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Subdivision of *Mycobacterium tuberculosis* into five variants for epidemiological purposes: methods and nomenclature

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SUMMARY

Virulent strains of *Mycobacterium tuberculosis* isolated from humans are divisible into five variants by using four tests: oxygen requirement (aerobic or microaerophilic), nitrate reductase activity, susceptibility to pyrazinamide (60 μg/ml) and susceptibility to thiophene-2-carboxylic acid hydrazide (5 μg/ml). The five variants are referred to as Classical human, Asian human, bovine, African I and African II. The relation of these variants to previously described types is discussed. This simple division has been shown to be useful in epidemiological studies.

INTRODUCTION

Like – but oh! how different!

Wordsworth, *The Mountain Echo*

The tubercle bacillus, discovered one hundred years ago by Robert Koch (1882), was named *Mycobacterium tuberculosis* by Lehmann and Neumann in 1896. For many years this species included both human and bovine types of tubercle bacilli. The bovine type was sometimes accorded separate species status as *M. bovis* but it did not achieve this officially until 1970 (Karlson & Lessel, 1970). Another species, associated with tuberculosis in Africans and named *M. africanum*, was proposed by Castets, Rist & Boisvert (1969). This separation into three species has been questioned by several workers using Adansonian classification (Tsukamura, 1967, 1976; David *et al.* 1978); DNA homology (Bradley, 1972; Baess, 1979) and immunodiffusion analysis (Stanford & Grange, 1974). Grange (1979) concludes that it is preferable to describe all these variants as *M. tuberculosis* with a type designation, e.g. human, bovine or African; and Tsukamura (1976) refers to them as the '*M. tuberculosis* complex'.

Other closely related mycobacteria have been isolated from the vole (*Microtus agrestis*) (Wells, 1936) and the dassie (cape hyrax, *Procavia capensis*) (Smith, 1960),

but not from humans. Indeed the former, which has been given the separate species epithet *M. microti* (Reed, 1957), is attenuated in man and has been used as a vaccine. Bacille Calmette-Guérin (BCG) was supposedly derived from a bovine strain but it differs in several important respects from both the bovine and the human types (Yates, Collins & Grange, 1978).

In addition, variations occur within the types referred to above. A variant of the human type, first described by Dhayagude & Shah (1948) in India, was further characterized by Grange *et al.* (1977, 1978), who noted that it was of a distinctive bacteriophage type and accounted for about 30% of cases of tuberculosis among Asian immigrants in London, but was comparatively rare among Europeans in the UK. Marks (1976) recognized two variants of the bovine type, European and Afro-Asian, which differed in their susceptibility to antituberculous drugs, especially pyrazinamide to which the former are resistant and the latter sensitive. Yates & Collins (1979) used four simple biochemical and cultural tests which enabled them to distinguish two variants of the human type and two of the bovine type. The former strains separated into European and Asian variants which correlated well with bacteriophage typing results and the ethnic group of the patients; the latter separated into European and Afro-Asian variants, which again correlated well with ethnic groups. The Afro-Asian variant was considered to be synonymous with *M. africanum*.

The work reported here is an extension of that of Yates & Collins (1979). Many more strains have been examined, including some from West African patients. Africans have been considered separately from Asians and the variant (or species) *M. africanum* has been reconsidered.

MATERIALS AND METHODS

Strains of tubercle bacilli

A total of 7428 strains, from the same number of patients, and accumulated in the period 1978–80, were examined. All were identified as tubercle bacilli by their macroscopic and microscopic appearance; their failure to grow within 21 days on Lowenstein–Jensen medium incubated at 25 °C; their failure to grow within 21 days at 37 °C on the same medium containing 500 μg/ml of *p*-nitrobenzoic acid; and growth but no pigment within 21 days on Lowenstein–Jensen medium incubated at 37 °C in an internally illuminated incubator (Collins & Lyne, 1979).

Suspensions of the strains were prepared and test media inoculated for sensitivity tests as described by Collins & Yates (1978).

Sensitivity to thiophene-2-carboxylic acid hydrazide (TCH)

TCH (Aldrich Chemical Co. Dorset) was dissolved in water and added to Lowenstein–Jensen medium to give a final concentration before inspissation of 5 μg/ml.

