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A NEW PRACTICAL CLASSIFICATION OF THE MYCOBACTERIA

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IN recent years, various techniques have been applied with great success to the problems of mycobacterial taxonomy. These methods are mostly too complex for general use and the identifications achieved often go beyond clinical requirements, but the knowledge gained has made it possible to design simpler and more practical means of classification. Such procedures having been effectively applied to British material in the Reference Laboratory (Marks, 1972), a study was undertaken of their suitability for other countries. Important modifications proved necessary, and the resulting new classification is presented here with a brief account of the methods recommended for its use. No consideration will be given to any test that is not essential to the classification, or to organisms that are not cultivable or are of purely veterinary interest.

MATERIALS AND METHODS

Origin of organisms studied. A total of 152 strains of mycobacteria other than tubercle bacilli was obtained from overseas colleagues. Those from Zaïre were environmental in origin but the rest were derived from clinical specimens. The countries of origin and the numbers of strains were Czechoslovakia (12), France (12), Germany (11), Japan (49), Kenya (12), South Africa (9), USA (15), Venezuela (12) and Zaïre (20).

Initial examination. Healthy cultures only were used. Dry or decrepit growth was first subcultured at 25°C and 37°C. The 25°C subculture was used if it showed equal or better growth than the other culture because some psychrophiles become converted to mesophiles when repeatedly grown at temperatures above their optimum. A film was stained by the Ziehl-Neelsen method to confirm the nature of the organism and freedom from contamination.

Temperature requirements. A 1-mm loopful of growth was thoroughly emulsified in 0.25 ml of sterile water or buffer, either by grinding or by mechanical means, e.g., violent shaking with glass beads or rapid eccentric magnetic stirring of nails. Slopes consisting of 2 ml of Löwenstein-Jensen medium in 7-ml screw-capped "bijou" bottles were inoculated with a 2-mm loop withdrawn edgewise. The slopes were incubated at 25°C, 37°C and 45°C,

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the temperatures being controlled to $\pm 0.2^\circ\text{C}$. A block thermostat with lid was made to the author's design by Grant Instruments, Cambridge, for use in providing the incubation temperature of 45°C . The test was read on the 3rd or 4th day, again at 7 days and then weekly until at least one slope showed growth at or near its maximum. If at the first reading growth was "mature", equal at 25°C and 37°C , and absent at 45°C , the test was repeated with a suspension diluted 1 in 100. "Mature" growth is here defined as equivalent to that attained by normal *Mycobacterium tuberculosis* after 3 weeks at 37°C in the standard test. In reading the temperature test, confluent or near-confluent growth—no matter how fine—was accepted, but trace or sparse growth was not. Care was taken not to pick up medium on sampling the culture, especially when the slope was soft, because its presence in the inoculum sometimes produced a confusing film of deposit. However, the appearance of such a film did not change as incubation continued. Inocula were confined to a circle 5–10 mm in diameter so that the edge of the area could be inspected at the time of reading.

Pigmentation (after Runyon, 1959). Two subcultures were made on egg-medium slopes and incubated at 37°C , one in continuous illumination and the other wrapped to exclude light. A 1-mm loopful of culture was used for each slope and the illumination was equivalent to a 25-watt lamp at 30–60 cm distance. Photochromic organisms produced a yellow, orange or red pigment in the light only, whereas scotochromogens produced pigment in the dark as well as under illumination, although often to a lesser degree. A heavy subculture of a pigmented photochromogen sometimes carried over enough precursor to give a little pigment in the dark. To guard against misleading results arising from this cause, the 37°C slope referred to under *Temperature requirements* was also wrapped if the culture under examination was pigmented; this had the effect of providing an additional test with a lighter inoculum. Psychrophiles usually grew well enough at 37°C for the pigment test, but if not the test was repeated at a lower temperature; the use of daylight illumination in a warm place on the laboratory bench was found convenient.

