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Differentiation of Clinical *Mycobacterium tuberculosis* Complex Isolates by *gyrB* DNA Sequence Polymorphism Analysis

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The discriminatory power of *gyrB* DNA sequence polymorphisms for differentiation of the species of the *Mycobacterium tuberculosis* complex (MTBC) was evaluated by sequencing and restriction fragment length polymorphism (RFLP) analysis of a 1,020-bp fragment amplified from clinical isolates of *M. tuberculosis*, *Mycobacterium bovis* (pyrazinamide [PZA] resistant as well as PZA susceptible), *Mycobacterium africanum* subtypes I and II, and *Mycobacterium microti* types vole and llama. We found sequence polymorphisms in four regions described previously and at one additional position. These differences in the *gyrB* sequences allow an accurate discrimination of *M. bovis*, *M. microti*, and *M. africanum* subtype I. The PZA-susceptible subtypes of *M. bovis* shared the *M. bovis*-specific substitution at position 756 with the PZA-resistant strains, but can be unambiguously differentiated by a characteristic substitution at position 1311. As a drawback, *M. tuberculosis* and *M. africanum* subtype II showed an identical *gyrB* sequence that facilitates discrimination from the other species, but not from each other. A PCR-RFLP technique applying three restriction enzymes could be shown to be a rapid and easy-to-perform tool for the differentiation of the members of the MTBC. Based on these results, we present a clear diagnostic algorithm for the differentiation of species of the MTBC.

The closely related species *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti* that form the *M. tuberculosis* complex (MTBC) are the causative agents of tuberculosis (TB) in humans and animals (20). Their close relationship has been demonstrated by DNA-DNA hybridization, by multilocus enzyme electrophoresis, and sequencing of the 16S ribosomal DNA (rDNA) gene and the 16S-to-23S rDNA internal transcribed spacer (ITS) (4, 5, 7, 11, 14, 19). Despite this close genetic relatedness, the members of the MTBC differ in their host range and pathogenicity (20). The natural habitat of *M. tuberculosis* and *M. africanum* is humans. According to the latest figures of the World Health Organization (22), *M. tuberculosis* infects more than one-third of the world's population. *M. africanum* has been described as representing up to 60% of the isolates obtained from patients with pulmonary TB in certain regions in Africa (6; V. Sticht-Groh, G. Bretzel, S. Rüsch-Gerdes, S. Bwire, and H. J. S. Kawuma, 28th World Conf. IUATLD/UICTMR, abstr. A169, 1994). Based on their biochemical characteristics, two major subgroups of *M. africanum* have

TABLE 1. Selected discriminatory phenotypic and genetic characteristics of the strains analyzed[a]

Organism and group (*n*)	Colony morphology (growth)	Growth in presence of TCH	Change of color of bromcresol medium	PZA sensitivity	Presence of *oxyR* mutation (G to A)	Spoligotype (characteristic features)
M. tuberculosis (*n*)	Eugonic	+	+	S	−	At least 1 of spacers 39–43 present
M. bovis						
Subsp. *bovis* (5)	Dysgonic	−	−	R	+	Spacers 39–43 not present
Subsp. *caprae* (5)	Dysgonic	−	−	S	+	Spacers 39–43 and 3–16 not present
Subsp. C (2)	Dysgonic	−	−	S	+	Spacers 18–43 and 3–16 not present
M. africanum						
Subtype I (5)	Dysgonic	−	−	S	−	At least 1 of spacers 33–36 and spacers 39–43 present
Subtype II (5)	Dysgonic	+	−	S	−	Spacers 33–36 not present and at least 1 of spacers 39–43 present
M. microti[b]						
Subtype vole (1)	ND	ND	ND	ND	−	Only spacers 37 and 38 present
Subtype llama (2)	ND	ND	ND	S	−	Spacers 37, 38, 26, 24, 23, and 5–7 present

[a] S, susceptible; R, resistant; +, positive test result; −, negative test result; ND, not determined.
[b] Biochemical tests of *M. microti* isolates could not be performed because of the limited growth on solid media.

