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DIFFERENTIAL IDENTIFICATION OF MYCOBACTERIA
VI. MYCOBACTERIUM TRIVIALE KUBICA SP. NOV.

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ABSTRACT. A new species of nonphotochromogenic mycobacteria is described. Most of the strains examined have been isolated from man; however, because of their lack of relationship to human disease, the name Mycobacterium triviale Kubica is proposed. The physical and biochemical characteristics, serologic activity, and thin layer chromatographic pattern of this new species serve to distinguish it from other nonphotochromogenic mycobacteria in Runyon's Group III.

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In 1958 the Georgia Department of Public Health directed our attention to certain nonphotochromogenic mycobacteria which had been isolated either from normal individuals or from former tuberculosis patients whose disease showed no evidence of recrudescence. In all cases these isolations consisted of no more than two positive cultures, each of which yielded one colony or at most only a few colonies. Over the next 7 years cultures of similar organisms were collected in this laboratory and none of them was shown or thought to be associated with disease in man.

In November 1966 we described our experience with 16 of these isolates and designated them as the "V" subgroup of Runyon Group III nonphotochromogens because of their distinct difference from other members of the nonpigmented, slowly growing mycobacteria (Jones et al. 1966). Our concern at that time was the fact that on both egg medium and

oleic acid agar the colonies of the "V" subgroup resembled those of M. tuberculosis. That a number of these isolations had come from former tuberculous patients suggested that some microbiologists might identify these organisms as tubercle bacilli purely on the basis of colonial morphology and the patient's clinical history. We called attention to the colonial similarity of this normally saprophytic mycobacterium to that of M. tuberculosis and urged diagnostic laboratories to use several valuable in vitro tests to separate the "V" subgroup mycobacteria from tubercle bacilli and from other mycobacteria showing variable pathogenic potential for man. Since these organisms were only sporadically isolated from man as a single colony or at most as a few colonies and under circumstances indicating no complicity in human disease, the species name Mycobacterium triviale Kubica sp. nov. is proposed. The description of authorship of the name is recognition by the seven authors of this manuscript that the name was proposed by Dr. Kubica and accepted by them. The type culture of the species is on deposit with the American Type Culture Collection (ATCC) as ATCC-23292. After a detailed numerical taxonomic examination, this culture was found to exhibit the same reaction pattern as the hypothetical median strain of the species (Kestle et al, 1967); other strains deposited earlier with the ATCC (Jones et al, 1966) varied from the median pattern in 1 or 2 properties.

Both on primary isolation and on subculture, when a barely turbid bacillary suspension is used as inoculum, fully matured colonies of M. triviale do not appear on solid media for more than a week. The colonies on egg medium are rough, dry, heaped-up, non-chromogenic, and acid-fast by either Ziehl-Neelsen or fluorochrome stains. On oleic acid agar, the characteristic rough R colony type (Jones et al, 1966; Fregnan and Smith, 1962; Kubica and Jones, 1965) is seen. M. triviale grows poorly, if at all, on modified corn meal agar and generally produces colonies only along the most heavily inoculated portion of the streak.

The slow growth rate, lack of chromogenicity, failure to produce any photo-activated pigment, and negative reaction in the niacin test serve to place this organism in the Runyon Group III nonphotochromogens. In addition to the niacin test, such characteristics as catalase hyperactivity, rapid Tween hydrolysis, arylsulfatase activity, and resistance to thiacetazone (amithiazone) help to separate M. triviale from M. tuberculosis.

M. triviale organisms have been facetiously referred to as the "slowly growing rapid growers" (Jones et al, 1966) because they have properties characteristic of many rapidly growing acid-fast bacilli: they may show a weakly positive arylsulfatase reaction at 3 days and a strongly positive reaction after 2 weeks, and most strains will grow on 5 % sodium chloride (Kestle et al, 1967). However, such characteristics as rate of growth, failure to grow on corn meal agar, negative iron uptake, very slow reduction of tellurite, and slow Tween opacity reaction serve to separate M. triviale from Runyon Group IV mycobacteria. Although a detailed series of in vitro properties were reported earlier (Kestle et al, 1967) to separate the "V" subgroup (M. triviale) from other slowly growing mycobacteria, an abbreviated chart of these properties is presented in Table 1. The chart shows characteristics of more differential value and the number of strains of each species studied.