Oxygen preference. Aerobes grow best at or adjoining the surface of semi-solid agar medium and micro-aerophiles grow uniformly deep or with a submerged band of denser growth (Marks, 1972). *M. tuberculosis* and *M. bovis* could be distinguished from one another by the oxygen-preference test and other mycobacteria could also be separated into two divisions. However, with "opportunistic mycobacteria" (defined later), a convenient alternative was to relate oxygen preference to the rate and appearance of growth on egg medium, information that could be obtained from the temperature test without extra work. Opportunistic mycobacteria that gave "mature", i.e., heaped, growth in 7 days or less at their optimal temperature were taken to be aerobes and those that needed a longer period or never attained it were taken to be micro-aerophiles. In cases of doubt, growth in semi-solid medium was examined. Organisms considered intermediate in their oxygen preference in the earlier classification are here classed as micro-aerophilic.

Tween hydrolysis (after Wayne, 1962). The formula of the substrate solution was Na_2HPO_4 0.575 g, KH_2PO_4 0.35 g, 10% Tween 80 solution 5 ml, and distilled water 100 ml. The pH was checked and if necessary adjusted to 7.0. After the addition of 2 ml of 0.1% freshly prepared neutral-red solution, 2-ml volumes were dispensed into 7-ml screw-capped bottles and autoclaved at 120°C for 15 min. These were stored, shielded from light, in the refrigerator and renewed monthly. Batches of neutral red were found to vary and the amount recommended could be adjusted to provide a suitable indicator colour. The buffer had an indefinite life and 10% Tween a life of many months, so that stocks of these ingredients could be held.

The Tween-test medium was inoculated with a heaped 2-mm loopful of growth from Löwenstein-Jensen medium, dispersing it to some extent against the bottle wall below the surface of the liquid. Bottles inoculated with aerobic mycobacteria were incubated at 37°C for 7 days, and those inoculated with micro-aerophiles were incubated for 14 days. Hydrolysis released acid which changed the colour towards red. It was convenient to relate results to the more stable colours of phenol-red standards, the latter at pH 7.2, 7.3 and 7.4 corresponding to weak, moderate and strong Tween hydrolysis, respectively. The phenol-red standards were stored in the dark.

Enzyme tests are liable to error and it was necessary to confirm the negative Tween test required for identification of the Avium-Intracellulare group by a repeat test on a healthy subculture.

Drug-sensitivity tests. The suspension prepared for the temperature test was diluted 1 in 5 and media were inoculated with it by means of a 2-mm loop withdrawn edgewise. Sensitivity was expressed as the ratio between the titration endpoint and the mode of the endpoints of a group of normal wild strains of *M. tuberculosis*. The range of drug concentrations was designed to allow a ratio of 16 for isoniazid and 8 for other drugs. Ratios of 4 or more in respect of streptomycin, PAS, isoniazid and rifampicin were accepted for the purposes of the present study as indicating resistance, and ratios of 3, obtained by interpolation, were accepted in respect of other drugs. Ratios of 3 and 2 respectively were termed "border-line". Pyrazinamide was tested by a special technique based on that described previously (Marks, 1964).

Egg medium. Löwenstein-Jensen medium was used without starch and inspissated by being warmed to and held at 75°C–85°C for 45 min. in an electric oven fitted with a fan circulator. Medium with a smooth shiny surface was necessary to show fine growth clearly, and it was found that other methods of inspissation sometimes failed to produce such a surface. Growth was inspected when necessary with a $\times 5$ lens, rotating the slope to vary the incidence of light on the edge of the inoculated area.

Screening. A suspension was prepared and inoculated as in the temperature test. Four slopes were used, namely, two plain, one with *p*-nitrobenzoic acid 500 μg per ml, and one with thiacetazone 10 μg per ml. One plain slope was incubated at 25°C and the control and drug slopes at 37°C; the control was maintained under illumination as described above. Further details have been published previously (Marks, 1974). Normal *M. tuberculosis* did not grow at 25°C in the test, or on the drug slopes, and it gave non-pigmented growth at 37°C. *M. bovis* behaved similarly except that trace growth sometimes occurred in the presence of *p*-nitrobenzoic acid or at 25°C.