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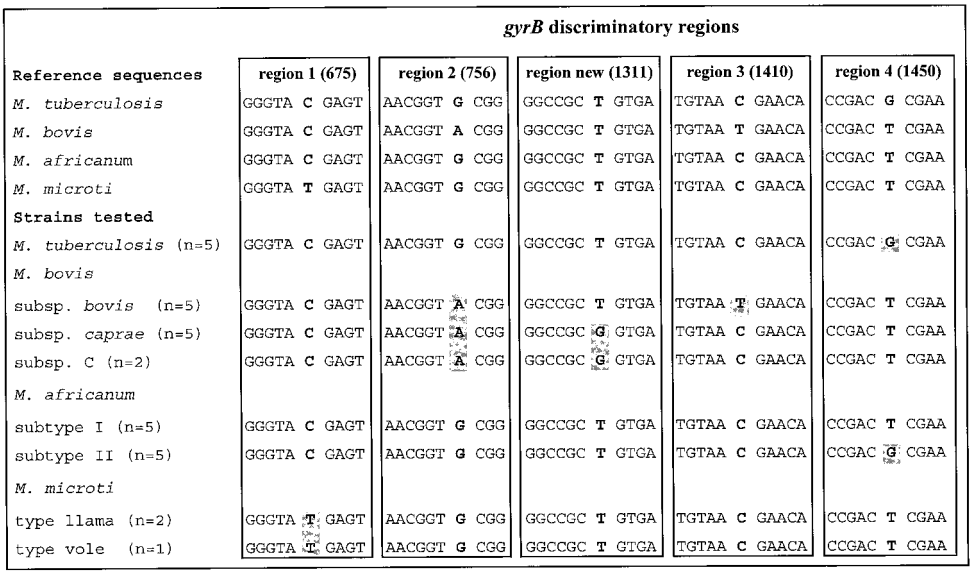

FIG. 1. DNA sequences of the four discriminatory regions in the *gyrB* gene described by Kasai et al. (9) and of one new region found in this study. Discriminatory base substitutions are shaded.

been described that correspond to their geographic origin in West or East Africa (subtypes I and II) (3, 14). *M. bovis* can cause disease in a wide range of domestic or wild animals, such as cattle or goats, as well as in humans (20). Resistance to pyrazinamide (PZA) is a major criterion for the differentiation of *M. bovis*, but some studies report susceptibility to PZA among *M. bovis* isolates (2, 21), and recently two PZA-susceptible subtypes of *M. bovis* have been described (14). *M. microti* has been reported to infect both small rodents like voles and, more recently, humans (19, 20).

All species of the MTBC are characterized by identical 16S rRNA gene and ITS sequences as well as by a number of specific repetitive elements, like the insertion sequence IS*6110* or the direct repeat (DR) locus, that allow a rapid identification of the MTBC by gene probes or PCR methods (12, 16). In contrast, routine differentiation is still based on a number of phenotypic characteristics and biochemical tests, such as nitrate reduction or niacin accumulation (20). These tests need sufficient bacterial growth, are time-consuming, do not allow an unambiguous species identification in every case, and may not be performed by every laboratory routinely. Hence, further methods allowing accurate and rapid species identification are urgently needed for clinical and epidemiological purposes. To solve this problem, over the last few years, several DNA-based techniques have been evaluated, and spoligotyping and other molecular methods have been demonstrated to be useful tools for rapid species differentiation (8, 12, 14, 15, 17–19). However, none of these molecular markers could be used solely, and no molecular technique facilitates the differentiation of all four species (e.g., differentiation of *M. tuberculosis* and *M. africanum* remained dependent on biochemical tests) (14). Recently, Kasai and coworkers (9) reported DNA sequence variations in the *gyrB* gene that may be useful for species differentiation of slowly growing mycobacteria and even for the differentiation of members of the MTBC.

The aim of this study was to evaluate the discriminatory power of *gyrB* sequence polymorphisms for differentiation of clinical MTBC isolates. The *gyrB* sequences of a 1,020-bp region comprising the four species-specific positions described by Kasai et al. (9) from clinical isolates of *M. bovis* (PZA resistant as well as PZA susceptible) and *M. africanum* subtypes I and II have been analyzed. Moreover, clinical isolates of *M. tuberculosis* and of *M. microti* types vole and llama have been included.