The features of M. triviale which are most reliable in separating it from M. avium and M. intracellulare (Battey bacilli) within Group III are strong nitrate reductase activity, rapid Tween 80 hydrolysis, strong catalase activity, slow reduction of tellurite, and growth on 5 % sodium chloride. M. triviale differs markedly from M. gastri in nitrate reductase, catalase activity, thiacetazone susceptibility, growth on 5 % sodium chloride, nitrite inhibition (Abbott et al, 1968), and other properties. M. xenopi and M. triviale differ distinctly in these properties: nitrate reduction, catalase, Tween 80 hydrolysis, growth at 5% sodium chloride, and colonial morphology (Jones et al, 1966; Pattyn 1966).

Differentiating M. triviale from organisms in the M. terrae complex of Group III is particularly difficult because of the problems encountered in resolving the M. terrae complex. In our laboratory, the latter group undoubtedly contains organisms identified as M. terrae Wayne (Wayne, 1966), M. nonchromogenicum or M. terrae Tsukamura (Tsukamura, 1965, 1966; Tsukamura and Tsukamura, 1967), and M. novum Tsukamura (Tsukamura, 1967). The tests most valuable in separating these species from one another and the percentage of each species yielding positive or negative reactions are shown in Table 2.

A total of 137 Group III strains, representing the various "species" discussed, were compared in an $n \times n$ series. In such a study the reaction pattern of each strain

Table 1. Tests of Value in Separating *M. triviale* from Other Slowly Growing Mycobacteria¹.

Runyon Group	Colony Type ²	Pigment ⁴	Niacin	Nitrate Red'n	Catalase		Tween hydrolysis (in days)	Arylsulfatase (3 day)	Growth observed on				Tellurite Reduction 3 days
					in mm	68°			Thiacetazone 10 mcg/ml	5% NaCl	Nitrite M/80	M/40	
I	<i>M. tuberculosis</i>	200 Rough R	N	+	>3+	<40	-	>10	-	-	-	-	-
	<i>M. bovis</i>	34 Rough R	N	-	-	<40	-	>10	-	-	-	-	-
	<i>M. kansasii</i>	136 Smooth K	P	-	>3+	>50	+	<5	-	-	-	-	-
	<i>M. marinum</i>	17 K-like	P	-	-	<40	-	<5	-	+	-	-	-
II	<i>M. sp. scrofulaceum</i>	83 Var. ²	S	-	+	>50	+	-10	-	+	-	-	-
	<i>M. sp. aquae</i>	128 Var.	S	-	-	>50	+	<5	-	+	-	+	-
	<i>M. avium</i>	106 Var.	N	-	-	<40	+	>10	-	+	-	+	+
III	<i>M. intracellulare</i>	224 Var.	N	-	-	<40	+	>10	-	+	-	+	+
	<i>M. xenopei</i>	20 Rough Xf ³	N/S	-	-	<40	+	-10	+	-	+	-	-
	<i>M. gastri</i>	16 K-like	N	-	-	<40	-	<5	-	-	-	-	-
	<i>M. terrae complex</i>	117 Var.	N	-	>1+	>50	+	<5	-	+	-	+	+
	<i>M. triviale</i>	27 Rough R	N	-	>1+	>50	+	<5	+	+	+	+	-

1. Listed reactions are those usually observed (>85%); variations should be expected with individual strains;

† indicates variable reactions observed.

2. As observed on oleic acid-albumin agar; Var.=variable; for details see Kubica and Jones, 1965.

3. See Pattyn, 1966.

4. N=nonphotochromogen; S=scotochromogen; P=photochromogen.

Table 2. Key tests of Value in Separating M. triviale from Organisms in the M. terrae Complex¹.