RESULTS

Classification procedure

It was possible to make the five primary divisions shown in table I by means of growth-temperature requirements. It was not necessary to specify relative degrees of growth in the "wide range" and "thermophile" divisions.

Classification proceeded with the use as required of the tests for pigment formation, oxygen preference and Tween hydrolysis. By these means 15 groups could be defined that embraced all the cultivable mycobacteria met in clinical bacteriology. They are presented in table II. The named organisms

TABLE I

Primary divisions of mycobacteria made on the basis of growth temperature requirements

Primary division	Degrees of growth at		
	25°C	37°C	45°C
Strict mesophiles	—	+	—
Psychrophiles	+	$\leq +$	—
Mesophiles	$< +$	+	—
Wide range	+	+	+
Thermophiles	—	+	+

TABLE II
Classification of the mycobacteria in clinical bacteriology

Primary division on basis of temperature requirement	Further distinguishing characters			Designation as species or group
	Pigment formation	Oxygen preference	Tween test	
Strict mesophiles	...	Aerobic	...	<i>M. tuberculosis</i>
	...	Micro-aerophilic	...	<i>M. bovis</i>
Psychrophiles	Photochromic	...	...	Marinum
	Scotochromic	...	...	Scotochromic psychrophiles
	Nonchromic	Aerobic	...	Chelonei
	Nonchromic	Micro-aerophilic	...	Ulcerans
Mesophiles	Photochromic	...	...	Kansaii
	Scotochromic	Aerobic	...	Flavescens
	Scotochromic	Micro-aerophilic	+	Gordonae
	Nonchromic	Aerobic	...	Fortuitum
	Nonchromic	Micro-aerophilic	+	Terrae
	Sc. or nonchr.	Micro-aerophilic	—	Avium-Intracellulare
Wide range	...	Micro-aerophilic	—	Avium-Intracellulare
	...	Aerobic	...	Smegmatis-Phlei
Thermophiles	...	...	...	Xenopi

Only characters essential to the classification are recorded. Drug-sensitivity tests were used to provide support for certain categories and in some instances to define subdivisions (see text). Unclassifiable strains had characteristics not listed in this table.

were both acid- and alcohol-fast; any mycobacteria that proved to be only acid-fast were included in the final "unclassifiable" group. *Nocardia* sometimes simulated mycobacteria and were then likely also to be considered unclassifiable; their identification will not be discussed further here. Brief notes on the individual groups follow. The term "tubercle bacilli" will be used for *M. tuberculosis* and *M. bovis* and the collective term "opportunistic mycobacteria" for the other organisms discussed. The drugs used to characterise certain of the organisms by their sensitivity pattern are those used in the treatment of tuberculosis.

Strict mesophiles

M. tuberculosis. For the purpose of routine practice, identification could be made by a screening procedure as described above, followed by a demonstration of eugonic (heaped) growth on glycerol-egg medium at 37°C. For a formal classification it was necessary to show that the organism was a strict mesophile giving superficial growth in semi-solid agar medium, and the supporting evidence of drug-sensitivity tests and of the absence of pigment was desirable. Opportunistic mycobacteria were distinguished from *M. tuberculosis* by their resistance to PAS or isoniazid or more usually to both; most were also highly resistant to thiacetazone and if not, with few exceptions, were pigmented.

Although *M. tuberculosis* is normally sensitive to all drugs it was considered that difficulty could arise from primary or acquired resistance. However, classification is usually applied to new isolates and primary resistance in these is seldom to more than one drug. The very rare cases of doubt could be resolved by testing the full range of drugs and relating the results to the patients' history and local experience of primary resistance.

