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Differentiation among Members of the *Mycobacterium tuberculosis* Complex by Molecular and Biochemical Features: Evidence for Two Pyrazinamide-Susceptible Subtypes of *M. bovis*

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The variations in biochemical as well as molecular characteristics among several members of the *Mycobacterium tuberculosis* complex that are not *M. tuberculosis* have been assessed to facilitate an unambiguous species identification. Altogether, 96 *M. tuberculosis* complex strains including 52 *M. bovis* isolates and 44 *M. africanum* isolates were analyzed by spoligotyping. The strains could be clustered into five spoligotype groups. All *M. bovis* isolates showed the typical absence of the spacers 39 to 43 and typical biochemical properties. However, within these strains we found a group of strains that had a spoligotype pattern which is clearly defined by the additional absence of spacers 3 to 16 and that were uncommonly susceptible to pyrazinamide (PZA). This spoligotype pattern has previously been described as being typical for a caprine genotype because of its predominant isolation from sheep and goats. Due to the clinical importance of PZA resistance, we propose two *M. bovis* subtypes: *M. bovis* subtype *bovis*, which is resistant to PZA, and *M. bovis* subtype *caprae*, which is susceptible to PZA. Two additional strains that clustered in group 3 showed biochemical and genetic properties typical for *M. bovis* and were also sensitive to PZA; thus, they may represent a third PZA-susceptible *M. bovis* subtype. The *M. africanum* isolates could be clustered into two spoligotype groups which can be differentiated from *M. bovis* by hybridization to spacers 39 to 43. These groups correspond to the previously described *M. africanum* subtypes I and II and can be clearly distinguished from each other by spoligotyping and resistance to thiophen-2-carboxylic acid hydrazide. Our results demonstrate that spoligotyping is a useful tool for differentiation of *M. bovis* and *M. africanum*. Moreover, we describe two PZA-susceptible *M. bovis* subtypes and describe a method that facilitates an unambiguous differentiation of the two *M. africanum* subtypes.

The *Mycobacterium tuberculosis* complex comprises the closely related species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti* (25, 27). These species are the causative agents of tuberculosis (TB) in humans and animals. Their near relatedness could be verified by the high degree of similarity at the DNA level (DNA relatedness of 85 to 100% [12]) by multilocus enzyme electrophoresis and sequencing of the 16S rRNA gene (rDNA) and the 16S-to-23S rDNA internal transcribed spacer (ITS) (8, 9, 15, 25).

Despite this genetically close relationship, the members of the *M. tuberculosis* complex differ in their epidemiology and in their importance for TB disease in humans. *M. tuberculosis* is the major cause of human TB and, according to the latest figures of the World Health Organization (29), infects more than one-third of the world's population. The natural habitat of *M. tuberculosis* is humans, but it can also infect animals which have contact with humans. *M. africanum* is predominantly isolated in different parts of Africa (4). In certain regions, *M. africanum* represents up to 60% of the isolates obtained from patients with pulmonary TB (11; V. Sticht-Groh, G. Bretzel, S. Rüsch-Gerdes, S. Bwire, and H. J. S. Kawuma, Abstr. 28th World Conf. IUATLD/UICTMR, abstr. A169). *M. microti* causes TB mainly in small rodents like voles (26, 27), but until now its importance for TB in humans has remained unclear. The recently described new taxon *M. canetti* can cause TB in humans, but so far only a few *M. canetti* strains have been isolated and its epidemiological contribution to TB in humans is uncertain (25). The host range of *M. bovis* is broad, and it can cause TB in various domestic or wild animals like cattle or goats, but it can also cause TB in humans (27). Bovine TB remains an important disease in many countries of the world, causing significant economic losses.

Routine identification of *M. tuberculosis* complex isolates can easily be performed with commercially available gene probes (22). Moreover, several repetitive elements like the direct repeat (DR) locus or the insertion sequence IS*6110* have been found to be exclusively present in members of the *M. tuberculosis* complex (16). However, differentiation among the most important mycobacterial pathogens for humans, *M. tuberculosis*, *M. africanum*, and *M. bovis* is based on several biochemical tests (27). *M. tuberculosis* is most easily identified by its special colony morphology (eugonic growth), by nitrate reduction, and by niacin accumulation. *M. bovis* shows dysgonic growth and is negative for nitrate reduction and niacin accumulation (27). A major criterion for the differentiation of *M. bovis* is its intrinsic resistance to pyrazinamide (PZA). However, some studies report susceptibility to PZA among *M. bovis* isolates (3, 28). In recent years we noticed in our laboratory a portion of approximately 5% *M. bovis* strains susceptible to PZA. Since some *M. africanum* strains with biochemical characteristics identical to those of *M. bovis* are mainly identified by their PZA sensitivity, differentiation between these strains and PZA-susceptible isolates of *M. bovis* becomes very difficult or even impossible.

