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Rapid Differentiation of Bovine and Human Tubercle Bacilli Based on a Characteristic Mutation in the Bovine Pyrazinamidase Gene

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Bovine tuberculosis (TB) caused by *Mycobacterium bovis* is an important veterinary disease that can also afflict humans. Although *M. bovis* shares an almost identical genome with *M. tuberculosis*, subtle differences in host specificity and several biochemical parameters can be used to distinguish the two closely related species. The current method for distinguishing *M. bovis* from *M. tuberculosis* relies on tedious testing of biochemical parameters, including natural resistance to pyrazinamide and defective pyrazinamidase (PZase) activity of *M. bovis* strains. In this study, we report the development of a rapid PCR–single-strand conformation polymorphism (SSCP) assay to differentiate *M. bovis* from *M. tuberculosis* strains, based on the detection of a single characteristic point mutation in the PZase gene (*pncA*) of *M. bovis*. Eighty-seven of 89 *M. bovis* strains could be distinguished from *M. tuberculosis* strains. Surprisingly, two animal isolates which were initially identified as *M. bovis* were shown to be *M. africanum* because they had a wild-type *pncA* sequence with positive PZase. These two *M. africanum* strains contain multiple (three and six) copies of insertion sequence IS*6110*, a feature they have in common with *M. tuberculosis*. The implication of this finding for the taxonomy of *M. tuberculosis* complex is discussed in relation to host preference and epidemiology. The development of a rapid PCR-SSCP test for distinguishing *M. bovis* from *M. tuberculosis* will be useful for monitoring the spread of bovine TB to humans in areas where bovine TB is endemic and for directing the treatment of human TB caused by *M. bovis*.

Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, is a significant veterinary disease that can spread to humans. *M. bovis* infection is responsible for about 7,000 new cases of human TB per year in Latin America (13). Disease caused by *M. bovis* in human immunodeficiency virus-positive individuals is also an increasing concern (6). Although the incidence of human *M. bovis* disease has been greatly reduced in developed countries since the introduction of milk pasteurization, it remains an important veterinary disease as well as a public health problem in certain parts of the world (5, 6, 24). Eradication of bovine TB or of human TB due to *M. bovis* infection has been hampered by the fact that *M. bovis* resides in many animal hosts, including opossums, badgers, deer, hares, and bison, that can spread it to cattle and humans. *M. bovis* and *M. tuberculosis* are closely related organisms and have almost identical genomes (2). Despite the high degree of DNA homology between the two organisms, *M. tuberculosis* causes disease almost exclusively in humans and rarely in other animals, whereas *M. bovis* can cause TB in a wide range of animal hosts, including humans (3, 24). In areas of endemicity where bovine and human TB coexist, the distinction of *M. bovis* from *M. tuberculosis* is important for monitoring the spread of *M. bovis* to humans. In addition, such a distinction has an impact on the treatment of the disease, since *M. bovis* strains are naturally resistant to pyrazinamide (PZA) (11); thus, human TB caused by *M. bovis* cannot be treated with PZA.

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Currently, strains of *M. bovis* and *M. tuberculosis* are distinguished by several biochemical parameters, including niacin accumulation, PZA susceptibility, pyrazinamidase (PZase) activity, nitrate reduction, and thiophene carboxylic acid hydrazide (TCH) susceptibility (3). These biochemical methods are tedious and time-consuming, requiring several weeks to complete. An immunological method of differentiating between *M. bovis* and *M. tuberculosis* based on detection of the protein antigen MPB70 in *M. bovis* has been reported (9), but its use and reliability are limited because this protein is also present in *M. tuberculosis* (9). The Gen-Probe system (San Diego, Calif.), based on the species-specific 16S rRNA gene, only defines the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*) to a species level but does not further differentiate among members of the *M. tuberculosis* complex. Genetic tests that use the copy number of insertion sequence IS*6110* (8) to distinguish between the two types of bacilli have also been attempted (20, 22). This approach relies on the observation that most strains of *M. bovis* have 1 to 6 copies of IS*6110*, whereas most *M. tuberculosis* strains have 10 to 16 copies (20–22). However, some *M. tuberculosis* strains also have only a few copies of IS*6110* (21) and overlap with *M. bovis*, and certain *M. bovis* strains have multiple copies of IS*6110* (22); thus, this method is unreliable. Recently, Rodriguez and colleagues have identified a 500-bp DNA fragment that appears to be present in *M. bovis* but not in *M. tuberculosis*; however, only a few *M. tuberculosis* and *M. bovis* strains were analyzed in that study, and further testing is needed to confirm the results (14).