Two special cases need mention. BCG emerged from classification as *M. tuberculosis* unless pyrazinamide, to which it is resistant, was used. In the writer's experience, tubercle bacilli isolated from local abscesses or regional adenitis after BCG vaccination have always been BCG, and a clinical history would therefore effectively prevent error. Special methods are available to support an identification of BCG if required (Marks *et al.*, 1971). The second special case, very rare in classification practice, was represented by a strain of *M. tuberculosis* highly resistant to isoniazid and having lost its catalase and become micro-aerophilic and dysgonic. However, new isolates of *M. bovis* differed in their lower level of isoniazid resistance which seldom attained and never exceeded a ratio of 4.

M. bovis. Members were micro-aerophilic. As with *M. tuberculosis* it was advisable to support the identification by sensitivity tests. Three varieties, namely the European, Afro-Asian and Africanum could be recognised within the species.

Members of the European variety were resistant to pyrazinamide. The most dysgonic of them sometimes appeared sensitive to all other drugs, but usually there was low-grade resistance to isoniazid of ratio 2-4 and, with PAS, a mixture of sensitive and resistant bacilli. A borderline result was occasionally obtained in tests with ethionamide or capreomycin. Opportunist mycobacteria were resistant to pyrazinamide but never had the PAS and isoniazid pattern of *M. bovis*.

Members of the Afro-Asian variety were sensitive to all drugs.

The Africanum variety was originally distinguished by its lack of virulence for rabbits but in the present classification it was recognised by a low-grade resistance to thiacetazone and sensitivity to other drugs. Some workers prefer to give the organism specific rank.

Psychrophiles

Marinum group. Members were photochromic and divisible into two subgroups, namely, *M. marinum* and a subgroup of unclassified organisms.

At 25°C *M. marinum* was sensitive to ethambutol, ethionamide and cycloserine, and gave results varying from borderline to ratio 4 with rifampicin. It was resistant to other drugs. All isolates from man were from clinically significant skin lesions.

The unclassified subgroup differed from *M. marinum* in drug-sensitivity pattern. These organisms were too rare for their significance to be assessed.

Scotochromic psychrophiles. No member of this group was thought significant and subdivision would therefore have offered no advantage.

Chelonei group. Members were aerobic nonchromic psychrophiles. They were mostly met as contaminants, but one entity within the group, *M. chelonei* (syn. *borstelense*), was occasionally responsible for infections in or just below the skin.

Ulcerans group. Members were micro-aerophilic and nonchromic and could be divided if required into two subgroups, namely, *M. ulcerans* (syn. *buruli*) and a subgroup of unclassified organisms.

M. ulcerans (syn. *buruli*) grew very slowly both at 25°C and at 37°C. It causes serious skin infection in certain tropical and subtropical countries. In these areas the more favourable temperature of 30°C is advised both for its isolation and for its sensitivity tests. The strains examined in the Reference Laboratory were sensitive to streptomycin, rifampicin and cycloserine, and gave a borderline result with capreomycin. They were resistant to PAS, isoniazid, ethionamide and thiacetazone, and gave variable results with ethambutol, probably owing to a population of mixed sensitivity.

The unclassified organisms grew better than *M. ulcerans* at 25°C and had different drug-sensitivity patterns. Almost all were non-significant.

Mesophiles

Kansasii group. Members were photochromic and could be divided into two subgroups, namely, *M. kansasii* and a subgroup of unclassified organisms.

M. kansasii was sensitive to rifampicin, ethionamide, ethambutol and cycloserine, and showed a mixture of sensitivity and resistance to streptomycin and capreomycin. It was resistant to PAS and isoniazid and gave variable results with thiacetazone. Most isolates were clinically significant.

The drug pattern of the unclassified organisms differed from that of *M. kansasii*. These organisms were rare and usually not significant. They included *M. simiae*.

Flavescens group. Members were aerobic and scotochromic. Besides *M. flavescens* they included a variety of other entities varying in drug-sensitivity pattern and degree of pigmentation. As none was clinically significant, subdivision of the group would have offered no advantage.