Species	Number of Strains	Arylsulfatase 2 weeks		Growth observed on indicated media ²							Colony ² type on OAA ²		Amidases ⁴	
		+	3+	LJ 40°C	Streptomycin 2 mcg/ml	5% NaCl	Oleate 0.05%	Malachite green	Methyl Violet	Pyronin B	Sm I	Rough R	Pyrazinamide	Nicotinamide
<u>M. triviale</u>	30	+(100)	+(77)	-(67)	-(93)	+(100)	-(85)	-(100)	-(100)	-(89)	-(96)	+(96)	-(1/10)	-(1/10)
<u>M. terrae</u> Wayne	90	+(70)	-(99)	+(74)	+(85)	-(90)	+(66)	+(93)	+(97)	+(99)	+ ³ (69)	- ³ (72)	-(5/15)	-(5/15)
<u>M. nonchromogenicum</u> Tsukamura	24	+(96)	-(79)	+(100)	-(64)	-(100)	+(75)	+(88)	+(96)	+(100)	+(92)	-(92)	+(15/20)	+(15/20)
<u>M. novum</u> Tsukamura	3	-(100)	-(100)	+(100)	+(100)	-(100)	-(100)	+(100)	+(100)	+(100)	-(100)	+(100)	No test	No test

1. Results are indicated as usually + or 0; number in parentheses indicates percentage of strains, showing this reaction.

2. For details see Kestle et al, 1967.

3. Indicates mixture of colony types often seen.

4. Figures in parentheses indicate (No. positive/No. tested).

in the numerous differential tests employed is compared to the reaction pattern of every other strain in the series, and the percentage of similarity (S) between the pairs of strains is recorded. In the final analysis, strains with similar S_i -values form clusters, and the internal similarity (S_i) within these clusters may be determined by averaging all the S -values. The S -value diagram for these 137 cultures is shown in Figure 1. In this same figure both the inter-taxon S -values and the S_i values are expressed as percentages. These expressions give a better idea of the interrelationships of the species within Group III. These interrelationships will be further explored later.

The low S_i value for the M. xenopi cluster ($83\% \pm 7$) should be discussed since our experience indicates that these organisms are a very homogeneous group. All the differential in vitro tests for classification were done at 37°C , without prior knowledge of strain identity. Because the optimal growth temperature for M. xenopi is 40°C , it is conceivable that these cultures were not at the peak of their metabolic activity when tested at 37°C . Further tests are underway using strains of M. xenopi grown at 40°C to determine whether the internal similarity of this species can be increased by utilizing cultures grown at their optimal temperature.

Since the Group III nonphotochromogens have been remarkably consistent in their ability to degrade nicotinamide and pyrazinamide (Bönicke, 1961), the amidase activity of all strains of M. triviale sp. nov. and selected representatives of the other "species" in Group III was investigated using Bönicke's method (1961). Only one strain of M. triviale demonstrated enzyme activity after 16 hours of incubation with the substrates. Schröder (1967 and personal communication) has confirmed these observations. On the other hand, the majority of strains of M. nonchromogenicum (terrae) received from Tsukamura demonstrated nicotinamidase-pyrazinamidase activity, whereas many of the strains identified by us as M. terrae Wayne showed little or no activity. (See Table 2.) That some strains of M. nonchromogenicum (terrae) Tsukamura showed no amidase activity whereas certain strains of M. terrae Wayne had nicotinamidase and pyrazinamidase activity suggested that these "aberrant strains" may be in the improper cluster. Close examination of Figure 1 also suggests that certain nonchromogenicum strains reacted more like terrae and vice versa; however, the strains of

Figure 1. S-value diagram showing species relationships within Group III. Figures in left-hand column (under each species name) refer to percent internal similarity (S_i) of species $\pm$ the standard deviation within the species. Other figures refer to percent of mean similarity (S) between any pair of species.

M. nonchromogenicum were maintained in this cluster because they have been received with this identification from Tsukamura. Despite thoughts (Tsukamura, 1966) that Tsukamura's and Wayne's new species may be identical, our experience suggests that there are some differences between the two; however, the differences may be so minor that these two species may turn out to be variants or subspecies of the same organism. Certainly more detailed studies of the M. terrae complex are needed to resolve this controversy.

Wayne informed us (personal communication) that his studies on our strains of M. triviale indicated strong nicotinamidase and pyrazinamidase activity. Wayne's (1966) method of studying amidase activity involves a 24-hour test and a different preparative system than that described by Bönicke (1961); therefore, we selected 10 strains of M. triviale and made a detailed study of their amidase activities from 2-24 hours. For comparative purposes, quantitative amidase studies also were done on a few strains of both M. triviale and M. avium. The results of these studies are summarized in Table 3 and Figure 2. As shown in Table 3, a few strains began to show amidase activity at 24 hours, but they showed considerably less activity than that described by Wayne. Schröder (personal communication) has confirmed these observations also. Quantitative studies showed (Figure 2) that M. avium had more than 10 times the pyrazinamidase and nicotinamidase activity of M. triviale.