M. bovis strains, in contrast to *M. tuberculosis* strains, are well known to be naturally resistant to PZA and lack PZase activity (11); this feature is commonly used to distinguish *M.*

TABLE 1. Mycobacterial strains and characteristics

Mycobacterium species	No. of strains	*pncA* mutation (C→G)	Source or description
M. bovis type strain	1	+	ATCC 19210
M. bovis BCG substrains[a]	3	+	A. Dannenberg and P. Converse
M. bovis strains	36	+	U.S. animal isolates
M. bovis strains	25	+	New Zealand animal isolates
M. africanum strains	2	−	New Zealand animal isolates
M. bovis strains	22	+	Isolates from humans
M. tuberculosis H37Rv	1	−	ATCC 27294
M. tuberculosis strains	14	−	Clinical isolates
M. africanum	1	−	ATCC 25420

[a] Tice, Tokyo, and Phipps.

bovis from *M. tuberculosis* strains (3). To investigate the possibility of developing a genetic test for rapid differentiation of *M. bovis* from *M. tuberculosis* strains, we have cloned the PZase gene (*pncA*) from *M. tuberculosis* and *M. bovis* and have found a single point mutation in the *pncA* gene that appears to be unique to *M. bovis* (17). This point mutation, resulting in a change of C to G at nucleotide position 169 of the *pncA* gene, causes the replacement of histidine (CAC) with aspartic acid (GAC) at amino acid position 57 in *M. bovis* PZase (17). This substitution has been confirmed to be the cause of defective PZase activity and natural PZA resistance in *M. bovis* strains, including BCG substrains, by transformation studies with the functional *M. tuberculosis pncA* gene (17). Preliminary sequence analysis of the *pncA* gene from several *M. bovis* strains, including BCG substrains (Pasteur, Copenhagen, and Glaxo) shows that they all have the same single C-to-G characteristic mutation (17). No other mutations were found in the *pncA* gene of these *M. bovis* strains, indicating that the "defective" *pncA* gene in *M. bovis* is stable and should be a useful feature for design of a genetic test for differentiation of *M. bovis* from *M. tuberculosis.*

To determine the feasibility of developing a rapid method for differentiating *M. bovis* from *M. tuberculosis* strains based upon this characteristic single point mutation in the *M. bovis pncA* gene, we have analyzed *M. bovis* isolates from various animal hosts, including humans, from different geographic regions and compared the results with data obtained from *M. tuberculosis*. We demonstrate that a PCR–single-strand conformation polymorphism (SSCP) assay based upon detecting the characteristic point mutation in the *M. bovis pncA* gene can be used for rapid differentiation of *M. bovis* from *M. tuberculosis.*

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA. The mycobacterial strains used in this study are listed in Table 1. Mycobacterial strains were grown in 7H9 liquid medium with albumin-dextrose-catalase enrichment (Difco) at 37°C for 2 to 4 weeks. Biochemical tests used to distinguish *M. bovis* from *M. tuberculosis*, including a PZase activity assay, were performed as previously described (3, 23). Genomic DNA for PCR and Southern analysis was isolated by the glass bead method. Briefly, 5 ml of bacterial cultures were concentrated by centrifugation, and the cell pellet was washed with distilled water twice and resuspended in 150 μl of water. The bacterial cells were heat-killed by incubating at 80°C for 20 min. One-third to 1/2 volume of glass beads (0.1 mm) was added to the bacterial suspension, followed by vortexing at high speed for 5 min. Bacterial lysates were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) three times. The supernatant containing genomic DNA was precipitated with 2 volumes of absolute alcohol. The genomic DNA was collected by centrifugation, washed with 70% alcohol, and resuspended in 100 μl of distilled water. Genomic DNA from 15 *M. bovis* veterinary isolates was kindly supplied by V. P. Shankar (Texas A&M University Health Science Center).