Identification of *M. africanum* is based on a composition of biochemical characteristics which comprise properties of *M.*

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tuberculosis as well as of *M. bovis*. Two major subgroups of *M. africanum* that differ in their biochemical characteristics have been described, and these correspond to their geographic origin in West or East Africa (subtypes I and II, respectively). Numerical analyses of biochemical characteristics revealed that subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles *M. tuberculosis* (4). The high degree of variability in phenotypic characteristics among *M. africanum* isolates complicates the clear identification of *M. africanum* and may lead to misclassification of clinical isolates. Nevertheless, it is important to distinguish members of the *M. tuberculosis* complex for epidemiologic and public health purposes. Hence, unequivocal criteria and methods that ensure an accurate and easy-to-perform differentiation of *M. tuberculosis* complex isolates, especially of *M. bovis* and *M. africanum*, are necessary.

More recently the spoligotyping method proved to be useful for the differentiation of *M. bovis*, *M. microti*, and *M. canetti* (13, 26, 27). This method is based on the detection of various nonrepetitive spacer sequences located between small repetitive units (DRs) in the DR locus of *M. tuberculosis* complex strains. Additionally, the *mtp40* sequence has been reported to be useful as a tool for the identification of *M. tuberculosis* and *M. africanum* (16). Furthermore, single point mutations in the *pncA* gene and the *oxyR* locus have been described to be characteristic for *M. bovis* (21, 23). The *pncA* gene encodes a pyrazinamidase. A single nucleotide change at position 169 that causes resistance to PZA has been found to be specific for *M. bovis* strains (21). In the *oxyR* locus a DNA sequence polymorphism has also been reported to be suited for the identification of *M. bovis* (23). With respect to other members of the *M. tuberculosis* complex, *M. bovis* contains an adenine instead of a guanine residue at position 285. This DNA sequence polymorphism can easily be detected by a PCR-restriction fragment length polymorphism (RFLP) analysis (23).

In the study described here we showed that the spoligotyping method in combination with other genetic markers and biochemical tests facilitates an accurate differentiation of *M. bovis* and *M. africanum* species. Furthermore, we could clearly demonstrate the presence of at least one unique subtype of *M. bovis*, characterized by its susceptibility to PZA and a typical spoligotype pattern.

MATERIALS AND METHODS

Strains analyzed. A total of 96 *M. tuberculosis* complex strains comprising 52 *M. bovis* and 44 *M. africanum* strains isolated in the years 1997 and 1998 have been analyzed in this study. The isolates have mainly been obtained from patients living in Germany. Four isolates were obtained from patients living in Sierra Leone, 19 isolates were obtained from patients living in Uganda, 1 isolate was obtained from a patient living in Cameroon, and 1 isolate was obtained from a patient living in Nigeria. Four strains isolated from cattle have been included. The human isolates have been obtained from different patients of different geographic origins. *M. tuberculosis* H37Rv served as a control strain.

Primary isolation and culturing of mycobacterial isolates were performed as described elsewhere (14). All isolates were identified as *M. tuberculosis* complex by using gene probes (ACCUProbe; GenProbe, San Diego, Calif.).

Biochemical tests and susceptibility testing. Biochemical analyses for differentiation included colony morphology, nitrate reduction on modified Dubos broth, niacin accumulation test (INH-test strips; Difco, Detroit, Mich.), growth in the presence of thiophen-2-carboxylic acid hydrazide (TCH; 2 μg/ml), and growth characteristics on Lebek medium and on bromcresol purple medium (induction of a pH-dependent change of color from blue to yellow, e.g., in the case of *M. tuberculosis* isolates) (7, 11, 14, 17). Lebek is a semisolid medium which can be used to test the oxygen preference of mycobacterial isolates. Aerophilic growth is indicated by growth on the surface and above the surface on the glass wall of the tube, whereas microaerophilic growth is indicated by growth below the surface. Drug susceptibility was determined by the proportion method on Löwenstein-Jensen medium and/or the modified proportion method in BACTEC 460TB. Drug susceptibility testing on Löwenstein-Jensen slants was performed according to the German Deutsches Institut für Normung guidelines.

