, butter, grass, manure, dust, metal cold water taps (Brem 1909), but have also been demonstrated in the tonsile, sputum, pus, blood, intestinal contents

of insects, and cow's milk (Albisiton 1930). The present study comprised 44 strains which were isolated from their natural habitat, and 7 strains obtained from the State Serum Laboratory, Copenhagen. Fifty strains were rapid growers and were placed in Runyon's Gr. IV. Only strain No. 17 which was isolated from sludge, was a slow grower and formed yellow pigment in the dark. This strain is pure is Runyon Gr. II. In this work the rapid growers were easily separated into 3 groups according to pigmentation and the influence of light as follows:

Group I:

Yellow pigment is formed in the dark. More pigment is formed after continued growth in the light (long exposure). Fifteen strains of the smooth type are is this group.

Group II:

Green or buff pigment is formed in the dark. Greyish black or yellow pigmentation occurs after continued growth in the light. Eighteen strains of smooth type colonies are in this group.

Group III:

Colonies are buff or colourless when grown in the dark, No influence of light after long exposure. Nine strains of smooth type colony and 9 strains of rough type colony are in this group.

Up to the present the unclassified mycobacteria have been insufficiently studied and the characteristics of the species may be variable, so that any of the above criteria may be used in an attempt to classify mycobacteria. The results indicated that every mycobacterium strain is able to hydrolyze starch, reduce nitrate to nitrite and break down urea, but unable to decompose casein, tyrosine and did not produce niacin, except M. tubercilosis human strain and M. borstelense var. niacinogenes (Bönicke 1966).

The question of classifying the mycobacteria by using the above criteria is still open. For instance, two cultures of M. fortuitum showed different reaction (Table 4). But some properties of mycobacteria (Kubika & Ringdon 1961), (Trukamura & Trukamura 1965) will distinguish them from the related strains.

It seems that every isolated strain of mycobacterium was a new species. Further study should be continued, especially with a view to finding a technique to differentiate the strains of atypical mycobacteria.

Summary :

Forty-four strains of atypical mycobacteria were isolated from different sources of natural habitat. All the strains are rapid growers, except one strain, which is slow. Fifty-one strains, including 7 strains from the State Serum Laboratory, Copenhagen, were studied and divided into three groups according to pigmentation and the influence of light. Biochemical properties were also studied. No attempt is made in this report to present the criteria for classification. The purpose is only investigation and comparison of their biochemical properties. The strains deserve furte her study of pathogenicity and hypersensitivity.

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