PCR-SSCP. The PCR was performed as described (15). The PCR primers for the SSCP were chosen so that the 180-bp PCR product amplified contains the characteristic *M. bovis* mutation (C to G) at nucleotide position 169 of the *M. tuberculosis pncA* gene (17). The forward primer (5′ATCAGCGACTACCTGG CCGA3′) was taken from bp 91 to 110 of the *pncA* gene, and the reverse primer (5′GATTGCCGACGTGTCCAGAC3′) was from bp 270 to 251 of the *pncA* gene (17). The PCR mixture contained the following reagents: 1× PCR buffer, deoxynucleoside triphosphates at 100 μmol, 0.5 μg of primers, 0.1 to 0.5 μg of mycobacterial genomic DNA, and 2.5 U of *Taq* DNA polymerase. The cycling parameters were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR-SSCP was carried out as described previously (12, 18). Briefly, PCR products (10 μl, containing about 0.5 to 1 μg of DNA) were denatured by boiling for 5 to 10 min in formamide dye (95% formamide, 10 mM sodium hydroxide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF), followed by cooling on ice for 5 to 10 min. The denatured PCR products were then loaded on a 20% polyacrylamide gel (16 by 20 cm) (containing 5% glycerol) that had been precooled at 4°C. The gel was run in 0.5× Tris-borate-EDTA buffer at a constant power of 5 W in a cold room overnight. The gel was then stained with ethidium bromide (0.5 μg/ml), and the SSCP bands were visualized under UV light.

DNA sequencing. The *M. tuberculosis pncA* gene is 558 bp (17). To determine the sequence of the *pncA* gene from various strains of *M. bovis* or *M. tuberculosis*, we amplified the *pncA* gene by PCR with the following forward and reverse primers. The forward primer (5′GCTGGTCATGTTCGCGATCG3′) was taken from 105 bp upstream of the start codon of the *M. tuberculosis pncA* gene, and the reverse primer (5′GCTTTGCGGCGAGCGCTCCA3′) was from 60 bp downstream of the stop codon of the *pncA* gene. *pncA* sequences from different mycobacterial strains were determined by PCR direct sequencing with the above forward and reverse primers in an automatic DNA sequencer (Johns Hopkins Genetic Core Facility).

Southern blot analysis. Genomic DNA from various *M. bovis* and *M. tuberculosis* strains was digested with *Pvu*II, run on a 0.8% Tris-borate-EDTA agarose gel, and blotted onto a nylon membrane as previously described (16, 25). The blot was probed with the ^{32}P-labelled 445-bp PCR fragment amplified from the IS*6110* of *M. tuberculosis* H37Rv by using the following primers. The forward primer was taken from bp 252 to 274, and the reverse primer was taken from bp 674 to 696, of the IS*6110* sequence as described previously (4).

RESULTS

Rapid differentiation of *M. bovis* from *M. tuberculosis* by PCR-SSCP. Sequencing of the PZase gene (*pncA* gene) from *M. tuberculosis* and *M. bovis* has identified a C-to-G mutation in the *pncA* gene in three *M. bovis* strains and three BCG substrains (17). In order to determine whether *M. bovis* strains isolated from various animal species from different geographic regions would also have the same characteristic mutation and could thus be rapidly distinguished from *M. tuberculosis*, we analyzed 89 *M. bovis* strains, including 3 other BCG substrains, by PCR-SSCP with primers that cover the characteristic point mutation in the *M. bovis pncA* gene. As controls, we included 15 *M. tuberculosis* strains for this study. The results of this PCR-SSCP analysis are presented in part in Fig. 1. Eighty-seven of 89 *M. bovis* strains, including the 3 BCG substrains (Tokyo, Tice, and Phipps), showed a PCR-SSCP banding pattern common to *M. bovis* strains but distinct from *M. tuberculosis* strains (Fig. 1), indicating that all these *M. bovis* strains have the same characteristic C-to-G mutation. Interestingly, however, two *M. bovis* strains showed the same wild-type SSCP pattern as did *M. tuberculosis* strains (Fig. 1). These two *M. bovis* strains, strains 20 and 25 (Fig. 1, lanes 35 and 40), were animal isolates from New Zealand. All 15 *M. tuberculosis* control strains had a PCR-SSCP banding pattern different from that of the *M. bovis* strains (partially presented in Fig. 1, lanes 6, 7, 16 through 19, 32 through 34, and 43 through 45).