Nitratase test

The organisms were grown for 18 days in 1 ml amounts of Middlebrook 7H9 medium (Difco). Sodium nitrate, 0·1 ml of 1 % solution, was added; the tubes were incubated for 4 h at 37 °C and then tested for nitrate reduction by the method described by Collins & Lyne (1979) using sulphanilamide, *N*-1-naphthyl-ethylenediamine di-HCl and zinc dust.

Oxygen requirement

A modification of the method of Marks (1976) was used. Tubes containing 12 ml of Kirchner medium enriched with 10 % horse serum and made semi-solid with 0·1 % pure agar (Oxoid) were inoculated with approximately 0·2 ml of suspension. After careful mixing to avoid aeration the tubes were incubated undisturbed for 21 days. Aerobic growth occurred at the surface extending into the medium to a depth of 5 mm. Micro-aerophilic growth occurred as a band, about 5 mm wide, and between 5 and 10 mm below the surface, but occasionally extending towards the surface.

Pyrazinamide sensitivity

A modification of the method of Marks (1964) was used. Kirchner medium (2 ml) containing 0·1 % agar and at pH 5·2 was layered onto butts of Lowenstein–Jensen medium (1 ml) at the same pH. Duplicate sets of medium with and without 60 μg/ml of pyrazinamide received 0·05 ml of the inoculum. Another set received 0·05 ml of the inoculum diluted 1:10 in Middlebrook 7H9 medium (Difco). Tests were incubated at 37 °C and read at 14 and 21 days. Strains showing growth from either inoculum in the pyrazinamide-free medium, but not in the medium containing the drug, were recorded as sensitive. False resistance was sometimes observed with the heavier inoculum.

RESULTS

Of the total of 7428 patients, 4723 had European names, 2662 had Asian names and 43 had African names. The Asians included patients from the Indian subcontinent, from East Africa, and a few from the Middle East.

Table 1 shows that the 7428 strains could be separated into two main groups by their oxygen requirements. Of these 7246 were aerobic and 182 were micro-aerophilic.

All of the aerobic strains reduced nitrate and were sensitive to pyrazinamide but could be separated into two variants according to their susceptibility to TCH. Of the 6436 strains that were resistant to TCH, 4324 (65·8 %) were from patients with European names. These are described as Classical human variants. Of the 810 that were sensitive to TCH, 426 (52·6 %) were from Asians and were assigned as Asian human variants. The associations of the strains to the ethnic origin of the patients was highly significant ($P < 0·001$).

All of the micro-aerophilic strains were sensitive to TCH but could be separated into three variants by their susceptibility to pyrazinamide and ability to reduce nitrate. Of the 86 strains which were resistant to pyrazinamide and did not reduce

Table 1. *Variants of tubercle bacilli and ethnic groups of patients*

	O_2	TCH	NO_3	PZ	Europeans	Asians	Africans	Totals
Classical bovine	M	–	–	R	77 (89·6)	7 (8·1)	2 (2·3)	86
African I	M	–	–	S	23 (30·3)	32 (42·1)	21 (27·6)	76
African II	M	–	+	S	9 (45)	6 (30)	5 (25)	20
Asian human	A	–	+	S	380 (46·9)	426 (52·6)	4 (0·5)	810
Classical human	(A)	+	(+)	(S)	4234 (65·8)	2191 (34·0)	11 (0·2)	6436
Totals					4723 (63·6)	2662 (35·8)	43 (0·6)	7428

A, aerobic; M, micro-aerophilic; R, resistant; S, sensitive; O_2, oxygen preference; TCH, thiophene-2-carboxylic acid hydrazide sensitivity; NO_3, nitratase; PZ, pyrazinamide sensitivity.

(+), (A), (S) tests not done on all TCH-positive strains.

( ) Figures in parentheses are percentages.

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nitrate, 77 (89·6%) were from European patients, and these variants were designated as Classical bovine. Of the 76 strains which were sensitive to pyrazinamide and did not reduce nitrate, 32 (42·1%) were from Asians and 21 (27·6%) from Africans. These were labelled African I variants.

Only 20 strains were sensitive to pyrazinamide and reduced nitrate. These were not significantly associated with any ethnic group (9 Europeans and 11 non-Europeans) but were called African II variants because of their general similarity to the African I variants.

DISCUSSION

Many species of mycobacteria contain a number of distinct variants that differ in their cultural and biochemical properties and in their virulence for experimental animals. *Mycobacterium tuberculosis* is no exception, but the great importance of this species as a pathogen of man and animals has led workers to allot separate species names to variants that, when examined by criteria applied to other species, are clearly recognizable as intraspecific variants of a single species.