Gordonae group. Members were micro-aerophilic, scotochromic and Tween positive. Almost all those met belonged to the species *M. gordonae*, a common contaminant. The group also contained a very rare pathogen, *M. szulgai* which required consideration when there was evidence of clinical significance. Its resistance to cycloserine exceeded that of *M. gordonae*, but otherwise special tests were needed for its identification.

Fortuitum group. Members were aerobic and nonchromic. The group contained a number of entities including *M. fortuitum* and *M. abscessus* (syn. *chelonei* subsp. *abscessus*) but their separation had no clinical advantage. Cultures of *M. abscessus* sometimes had a faint pink colour which was absent or inconspicuous in the standard pigment test. Members of the group were mainly contaminants but were occasionally the cause of superficial abscesses following vaccinations, injections or penetrating injury and they rarely caused

lymphadenitis. They occasionally occurred in lung infections, almost all of which seemed no more than harmless colonisations of already damaged lung.

Terrae group. Members were micro-aerophilic, nonchromic and Tween positive. In instances where supporting evidence was needed to distinguish doubtful members from the Fortuitum group, it was useful to note that the latter were almost always resistant to ethambutol and were very often quite weak or lacking in the ability to hydrolyse Tween; members of the *Terrae* group were frankly Tween positive and almost always sensitive to ethambutol. Besides *M. terrae*, the group contained a number of other entities, but as none had clinical significance further subdivision offered no advantage.

Avium-Intracellulare group. Members were Tween-negative micro-aerophiles, either mesophilic or wide-range in their temperature requirement. They were either nonchromic or scotochromic. The group included the species *M. avium*, *M. intracellulare* and *M. scrofulaceum*. Isolates from surgical material and aspirates were usually significant, and those from sputum were often significant, but any obtained from other sources, e.g., urine, were usually not significant. Subdivision of the group into species and the latter into biotypes was considered useful in epidemiological studies, but not for clinical purposes.

The negative Tween test was considered critical for the identification and, to guard against error, confirmation with a subculture was advisable.

Wide range

Avium-Intracellulare group. This has been described above.

Smegmatis-Phlei group. Members were aerobic. They included *M. smegmatis*, *M. phlei* and other entities, but as they were rarely if ever significant their subdivision was unnecessary.

Thermophiles

Xenopi group. The absence of growth at 25°C distinguished these organisms from all other opportunist mycobacteria. Otherwise they most closely resembled Avium-Intracellulare organisms. The group could be divided into two subgroups, namely, *M. xenopi* and a subgroup of unclassified organisms.

M. xenopi was sensitive to cycloserine and ethionamide, possessed doubtful or weak resistance to streptomycin, capreomycin and rifampicin and was resistant to PAS, isoniazid, ethambutol, thiacetazone and pyrazinamide. Sensitivity to erythromycin was demonstrated by means of a disk impregnated with 5 µg of antibiotic. Resistance to isoniazid was usually at ratio 4 and did not exceed ratio 8. In contrast, Avium-Intracellulare organisms were usually more resistant to isoniazid and resistant to erythromycin. *M. xenopi* is a potential pathogen, but the proportion of isolates with clinical significance varied greatly in different areas. Infections encountered were limited to adults and almost always simulated pulmonary tuberculosis.

The unclassified subgroup accommodated any thermophile differing from *M. xenopi*. Strains were too rare for significance to be assessed.

Classification of British opportunist mycobacteria in 1974

In 1974 the Reference Laboratory classified 310 British opportunist mycobacteria (counting one strain only for each of two outbreaks of laboratory contaminants). Of these only two strains could not be assigned to the named groups. One was acid- but not alcohol-fast and the other was a Tween-positive, micro-aerophilic, wide-range organism. Unclassifiable strains were of no clinical significance.