Spoligotyping analysis. Spoligotyping of isolates was performed as described by Kamerbeek et al. (13). Briefly, one loopful of bacteria was suspended in distilled water (400 μl), subjected to sonication for 15 min, and boiled for 20 min in a water bath. This suspension was directly used for PCR amplification of the DR region (18). The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides derived from previously described DR spacer sequences which were bound on a nylon membrane by reverse line blotting (Isogen, Maarssen, The Netherlands). Safety measures were taken according to the German DIN guidelines (6).

PCR amplification conditions. For amplification of the *mtp40* sequence the primers PT-1 (5′-CAACGCGCCGTCGGTGG-3′) and PT-2 (5′-CCCCCCACG GCACCG-3′) were used to amplify a 396-bp fragment (5). Three microliters of the bacterial suspension (see spoligotyping analysis) were used for PCR. The 50-μl reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate (Boehringer, Mannheim, Germany) at a concentration of 200 μM, 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany). The reaction mixture was overlaid with mineral oil (Perkin-Elmer, Foster City, Calif.). PCR amplifications were performed in a Perkin-Elmer Cetus 480 instrument by the following protocol: initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 69°C for 1 min, and extension at 72°C for 1.5 min; and a final extension at 72°C for 5 min.

Amplification of the ITS was carried out with the primers ITS-1 (5′-GATTG GGACGAAGTCGTAAC-3′) and ITS-2 (5′-AGCCTCCCACGTCCTTCATC-3′) as described previously (19). A 673-bp region of the *pncA* gene was amplified by using the primers *pncA*-1 (5′-GCTGGTCATGTTCGCGATCG-3′) and *pncA*-2 (5′-CAGGAGCTGCAAACCAACTCG-3′) as described by Sreevatsan et al. (24).

DNA sequencing analysis. Direct sequencing of ITS and *pncA* PCR fragments was performed by cycle sequencing by using the BigDye RR Terminator Cycle Sequencing Kit (Perkin-Elmer) and the ABI Prism 377 DNA sequencer (Perkin-Elmer) as instructed by the manufacturer. The PCR primers were also used as sequencing primers. The DNASIS program V2.1 (Hitachi, San Bruno, Calif.) was used for DNA sequence comparisons. DNA sequences were compared with the most up-to-date version of the GenBank nonredundant data bank by using the BLASTN algorithm (1).

PCR-RFLP analysis of the *oxyR* DNA polymorphism at position 285. The PCR-RFLP analysis of *oxyR* was performed as described by Sreevatsan et al. (23). Briefly, the presence of the *M. bovis*-specific mutation at *oxyR* nucleotide 285 was analyzed by amplification of a 548-bp fragment of *oxyR* by using the primers *oxyR*-1 (5′-GGTGATATATCACACCATA-3′) and *oxyR*-2 (5′-CTATG CGATCAGGCGTACTTG-3′), followed by restriction of the PCR fragment with *Alu*I and separation of the resulting DNA fragments by agarose gel electrophoresis (23).

RESULTS

Spoligotyping analysis, susceptibility to PZA, and source of isolates. All isolates have been analyzed by spoligotyping and were clustered in similarity groups by the presence of characteristic spacers. Among the 96 strains analyzed five spoligotype groups each comprising at least two isolates could be found (Fig. 1). The spoligotypes among isolates of one group shared common features that led to a clear distinction from the spoligotypes of other isolates. However, in general we found a high degree of variability of spoligotype patterns among the strains analyzed, suggesting that these isolates were not epidemiologically related. All 52 *M. bovis* isolates had spoligotype patterns which showed the typical absence of spacers 39 to 43 (groups 1 to 3). Surprisingly, among these strains we found a distinct group of 15 isolates (group 2) that had a spoligotype pattern which is clearly defined by the additional absence of spacers 3 to 16 (Fig. 1). All isolates that clustered in group 1 were resistant to PZA, whereas the strains of group 2 were found to be susceptible to PZA (Table 1). All isolates of group 1 were obtained from human patients. Of the 15 isolates of group 2, 12 were obtained from humans but 3 were obtained from cattle. Group 3 comprises two isolates which showed a hybridization signal only to spacer 2 and spacers 17 to 24 (Fig. 1). Both isolates were susceptible to PZA. One isolate was obtained from a human and one was obtained from a cow.