Sequence analysis of the *pncA* gene in *M. bovis* and *M. tuberculosis.* To confirm that *M. bovis* strains with the same PCR-SSCP banding pattern have the same point mutation, we determined the *pncA* sequence for six *M. bovis* strains. All showed the same characteristic C-to-G mutation in the *pncA* gene (Fig. 2). However, the two *M. bovis* strains that showed

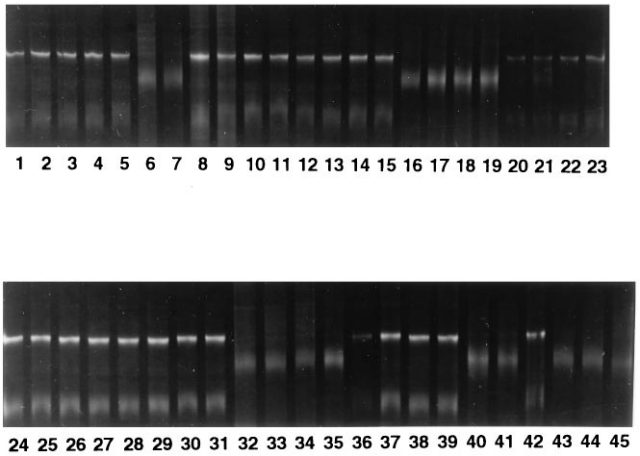

FIG. 1. Rapid differentiation of *M. bovis* from *M. tuberculosis* strains by PCR-SSCP. Various *M. bovis* and *M. tuberculosis* strains were subjected to PCR-SSCP analysis with primers that span the characteristic C-to-G mutation at nucleotide position 169 of the *pncA* gene. Lane 1, BCG Tokyo; lane 2, BCG Pasteur; lanes 3 to 5, 8 to 15, 24 to 31, 36 to 39, and 42, New Zealand *M. bovis* strains; lane 6, *M. tuberculosis* H37Rv; lane 7, *M. tuberculosis* Erdman; lanes 16 to 19, 32 to 34, 41, and 43 to 45, *M. tuberculosis* clinical isolates; lanes 20 to 23, U.S. *M. bovis* strains; lanes 35 and 40, New Zealand *M. bovis* 20 and 25, respectively.

the typical *M. tuberculosis* SSCP banding pattern both had the wild-type *M. tuberculosis pncA* sequence. Sequence analysis of 15 *M. tuberculosis* strains showed that none of them had the C-to-G mutation in the *pncA* gene. *M. africanum*, a member of the *M. tuberculosis* complex, was found to have a wild-type *pncA* sequence like that in *M. tuberculosis* (Table 1).

Strain typing and biochemical analyses of the *M. bovis* strains. In order to gain insight into the identity of the 2 *M. bovis* strains that do not have the characteristic *pncA* mutation found in the other 87 *M. bovis* strains, we performed strain typing on a panel of *M. bovis* strains using IS*6110* as a probe. In contrast to most *M. bovis* strains, which contain a single copy of IS*6110* (4, 21, 22), the two strains with a wild-type *pncA* sequence, strains 20 and 25, had three and six copies of IS*6110*, respectively (Fig. 3, lanes 8 and 9). However, several other *M. bovis* strains that contain the characteristic *pncA* mutation also had multiple copies of IS*6110* (data not shown), indicating that not all *M. bovis* strains with multiple copies of IS*6110* have the wild-type *pncA* sequence.

Biochemical testing of the two New Zealand *M. bovis* strains with the wild-type *pncA* sequence showed that they are positive for PZase and susceptible to PZA—a feature they have in common with *M. tuberculosis*. However, with respect to other biochemical parameters, they behaved like typical *M. bovis*: they are negative for niacin accumulation and nitrate reduction and are susceptible to TCH. These results indicate that these 2 "*M. bovis*" strains are in fact *M. africanum*, which is usually a human pathogen but has also recently been reported to cause TB in pigs and cattle (1).

DISCUSSION

In this study we have developed a rapid test for distinguishing *M. bovis* from *M. tuberculosis* strains by PCR-SSCP based on detection of a single characteristic mutation in the *M. bovis pncA* gene. Using this SSCP method, we could rapidly differentiate 87 of 89 *M. bovis* strains from the *M. tuberculosis* strains. These 87 *M. bovis* strains, most of which have previously been shown to have different restriction fragment length

