Journal of Clinical Microbiology

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S E Hoffner, S B Svenson, R Norberg, et al.
1993. Biochemical heterogeneity of Mycobacterium tuberculosis complex isolates in Guinea-Bissau.. J. Clin. Microbiol. 31(8):2215-2217.

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1993, p. 2215–2217
0095-1137/93/082215-03$02.00/0

Vol. 31, No. 8

Biochemical Heterogeneity of *Mycobacterium tuberculosis* Complex Isolates in Guinea-Bissau

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Received 9 December 1992/Accepted 17 May 1993

Fifty-six strains of the *Mycobacterium tuberculosis* complex from patients in Guinea-Bissau were examined by using four biochemical tests (niacin production, nitrate reductase, pyrazinamidase, and resistance to thiophen-2-carboxylic acid hydrazide). The isolates were divided into five different biovars within a spectrum ranging from classical human *M. tuberculosis* to classical *M. bovis*.

Tuberculosis is a common disease in Guinea-Bissau. Although the annual incidence is not known, it is estimated to be one of the highest in the world. The present epidemic of human immunodeficiency virus type 2 infection (12) and the emerging epidemic caused by human immunodeficiency virus type 1 in Guinea-Bissau also most likely will cause an increase in the incidence of tuberculosis. In this report, we describe the use of some biochemical markers for epidemiological subtyping of *Mycobacterium tuberculosis* complex strains isolated from sputum samples from patients with tuberculosis in Bissau which might be relevant for epidemiological studies in Africa. This study is the first to investigate *M. tuberculosis* complex isolates from Guinea-Bissau.

During four periods (1989 to 1991), 288 sputum samples were collected from the same number of patients who were referred to the Laboratorio Nacional de Saude Publica, Bissau, Guinea-Bissau. The patients had been referred for sputum smear examination by either peripheral health care clinics or hospitals in Bissau. The samples were kept at +4°C and transported (by air, in Frigolite casings containing cold clumps) to the National Bacteriological Laboratory, Stockholm, Sweden, for mycobacterial culture and further examination.

Before culture, the samples were decontaminated from nonmycobacterial microorganisms by the sodium lauryl sulfate method (7). Aliquots (0.5 ml) of the homogenized specimen were inoculated into one tube with conventional Löwenstein Jensen (LJ) egg medium and one containing LJ medium with 0.6% pyruvate (LJpyr). In addition, samples were concentrated by membrane filtration and cultured on LJ medium. The tubes were incubated at 37°C for 7 weeks and examined weekly. Growth of mycobacteria was confirmed by acid-fast microscopy.

All isolates were characterized by standard biochemical methods (11). Resistance to thiophen-2-carboxylic acid hydrazide (TCH) (5 mg/liter) was determined by radiometric respirometry (BACTEC system; Becton Dickinson and Co., Cockeysville, Md.). The BACTEC method has previously been shown to agree with results obtained with LJ medium (3, 8). The BACTEC system was also used for susceptibility testing of *M. tuberculosis*. It is well established that this method is in good agreement with reference methods using solid media (9, 14). Detection of niacin was performed as described by Wayne (19), and nitrate reductase and pyrazinamidase tests were done as described by Kent and Kubica (11). The test for the *M. tuberculosis* complex included a nucleic acid probe using the Accuprobe system (Gen-Probe, San Diego, Calif.).

A total of 58 strains belonging to the *M. tuberculosis* complex were isolated. These isolates showed typical macroscopic and microscopic appearance and growth characteristics. Of the 58 isolates, only 1 was resistant to any of four major antituberculous drugs (isoniazid) (5).

Fifty-six *M. tuberculosis* complex isolates were further characterized biochemically. By the four biochemical tests (TCH resistance, niacin production, and nitrate reductase and pyrazinamidase tests), they were assigned to five different biovars (Table 1).

One strain conformed to the criteria for *M. bovis* (susceptible to TCH and negative in the other tests), and 19 isolates fully agreed with the criteria for *M. tuberculosis* (resistant to TCH and positive in the other tests). The remaining isolates were allocated into three distinct biovars representing a spectrum between the classical human and bovine tubercle bacilli (Table 1). With the biochemical methods used by us, two biovars (our biovars 2 and 3) corresponded to the so-called African I variant and one biovar (our biovar 4) corresponded to the African II variant described by Collins et al. (2).