MATERIALS AND METHODS

Strains analyzed. A total of 30 MTBC strains comprising 5 *M. tuberculosis* strains (isolated in 1999 from patients living in the area of Hamburg, Germany), 12 *M. bovis* strains (isolated in 1998 and 1999 from patients living in different parts of Germany [one strain obtained from cattle]), 10 *M. africanum* strains (isolated in 1998 from patients living in Germany, Uganda, Cameroon, and Nigeria), and 3 *M. microti* strains (isolated in 1999 from patients living in different parts of Germany) were analyzed in this study (Table 1). The *M. bovis* and *M. africanum* strains are a subset of a collection described previously (14). The main biochemical and genetic characteristics are summarized in Table 1. Furthermore, reference strains *M. tuberculosis* H37Rv and *M. bovis* BCG, as well as five drug-resistant *M. tuberculosis* strains (resistance patterns ranged from single resistance to isoniazid to multidrug resistance to isoniazid, rifampin, ethambutol, and pyrazinamide) isolated in 1998 from patients living in different parts of Germany and five susceptible strains isolated in 1998 and 1999 from patients living in Belgrade, Yugoslavia, were analyzed by *gyrB* PCR-restriction fragment length polymorphism (RFLP).

Primary isolation and culturing of mycobacterial isolates were performed as described elsewhere (10). All isolates were identified as MTBC by using ACCUProbe gene probes (GenProbe, San Diego, Calif.).

Biochemical tests and susceptibility testing. Biochemical analyses for differentiation included colony morphology, nitrate reduction on modified Dubos broth, the niacin accumulation test (INH-test strips; Difco, Detroit, Mich.), and growth in the presence of thiophen-2-carboxylic acid hydrazide (TCH; 1 μg/ml). Growth characteristics on Lebek medium and on bromcresol purple medium were determined as described previously (14). Drug susceptibility was determined by the proportion method on Löwenstein-Jensen medium according to the Deutsches Institut für Normung (DIN) guidelines and/or the modified proportion method in the BACTEC 460TB system according to the manufacturer's instructions.

PCR amplification conditions. The primers MTUB-f and MTUB-r (9) were used for amplification of a 1,020-bp fragment of the *gyrB* gene. Three microliters of a sonicated and heat-inactivated bacterial suspension (13) was used for PCR. The 50-μl reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate (dNTP) (Boehringer, Mannheim, Germany), 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany). PCR amplifications were performed in a PTC-100 thermocycler (MJ Research/Biozym, Hessisch Oldendorf, Germany) by the protocol described by Kasai et al. (9), except at an annealing temperature of 65°C.

DNA sequencing analysis. Direct sequencing of the *gyrB* PCR fragments was performed by cycle sequencing with the BigDye RR Terminator Cycle Sequencing kit (Perkin-Elmer, Foster City, Calif.) and the ABI Prism 377 DNA sequencer (Perkin-Elmer) as instructed by the manufacturer. The PCR primers were used as sequencing primers too. 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 NR data bank by using the BLASTN algorithm (1).

PCR-RFLP analysis of the *oxyR* DNA polymorphism at position 285 and of the *gyrB* DNA polymorphisms. The PCR-RFLP analysis of *oxyR* was performed according to Sreevatsan et al. (17). DNA polymorphisms in the 1,020-bp *gyrB* fragment amplified with the primer pair MTUB-f and MTUB-r were analyzed by restriction with *Rsa*I, *Sac*II, and *Taq*I in a volume of 10 μl, respectively, as instructed by the manufacturer (New England BioLabs, Schwalbach, Germany). The total reaction mixture was analyzed by 2% agarose gel electrophoresis in Tris-acetate buffer.

RESULTS AND DISCUSSION

Variations in the *gyrB* DNA sequence in a 1,020-bp region have been analyzed with a collection of MTBC isolates that had also been analyzed for their biochemical and other genetic characteristics in this study and (partially) in our previously published work (14). The most discriminatory characteristics are listed in Table 1. The strain collection comprises clinical isolates of *M. tuberculosis*, the PZA-resistant and -susceptible subspecies of *M. bovis*, *M. africanum* subtypes I and II, and *M. microti* types vole and llama and thus should be well suited for the evaluation of the discriminatory power of *gyrB* sequence polymorphisms for differentiation of the MTBC. The strains were assigned to the respective species according to their biochemical and genetic characteristics as listed in Table 1.