In this study we have subdivided *M. tuberculosis* into five variants by using four reliable and simple tests that can easily be carried out in any public health laboratory. Further subdivisions are obtainable by bacteriophage typing (Grange *et al.* 1977), mass spectroscopy (Weiten *et al.* 1981) and amino acid utilization (Barrow, 1981). These discriminative tests are indispensable for solving certain epidemiological problems, such as the spread of tuberculosis from badgers to cattle (Barrow, 1981), but they are costly and time consuming. On the other hand, the tests described in this study are easily performed on thousands of cultures received by busy reference centres and have proved to be of epidemiological usefulness in studies on tuberculosis in South-East England (Collins, Yates & Grange, 1981; Yates, Collins & Grange, 1982; Grange, Collins & Yates, 1982).

The two 'classical' types of *M. tuberculosis* are the human and the bovine. This nomenclature was introduced by Smith (1898), although with some reluctance as he realized that this would anticipate assumptions that the types were limited to the host whose names they bore. (Smith was right, for in 1901, Robert Koch himself made this erroneous assumption (Report, 1902).)

The Asian human strains differ from the classical human strains in being susceptible to hydrogen peroxide, attenuated in the guinea-pig, sensitive to TCH, of a characteristic phage type (type I), possessing a characteristic lipid in their cell walls (the attentuation indicator lipid) and being more resistant to para-aminosalicylic acid and thiacetazone (Grange *et al.* 1978). Although some discrepancies occur whichever test is used, the easiest way to differentiate between the strains is to test for susceptibility to TCH (Grange *et al.* 1977; Yates & Collins, 1979).

Marks (1976) divided the 'species' *M. bovis* into the European and Afro-Asian varieties, the former differing from the latter in being resistant to pyrazinamide. Subsequently Collins *et al.* (1981) used the terms 'Classical' and 'Afro-Asian bovine' strains. The term 'Classical' is preferable to 'European' as this variant

is not restricted to Europe. Indeed Smith's (1898) pioneering work was performed in the USA. Although we (Collins *et al.* 1981) followed the suggestion of Marks (1976) and regarded the Afro-Asian strain as being a variant of the bovine type, there is no evidence that this type is associated with cattle. Thus the term 'bovine' is best omitted.

The strains termed *M. africanum* by Castets and his colleagues (1969) were at first regarded as a geographically localized variant of the human tubercle bacillus. In the same year Meissner & Schröder (1969) described the properties of a group of strains from Africa which were 'intermediate' in their properties between human and bovine types and which they termed African strains. Subsequently Prat and his colleagues (1974) demonstrated a considerable degree of heterogeneity among this group of strains and considered that they represented a continuous spectrum of variation linking the human and bovine strains. On the other hand, David and his co-workers (1978) found, by numerical analysis, that the strains tended to form two clusters, one related to the human type and the other to the bovine type. Those resembling the bovine type were more frequent in West Africa (Yaounde, Dakar and Mauretania) while those resembling the human type occurred in the more easterly regions (Burundi and Rwanda) (Clavel, 1975; David *et al.* 1978).

The relationship of the 'African' strains to those known as *M. africanum* is largely a question of definition. All 26 African patients in the present study infected with such strains were from West Africa (Ghana and Nigeria). Strains of *M. africanum* from this region resemble the African I strains in being sensitive to TCH and pyrazinamide, and in being nitratase negative. The African II strains showed no preference for any ethnic group although, by being nitratase positive, they would be identified as the East African type of *M. africanum*. Some of the strains of this type were isolated from Asians, amongst whom there may have been immigrants from Uganda. Although most strains from Ugandan Africans and Asians appear to be classical or Asian human strains (Grange *et al.* 1977), a strain with the properties of the African II type has been isolated from a Ugandan Asian lady who had recently emigrated to Scotland (MacLeod, 1977).

Geographical differences in the distribution of variants of a mycobacterial species are not restricted to *M. tuberculosis* but have also been described in *M. chelonei* (Stanford *et al.* 1972; Grange, 1981) and *M. kansasii* (Engel *et al.* 1980). In *M. tuberculosis*, the variants may be due to divergent evolution in geographically distinct regions (Grange *et al.* 1978).

In conclusion, we propose a set of simple tests for the subdivision of *M. tuberculosis* into five major types which we consider represent important evolutionary variations within this species. This subdivision correlates closely with the variants recognized by other workers and is of proven epidemiological usefulness.

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