In contrast to the *M. bovis* strains analyzed, all 44 *M. africanum* isolates showed a hybridization signal for at least three of the spacers from spacers 39 to 43, reflecting the typical *M. tuberculosis* spoligotype pattern (groups 4 and 5). However, 20

FIG. 1. Spoligotypes and spoligotype groups among the *M. tuberculosis* complex isolates analyzed.

isolates additionally hybridized to at least two of the spacers from spacers 33 to 36, which are usually present in *M. bovis* strains, and thus were clustered in group 4. In contrast, hybridization to these spacers did not occur for the other 24 *M. africanum* isolates, which, consequently, have been clustered in group 5. All *M. africanum* isolates were susceptible to PZA (Table 1) and have been obtained from human patients. Besides strains isolated from German patients, among the isolates in group 4, 4 isolates were obtained from patients living in Sierra Leone (West Africa), 1 isolate was obtained from a patient living in Cameroon (West Africa), and one isolate was obtained from a patient living in Nigeria (West Africa), whereas 19 isolates of group 5 have been obtained from patients living in Uganda (East Africa).

TABLE 1. Biochemical and genetical characteristics of *M. tuberculosis* H37Rv and the strains analyzed[a]

Organism and group	PZA sensitivity	Niacin accumulation	Nitrate reduction	Growth in presence of TCH	Change of color of bromcresol medium	Colony morphology (growth)	Growth on Lebek medium	Presence of *mtb40*	Presence of *pncA* mutation (C to G)	Presence of *oxyR* mutation (G to A)
M. tuberculosis H37Rv ($n = 1$)	S	+	+	+	+	Eugonic	Aerophilic	+	–	–
M. bovis										
Group 1 ($n = 35$)	R (100)[b]	– (100)	– (100)	– (100)	– (100)	Dysgonic (100)	Microaerophilic (100)	– (100)[c]	+ (100)[d]	+ (100)[d]
Group 2 ($n = 15$)	S (100)	– (100)	– (100)	– (94), + (6)[e]	– (100)	Dysgonic (100)	Microaerophilic (100)	– (100)	– (100)[d]	+ (100)[d]
Group 3 ($n = 2$)	S (100)	– (100)	– (100)	– (100)	– (100)	Dysgonic (100)	Microaerophilic (100)	– (100)	– (100)	+ (100)
M. africanum										
Group 4 ($n = 20$)	S (100)	+ (55), – (45)	+ (65), – (35)	– (85), + (15)[e]	– (100)	Dysgonic (100)	Microaerophilic (100)	+ (70), – (30)	– (100)[d]	– (100)[d]
Group 5 ($n = 24$)	S (100)	+ (30), – (70)	+ (100)	+ (100)	– (100)	Dysgonic (100)	Microaerophilic (100)	+ (100)	– (100)[d]	– (100)[d]

[a] Abbreviations and symbols: S, susceptible, R, resistant; +, positive test result; –, negative test result.
[b] Values in parentheses are percentage of isolates.
[c] Ten randomly chosen strains were tested.
[d] Five randomly chosen strains were tested.
[e] Strains were resistant to isoniazid and cross-resistant to TCH.

Correlation between clustering in spoligotype groups and biochemical results. To address the question of whether the differentiation of the strains by spoligotyping correlates with the phenotypic characteristics, analyses of the biochemical properties of these strains have been performed. *M. tuberculosis* H37Rv was additionally analyzed for demonstration of characteristics typical of those of *M. tuberculosis*.

M. tuberculosis H37Rv had eugonic growth and was positive for niacin accumulation and nitrate reduction, and growth was not inhibited by the presence of TCH (Table 1). It showed aerophilic growth on Lebek medium and induced a change of color of bromcresol medium. In contrast, all other *M. tuberculosis* complex strains tested in this study had dysgonic growth and microaerophilic growth on Lebek medium and induced no color change of bromcresol medium (Table 1).