The primer pair MTUB-f and MTUB-r was used for amplification of a 1,020-bp fragment of the *gyrB* gene that comprises the four discriminative regions at positions 675, 756, 1,410, and 1,450. A PCR fragment of the correct size was obtained from all MTBC strains analyzed, but not from 78 validly described non-MTBC species (according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen "Bacterial nomenclature up-to-date" [http://www.dsmz.de/bactnom/bactname.htm]) (data not shown). Hence, our data confirm that the primer pair MTUB-f and MTUB-r allows the MTBC-specific amplification of a part of the *gyrB* and may be also used for identification of MTBC isolates.

The DNA sequences of all fragments were determined and compared with each other and with the sequences stored in the International Nucleotide Sequence Database. Sequence polymorphisms were found at the four positions described previously (9) and additionally at position 1311 of the *gyrB* sequence (Fig. 1). In accordance with Kasai and coworkers (9), our data confirm that all *M. bovis* isolates could be identified by a G-to-A substitution at position 756 and that *M. microti* isolates of the vole and llama types can be differentiated from the other MTBC species by a single base substitution at position 675 (T instead of C). However, considering the other variable sites, the situation found here was more complex as described by Kasai et al. (9). At position 1410, only the PZA-resistant strains of *M. bovis* showed the C-to-T substitution proposed for differentiation of *M. bovis*. In contrast, the *gyrB* sequences of the PZA-susceptible *M. bovis* strains were C, as is found in all other isolates. However, the PZA-susceptible *M. bovis* isolates showed a characteristic and previously unknown T-to-G mutation at position 1311 representing a unique identification sequence for these subspecies. The data presented demonstrate that both PZA-resistant and PZA-susceptible subtypes of *M. bovis* can be differentiated from the other species by single base substitutions at position 756 of the *gyrB* gene and from each other by specific substitutions at position 1410 (PZA resistant) and position 1311 (PZA susceptible), respectively. Considering *M. africanum* and *M. tuberculosis*, only *M. africanum* subtype I isolates can be differentiated by a unique *gyrB* sequence. In contrast, *M. tuberculosis* and *M. africanum* subtype II isolates possessed an identical *gyrB* sequence that allows a differentiation of these two species from the other members of the MTBC by the T-to-G substitution at position 1450, but not from each other. These data confirm the close relationship between *M. tuberculosis* and *M. africanum* type II that is also expressed by their biochemical characteristics as described elsewhere (3, 6, 14). However, with the *gyrB* polymorphisms described here, we present the first molecular marker for differentiation of *M. africanum* type I from the other members of the MTBC.

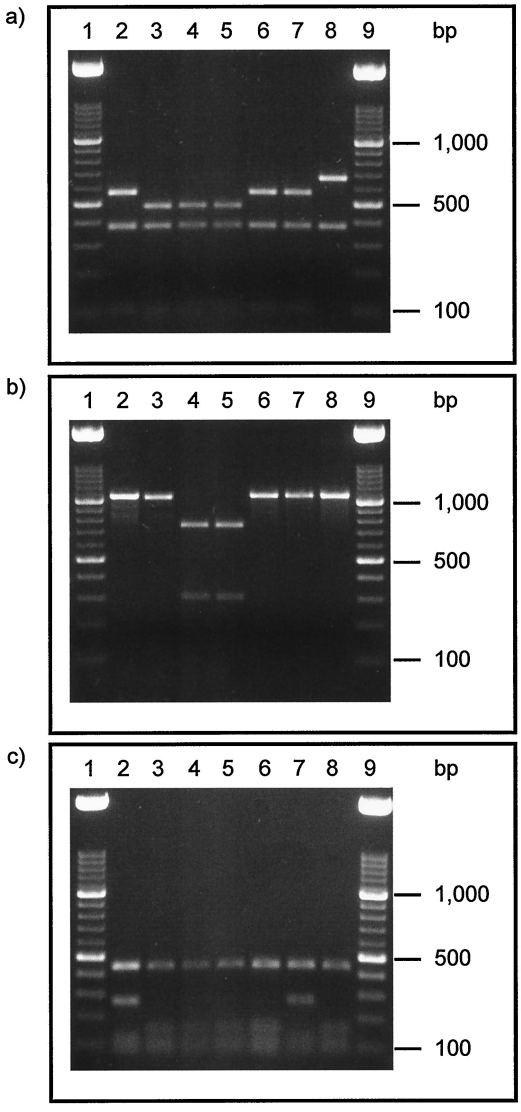

FIG. 2. RFLP patterns of PCR products obtained by *Rsa*I digestion (a), *Sac*II digestion (b), and *Taq*I (c) digestion of the 1,020-bp *gyrB* PCR fragment. Lanes: 1 and 9, 100-bp ladder; 2, *M. tuberculosis*; 3, *M. bovis* resistant to PZA; 4 and 5, *M. bovis* susceptible to PZA; 6, *M. africanum* subtype I; 7, *M. africanum* subtype II; 8, *M. microti*.