The 16-hour amidase test, as performed here, has been of definite value in separating M. triviale from most other Group III strains. However, Wayne's report of strong amidase activity for M. triviale in his test system and our own observation of some positive enzyme reactions after 24 hours suggest the need for further investigations of cell wall permeability and genetic studies of enzyme repressor mechanisms.

The serologic relationship of M. triviale to Group II and other Group III mycobacteria was investigated. A partially purified protoplasmic extract of cells of various species of mycobacteria was prepared by the method of Counts and Kubica (1968). Antiserum against such extracts was prepared in rabbits, and serologic reactions were studied by the double diffusion in agar gel method. Although some bands were common to other species of mycobacteria, several specific bands appeared in the M. triviale system

Table 3. Enzymatic breakdown of 10 amides by 10 strains of M. triviale at different reaction times.

No. of Strains	Reaction Time in Hours	Amides Tested*									
		I	II	III	IV	V	VI	VII	VIII	IX	X
		(mcg/ml of Ammonia)									
9	2-16	-	-	-	-	-	-	-	-	-	-
1	16	-	-	-	-	1.5	1	-	-	-	-
3	24	-	-	-	-	-	-	-	-	-	-
3	24	-	-	-	-	1-3	1-3	-	-	-	-
1	24	-	-	3	-	-	1	1	-	-	-
1	24	-	-	1	-	-	-	-	-	-	-
1	24	3	-	-	-	-	1	1	-	-	-
1	24	1.5	-	1.5	-	-	-	-	-	-	-

* I=acetamide; II=benzamide; III=urea; IV=isonicotinamide; V=nicotinamide; VI=pyrazinamide; VII=salicylamide; VIII=allantoin; IX=succinamide; X=malonamide.

Figure 2. Quantitative observation of the amount of ammonia released from pyrazinamide by *M. avium* and *M. triviale* as a function of time. Although not depicted here, reaction curves for nicotinamide were essentially the same as those for pyrazinamide.

Figure 3. Serologic relationship of several mycobacteria by double diffusion in agar gel. Center well contains rabbit-anti-*M. triviale* gamma globulin. Outer wells contain protoplasmic extracts of various species of mycobacteria: A = *M. avium*; B = *M. sp. aquae*; C = *M. terrae*; D and E = *M. gastri*; F = *M. triviale*.

Figure 4. Thin layer chromatography of alcohol-ether soluble lipids from various species of Runyon Group III mycobacteria. In decreasing order of observed intensity, separated bands are indicated in solid black, cross-hatched or dotted areas.

(Figure 3), indicating a serologic difference between this organism and other species within Runyon's Group III. Species representative of all slowly growing mycobacteria are not shown in Figure 3; however, it was always possible to make an absolute serologic identification of strains of M. triviale using this gel diffusion method. An earlier report by Jones et al. (1966) suggested a similar observation when they used fluorescent antibody and agglutination tests. When the protoplasmic antigen of M. triviale was reacted in gel diffusion tests with the sera of 10 employees from this laboratory, every serum showed a specific positive reaction. This finding suggests either widespread natural experience with M. triviale or universal laboratory infection with this same species. The latter hypothesis is not tenable when one considers that the personnel are more heavily exposed at work to M. tuberculosis than to M. triviale and that only 60% of the group show either a positive gel diffusion test or a positive skin test reaction to PPD-S; furthermore, five of the 6 people in this latter group had positive skin hypersensitivity reactions for years before working in this laboratory. Exposure to M. triviale in the natural environment seems to be the most plausible explanation for the positive gel diffusion reactions.

An alcohol-ether extract of representative strains of various species within the Runyon Group III was investigated by the thin layer chromatography (TLC) method (Reiner et al., 1967). Marks and Szulga (1965) and Szulga et al. (1966) reported differential TLC patterns for various mycobacterial species. Although our methods differ from those of Marks and Jenkins, we can corroborate their observations. Figure 4 is a tracing of a thin layer chromatogram of various "species" of Group III nonphotochromogens. Many bands are shared by the entire group; however, the presence or absence of certain specific bands offered further confirmation that Mycobacterium triviale differed from other described species in Group III.

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