Details of the laboratory's experience in 1974 are as follows: 5 *Marinum* group (all subgroup *M. marinum*); 6 scotochromic psychrophiles; 9 *Chelonei* group; 5 *Ulcerans* group (all unclassified subgroup); 68 *Kansasii* group (66 subgroup *M. kansasii* and 2 unclassified subgroup); 4 *Flavescens* group; 35 *Gordonae* group; 31 *Fortuitum* group; 13 *Terrae* group; 67 *Avium-Intracellulare* group; 2 *Smegmatis-Phlei* group; 63 *Xenopi* group (62 subgroup *M. xenopi* and 1 unclassified subgroup); 2 unclassifiable mycobacteria.

DISCUSSION

The classification described was based on four characters—temperature requirement, pigmentation, oxygen preference and Tween hydrolysis. The simplicity of this scheme had a corresponding danger in that in a situation in which demarcation was arbitrary and measurement was subjective there was nevertheless little latitude for error. Therefore, although in practice dilemmas were not common, some discussion of the best way to deal with them is merited.

Ambiguities in temperature requirement could usually be resolved by repeating the test with a lighter inoculum. This was essential when the first (mid-week) reading showed equal and mature growth at 25°C and 37°C but none at 45°C. A few organisms were met which seemed to be genuinely on the borderline between psychrophiles and mesophiles. Correlation with other methods indicated that these were best considered to be mesophiles, unless they were photochromogens, which indicated that they belonged to the *Marinum* group.

Pigmentation varied under different conditions. For example, it was enhanced by some drugs and depressed by others; it could also be depressed by growth at a suboptimal temperature. The standard test was reliable if the precautions recommended were taken.

Oxygen preference was accurately assessed unless the inoculum was poorly viable or too light; in such instances aerobes gave sparse deep growth in semi-solid medium and delayed, irregular or non-confluent growth on egg medium. When growth was inadequate the test was repeated with a standard inoculum of a healthy culture. On rare occasions it was difficult to distinguish on egg medium between the *Flavescens* and *Gordonae* groups or between *Fortuitum* and *Terrae*. Such instances were never clinically relevant but if necessary the matter could be resolved by the use of semi-solid medium or by the study of supplementary characters such as the drug-sensitivity pattern.

Positive Tween tests were very rarely false although contamination was theoretically possible and any unexpected result would have been investigated.

A negative result would be relevant only in respect of the Avium-Intracellulare group and should be confirmed with a healthy subculture.

Culture at 25°C was needed for the present classification. In some hot countries the lack of air-conditioning or of a cooled incubator might prevent the use of this temperature. Although much less satisfactory, a 30°C incubator could be used if, with the help of a hand lens ($\times 5$), five grades of growth were recognised and psychrophiles were defined as showing growth two or more grades higher at 30°C than at 37°C. Suggested grades for confluent growth are (1) seen with difficulty with the lens, (2) seen easily with the lens but not easily with the naked-eye, (3) visible at arm's length, (4) intermediate, and (5) mature growth as defined above. *M. xenopi* has not been reported in tropical countries and is therefore unlikely to pose a problem in consequence of its growth at 30°C. This was faint, and the species could be distinguished from wide-range, Avium-Intracellulare organisms by the very pronounced effect on growth of increased temperature and by the drug-sensitivity pattern. Problems that remain are those relating to screening and to the definition of strict mesophiles, both of which procedures employ the 25°C test. Replacement of the latter by a test with a slope at 30°C containing sodium salicylate 500 μg per ml is recommended; with the standard inoculum it did not sustain more than a trace of growth of tubercle bacilli, but allowed the growth of opportunist mycobacteria. A lens was necessary to read the test. Replacement of the 25°C test as described made the supporting role of drug-sensitivity pattern all the more important.

SUMMARY

A new classification is presented for mycobacteria cultured from clinical specimens. Five categories were defined on temperature requirement, and these were subdivided into 14 groups by pigmentation, oxygen preference and Tween hydrolysis. Support for some of the groups and for the recognition of certain important species within others was provided by drug-sensitivity tests. Less than 1.0% of the opportunist mycobacteria met in Britain eluded classification by these means.

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