We also have evaluated a PCR-RFLP assay for rapid detection of the DNA sequence polymorphisms in the *gyrB* gene that can be used for differentiation of the MTBC species. As described by Kasai et al. (9), the restriction enzymes *Rsa*I (targeting the polymorphisms at positions 675 and 756) and *Taq*I (targeting the polymorphism at position 1450) were used for digestion of the 1,020-bp PCR fragment. In addition, we used *Sac*II to detect the substitution at position 1311 that is characteristic of PZA-susceptible *M. bovis* isolates. As shown in Fig. 2a, PZA-resistant and -susceptible isolates of *M. bovis* and *M. microti* could be identified by their specific *Rsa*I RFLP

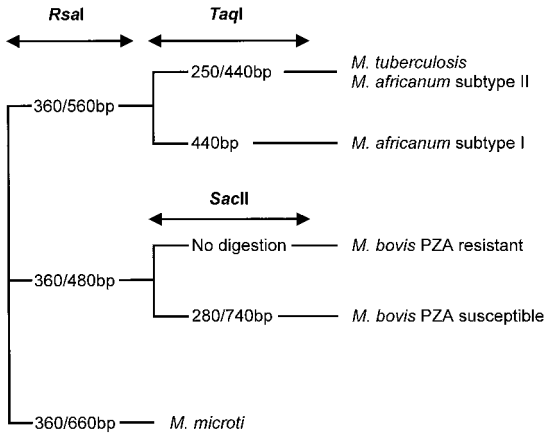

FIG. 3. Diagnostic algorithm of RFLP patterns for the differentiation of members of the MTBC.

patterns (360 and 480 bp for *M. bovis*, 360 and 660 bp for *M. microti*). The PZA-susceptible isolates of *M. bovis* could unambiguously be identified by showing two *Sac*II restriction fragments, whereas the *gyrB* fragment of all other species remained uncleaved (Fig. 2b). *M. africanum* type I can be differentiated from *M. tuberculosis* and *M. africanum* type II by the characteristic *Taq*I RFLP pattern (Fig. 2c). Based on the clearly visible restriction fragments, a diagnostic algorithm was developed that allows an easy differentiation of the MTBC species (Fig. 3). To further confirm the differentiation system presented, reference strains *M. tuberculosis* H37Rv and *M. bovis* BCG as well as five drug-resistant *M. tuberculosis* strains from patients living in Germany and five susceptible strains isolated from patients living in Belgrade, Yugoslavia, were analyzed by the *gyrB* PCR-RFLP technique. All *M. tuberculosis* strains showed the typical *M. tuberculosis*-specific *Rsa*I-*Taq*I RFLP patterns, just as *M. bovis* BCG showed *Rsa*I-*Sac*II RFLP patterns typical for PZA-resistant *M. bovis* isolates (data not shown).

Thus, the *gyrB* PCR-RFLP using the combination of restriction enzymes presented in this study is a rapid and easy-to-use technique to discriminate between *M. tuberculosis*/*M. africanum* type II, *M. africanum* type I, *M. microti*, *M. bovis*, and the *M. bovis* subtypes. In contrast to the spoligotyping method and DNA sequencing of the *gyrB* gene, just PCR and easy-to-perform agarose gel electrophoresis are necessary for a highly discriminatory differentiation of the MTBC, making this technique feasible in a wide variety of laboratories.

In conclusion, the DNA sequence polymorphism in the *gyrB* gene represents a unique marker that facilitates the differentiation of the MTBC by DNA sequencing or a simple PCR-RFLP analysis. This technique complements the collection of molecular differentiation techniques and may be used in addition to other methods or alone, replacing the more time-consuming biochemical test. However, the differentiation of *M. tuberculosis* and *M. africanum* type II so far cannot be achieved by analysis of molecular markers and remains based on phenotypic characteristics, such as growth characteristics on bromcresol purple medium (14).

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