DNA-DNA homology data support the assignment of *M. tuberculosis*, *M. africanum*, *M. microti*, and *M. bovis* to a single species (1, 10). It is not clear whether they should be reduced to subspecies or biovar status, and no formal proposals to reduce them to species synonymy have been published, although Tsukamura has suggested on several occasions that they be considered one species and has coined the term "TB complex" (15, 16, 17). Grange (6) concluded that it is preferable to describe them as variants (bovine, human, and African) of *M. tuberculosis*.

Prat and colleagues (13) found a considerable degree of heterogeneity among African strains and concluded that they represent a continuous spectrum linking the classical human and bovine variants. On the basis of numerical analysis of biochemical characteristics, David and coworkers (4), on the other hand, concluded that African strains tend to form two clusters, one related to the human type and one related to the bovine type. They did, however, state that the study of more strains might cause the clusters to fuse. In a recent study by van Soolingen and coworkers (18), African isolates of the *M.*

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TABLE 1. Biovars of 56 *M. tuberculosis* complex isolates

Biovar	Test Result				No. of isolates
	TCH[a]	NO_3[b]	Nia[c]	PZ[d]	
1[e]	S	−	−	−	1
2	S	−	−	+	6
3	S	−	+	+	9
4	S	+	+	+	21
5[f]	R	+	+	+	19

[a] S, susceptible; R, resistant.
[b] NO_3, nitrate reductase.
[c] Nia, niacin production.
[d] PZ, pyrazinamidase.
[e] Classical *M. bovis*.
[f] Classical human *M. tuberculosis*.

tuberculosis complex were more homogeneous according to restriction fragment length polymorphism patterns than were European isolates. This is in contrast to the larger biochemical variation among African isolates reported here, although the nucleic acid probe specific for the *M. tuberculosis* complex was positive for all isolates. Further studies on correlations between different biovars and restriction fragment length polymorphism patterns thus are important.

In Europe, the dominant variant within the *M. tuberculosis* complex is classical human *M. tuberculosis*, followed by classical *M. bovis*. All of the tests used in this study discriminate between these two variants or biovars. However, the only single test that assigned isolates to any specific group (classical *M. tuberculosis*) was TCH resistance. None of the biochemical tests could be used as a single confirmatory test for the entire *M. tuberculosis* complex.

In Europe, *M. tuberculosis* complex isolates from cattle and deer are practically always classical *M. bovis*. Thus, epidemiological subtyping of the *M. tuberculosis* complex into *M. bovis* is useful for epidemiological studies of tuberculosis in cattle, in particular to study transmission from cattle to humans. If classical *M. bovis* is also the predominant variant in cattle in Africa, it appears from this study that tuberculosis derived from cattle is rare in Guinea-Bissau. However, more detailed study of the biovar(s) of isolates from cattle in Africa is needed (First International Conference on Animal Tuberculosis in Africa and the Middle East, April 1992).

It is important to note that a considerable number of isolates belonging to biovar groups 1 to 3 were recovered only on LJpyr (Table 2), while equal numbers of isolates from groups 4 and 5 were recovered on LJ medium and LJpyr. Classical *M. bovis* grows better with LJpyr; and from this study it appears that the same is also true for other biovars, in particular for those biochemically most closely related to *M. bovis*. Hence, since 20 (36%) of the isolates, mainly those belonging to three of the five biovars described here, grew only on LJpyr, this medium should be included to ensure the isolation of these biovars.

TABLE 2. Recovery of *M. tuberculosis* complex isolates on LJ medium and LJpyr

Biovar	No. of strains that grew on:			Total no. of isolates
	LJ medium only	LJpyr only	Both media	
1 (*M. bovis*)		1		1
2	1	5		6
3		6	3	9
4	2	3	16	21
5 (*M. tuberculosis*)	2	5	7	14[a]

[a] An additional five strains belonging to this group were recovered only after concentration of the sample by membrane filtration (and subsequent incubation on LJ medium) and not in any of the two tubes used in this experiment.