All *M. bovis* strains analyzed (groups 1 to 3) were negative for niacin accumulation and nitrate reduction, and did not grow in the presence of TCH (one strain did grow in the presence of TCH due to cross-resistance to isoniazid). Among the *M. africanum* strains clustered in groups 4 and 5, variable results were observed for niacin accumulation and nitrate reduction (Table 1). However, growth of all strains clustered in group 4 was inhibited in the presence of TCH (except strains which were resistant to isoniazid), whereas all strains of group 5 were resistant to TCH.

Presence of *mtb40* sequence. The presence of the *mtb40* sequence has been tested by amplification of a 396-bp fragment with the primers PT-1 and PT-2. A DNA fragment of the correct size was amplified from the genome of *M. tuberculosis* H37Rv, from the genomes of 70% of *M. africanum* group 4 strains, and from the genomes of 100% of *M. africanum* group 5 strains (Table 1). In contrast, no amplification product has been observed from the genomic DNAs of all *M. bovis* isolates (groups 1 to 3).

Sequencing data and PCR-RFLP analysis of *oxyR*. Analyses of the ITS and the *pncA* gene sequences and PCR-RFLP analysis of the *oxyR* gene have been performed for five randomly chosen strains in each of spoligotype groups 1, 2, 4, and 5, and of the two strains in group 3. No variation in the ITS sequences could be found among the strains analyzed (data not shown). Comparison of the sequences to those in the International Nucleotide Sequence Database revealed that all sequences were identical to those previously published for *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* (9) (accession nos. L26327, L26328, L26329, and L26330, respectively; data not shown).

All spoligotype group 1 strains tested (which were resistant to PZA) had the C-to-G mutation at position 169 in the *pncA* gene, which had been described to be characteristic for *M. bovis*, whereas the other isolates tested (which were susceptible to PZA), including *M. tuberculosis* H37Rv, had the wild-type *pncA* sequence (Table 1).

The presence of the *M. bovis*-specific mutation at *oxyR* nucleotide 285 was analyzed by PCR-RFLP analysis. By this test, the presence of the G-to-A mutation at nucleotide 285 was indicated by a three-band pattern, whereas its absence was shown by only one visible restriction fragment. All *M. bovis* group 1, 2, and 3 isolates tested had the expected three-band pattern. In contrast, the *M. africanum* isolates of groups 4 and 5 analyzed and *M. tuberculosis* H37Rv showed the typical one-band pattern.

DISCUSSION

In this report we present clear criteria for the differentiation among clinical isolates of *M. tuberculosis*, *M. bovis*, and *M. africanum*, which are the members of the *M. tuberculosis* complex and which are the highmost important causes of TB in humans. Moreover, we distinguish three subtypes of *M. bovis*, one that is resistant to PZA and that has the normal phenotype of *M. bovis* and two that are susceptible to PZA. Among the *M. africanum* strains we describe two clearly defined subtypes which are distinguishable by molecular and biochemical characteristics.

All *M. bovis* and *M. africanum* isolates tested showed dysgonic growth and microaerophilic growth on Lebek medium and did not induce a change of color of the bromcresol medium, thus being clearly distinguishable from *M. tuberculosis*. Differentiation among these strains can be performed by a combination of tests that indicate molecular and phenotypic characteristics. In accordance with previously published data (13, 16, 27), all *M. bovis* isolates included in this study are characterized by the absence of the spacers 39 to 43 in their spoligotype patterns (in contrast to *M. africanum*), the absence of the *mtp40* sequence, and negative test results for niacin accumulation and nitrate reduction. Resistance to PZA has been described as a major criterion for the differentiation of *M. bovis* (22, 27). However, in this study a second group of *M. bovis* strains (group 2) has been found. Members of this group are susceptible to PZA and can clearly be distinguished from the other *M. bovis* strains by a lack of spacers 3 to 16 in the spoligotype pattern. These isolates as well as the PZA-susceptible *M. africanum* strains and *M. tuberculosis* H37Rv had a wild-type *pncA* sequence, confirming the correlation between the mutation and the resistance pattern. Considering the *oxyR* locus, group 2 isolates possessed the mutation at nucleotide 285, which is characteristic for *M. bovis*. In conclusion, most of the biochemical and growth characteristics as well as genetic features (spoligotype, *mtp40*, and *oxyR*) of the subtype 2 strains are identical with those of the PZA-resistant *M. bovis* isolates. The only character shared with *M. tuberculosis* and *M. africanum* is the wild-type *pncA* sequence.