In conclusion, a few simple, easily performed tests may be used for epidemiological studies of tuberculosis in Africa. On the basis of the high degree of DNA homology, as well as the apparent continuous spectrum of biochemical heterogeneity within the *M. tuberculosis* complex, we propose that mycobacteria belonging to the *M. tuberculosis* complex be regarded as belonging to biovars within one species. Such a definition should—in particular, from an epidemiological point of view—be more relevant, especially in areas where a high level of transmission between animals and humans is likely to occur.

We thank Erik Hoffmann for skillful technical assistance.
This study was supported by the Swedish Heart Lung Association.

REFERENCES

1. **Baess, I.** 1979. Deoxyribonucleic acid relatedness among species of slowly-growing mycobacteria. Acta Pathol. Microbiol. Scand. Sect. B **87**:221–226.
2. **Collins, C. H., M. D. Yates, and J. M. Grange.** 1982. Subdivision of Mycobacterium tuberculosis into five variants for epidemiological purposes: methods and nomenclature. J. Hyg. **89**:235–242.
3. **Collins, T., and P. N. Levett.** 1989. Radiometric studies on the use of selective inhibitors in the identification of *Mycobacterium* spp. J. Med. Microbiol. **30**:175–181.
4. **David, H. L., M. T. Jahan, A. Jumin, J. Grandry, and E. H. Lehman.** 1978. Numerical taxonomy analysis of *Mycobacterium africanum*. Int. J. Syst. Bacteriol. **28**:467–472.
5. **Dias, F., S. Ghebremichael, S. E. Hoffner, L. Martins, R. Norberg, and G. Källenius.** 1993. Drug susceptibility in *Mycobacterium tuberculosis* of a sample of patients in Guinea-Bissau. Tubercle **74**:129–130.
6. **Grange, J. M.** 1979. The changing tubercle. Br. J. Hosp. Med. **22**:540–548.
7. **Groothuis, D. G., and M. D. Yates (ed.).** 1991. Manual of diagnostic and public health mycobacteriology, 2nd ed. Bureau of Hygiene and Tropical Diseases, London.
8. **Hoffner, S. E.** Unpublished data.
9. **Hoffner, S. E., and G. Källenius.** 1988. Susceptibility of streptomycin-resistant *Mycobacterium tuberculosis* strains to amikacin. Eur. J. Clin. Microbiol. Infect. Dis. **7**:188–190.
10. **Imaeda, T.** 1985. Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. Int. J. Syst. Bacteriol. **35**:147–150.
11. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology. A guide for the level III laboratory. Centers for Disease Control, Atlanta.
12. **Nauclèr, A., P. Albino, A. P. da Silva, P. A. Andreasson, S. Andersson, and G. Biberfeld.** 1991. HIV-2 infection in hospitalized patients in Bissau, Guinea-Bissau. AIDS **5**:301–304.
13. **Prat, R., N. Rist, N. Dumitrescu, A. Mugabushaka, S. Clavel, and C. Duponchel.** 1974. Special characteristics of the cultures of tubercle bacilli isolated in Ruanda. Bull. Int. Union Tuberculosis **49**:53–62.
14. **Snider, D. E., R. C. Good, J. O. Kilburn, I. F. Laskowski, R. H. Lusk, J. J. Marr, Z. Reggiardo, and G. Middlebrook.** 1981. Rapid drug-susceptibility testing of *M. tuberculosis*. Am. Rev. Respir. Dis. **123**:402–406.
15. **Tsukamura, M.** 1967. Identification of mycobacteria. Tubercle **48**:311–338.

16. **Tsukamura, M.** 1984. Intermediate between *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Microbiol. Immunol. **28:**961–963.
17. **Tsukamura, M., and S. Mizuno.** 1968. "Hypothetical mean organisms" of mycobacteria. A study of classification of mycobacteria. Jpn. J. Microbiol. **12:**371–384.
18. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
19. **Wayne, L. G.** 1985. The "atypical" mycobacteria: recognition and disease association. Crit. Rev. Microbiol. **12:**185–222.