M. bovis isolates with this specific spoligotype pattern have previously been described as being of the caprine genotype since they have been isolated predominantly from sheep and goats in Spain (2, 10). In our study, we can verify the observation of Guitiérez et al. (10) that strains of this genotype can also cause TB in humans, further confirming the clinical importance of the caprine genotype of *M. bovis*. Moreover, the results presented here demonstrate that strains of this subtype also can cause TB in cattle. Beyond the spoligotype patterns, the major differences between the bovine and caprine genotypes of *M. bovis* are their natural reservoirs and the differences in their susceptibilities to PZA. Since resistance to PZA is an important fact in terms of choosing appropriate therapy for patients with TB, a clear differentiation and denomination of both subtypes of *M. bovis* may be crucial for the clinician and should be performed in the routine laboratory. As a consequence, we propose the differentiation of two subtypes of *M. bovis*: *M. bovis* subtype *bovis*, which is resistant to PZA and whose natural reservoir is cattle, and *M. bovis* subtype *caprae*, which is sensitive to PZA and whose natural reservoir is sheep and goats. Routine differentiation of these two subtypes is also essential for the analysis of their importance for the epidemiology of TB in humans.

The two isolates of group 3 were also found to be susceptible to PZA. They were indistinguishable from the other *M. bovis* subtypes by their phenotypic properties but can be clearly differentiated by their characteristic spoligotype patterns. Both strains had a wild-type *pncA* gene and the *M. bovis*-specific mutation in *oxyR*. The isolates had been obtained from a human and a cow. These data indicate that the group 3 strains

may represent a third subtype of *M. bovis* sensitive to PZA, may be of clinical importance, and may have cattle as a natural reservoir.

The differentiation of *M. africanum* on the basis of results of biochemical methods is hampered by the variability of biochemical characteristics of *M. africanum* isolates (11). Two major subgroups of *M. africanum* have been described by David et al. (4) and more recently by Haas et al. (11) and the subgroups corresponded to the geographic origins of the strains (West Africa, subtype I; East Africa, subtype II). However, due to variations in their phenotypic characteristics, unambiguous identification of these subtypes is not possible. In this study we define two subtypes of *M. africanum* (spoligotype groups 4 and 5) which can clearly be differentiated by spoligotyping and growth characteristics in the presence of TCH. By considering the biochemical properties, the *M. africanum* spoligotype group 4 strains correspond to the *M. bovis*-related subtype I strains and group 5 represents the *M. tuberculosis*-related subtype II strains (4, 11). This classification is further confirmed by the geographic origin of the *M. africanum* strains analyzed; six isolates of group 4 had been isolated from patients living in West Africa (Sierra Leone, Nigeria, and Cameroon), whereas 19 isolates of group 5 had been isolated from patients living in East Africa (Uganda). Hence, *M. africanum* subtype I seems to be predominant in West Africa, and subtype II seems to be predominant in East Africa. In this study we could clearly demonstrate that a differentiation between *M. africanum* subtypes I and II, as well as between both *M. africanum* subtypes and *M. bovis* subsp. *caprae* isolates (which in part have identical biochemical characteristics), can be achieved by means of spoligotyping. This might have implications for further investigations of the epidemiology and clinical importance of *M. africanum* subtypes I and II in different parts of Africa.

The DNA sequences of the ITS have been found to be identical among all spoligotype groups described in this study and to the ITS sequences of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* already stored in the GenBank database, further confirming the sequence conservation among the members of the *M. tuberculosis* complex and the close relationship among the members of the *M. tuberculosis* complex (9, 15, 25). This situation is contrary to that for other slowly growing mycobacterial species, the ITSs of which have been described to show high degrees of sequence variation and to be a valuable tool for the differentiation of closely related species (19, 20).

In conclusion, the spoligotyping method represents a unique molecular method for differentiation among isolates of *M. bovis* and *M. africanum*, as well as of *M. microti* and *M. canetti*, as documented elsewhere (25, 26). Since spoligotyping is PCR based, it does not need a well-grown bacterial culture, and thus differentiation results can rapidly be achieved.

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