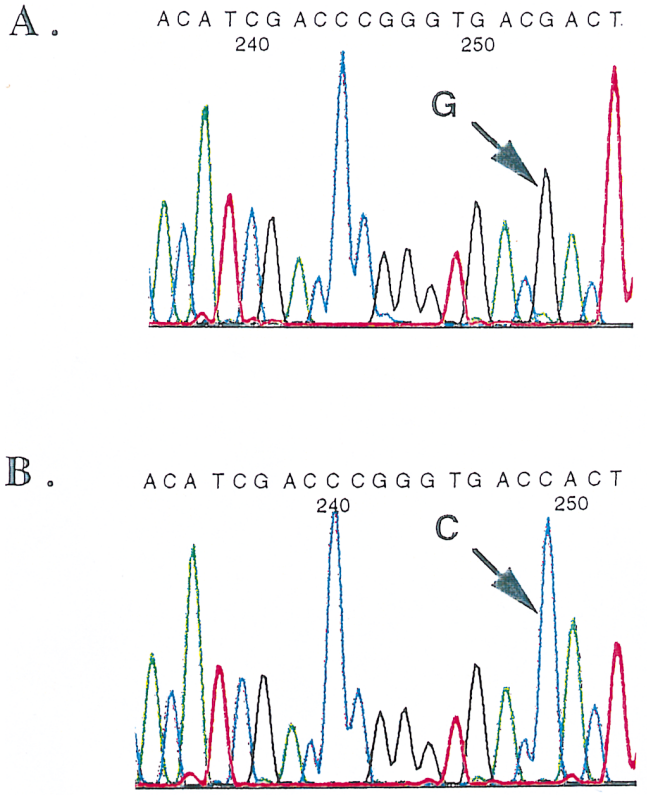

FIG. 2. Demonstration of the characteristic point mutation in the *M. bovis pncA* gene. (A) The single characteristic point mutation of C to G at nucleotide position 169 of the *pncA* gene from the *M. bovis* type strain, ATCC 19210. (B) The wild-type *pncA* sequence in the *M. tuberculosis* strain H37Rv. The position where the point mutation occurs is marked by arrows.

polymorphism (reference 4 and data not shown), all had the same SSCP pattern (Fig. 1). Sequence analysis of six *M. bovis* strains with the same SSCP pattern showed that they all had the same characteristic C-to-G point mutation at nucleotide position 169 of the *pncA* gene (Fig. 2). Fifteen *M. tuberculosis* strains, used as controls, had an SSCP pattern different from that of *M. bovis* strains (Fig. 1). Sequencing of the *pncA* gene

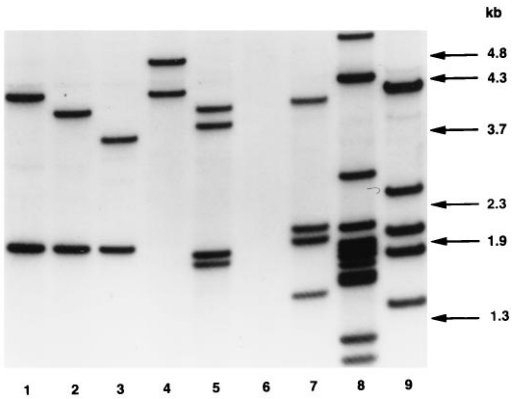

FIG. 3. Strain typing analysis of *M. bovis* strains by Southern blotting with IS*6110* used as a probe. Lanes 1 to 5, New Zealand *M. bovis* strains 14, 5, 17, 23, and 27, respectively; lane 6, gap; lane 7, *M. bovis* AN5; lanes 8 and 9, New Zealand *M. bovis* strains 25 and 20, respectively. Because the 445-bp PCR DNA probe contains a single *Pvu*II site used to cut the genomic DNA, each copy of IS*6110* is shown as two bands in the figure.

from the 15 *M. tuberculosis* strains revealed that they all had the wild-type *pncA* sequence, as expected. The two "*M. bovis*" strains that showed the same SSCP pattern as *M. tuberculosis* had a wild-type *M. tuberculosis pncA* sequence. Strain typing analysis showed that these two "*M. bovis*" strains contained multiple copies of IS*6110* (Fig. 3). However, not all *M. bovis* strains with multiple copies of IS*6110* analyzed had a wild-type *pncA* sequence.

The two animal isolates (strains 20 and 25) with a wild-type *pncA* sequence were initially identified as *M. bovis*, because they are susceptible to TCH, negative for niacin accumulation, and negative for nitrate reductase. Strain 20 is a native New Zealand strain isolated from a cow on a coastal farm, while strain 25 was accidentally imported into both New Zealand and Sweden in deer obtained from the United Kingdom (4). However, this study has shown that these two "*M. bovis*" strains have a wild-type *pncA* gene with positive PZase activity; thus, they should be more appropriately identified as *M. africanum*, a variant between *M. tuberculosis* and *M. bovis* (4). Based on nitrate reductase activity, *M. africanum* can be divided into two types, African I (nitrate reductase negative) and African II (nitrate reductase positive) (4). Because the two *M. bovis* strains found in this study are nitrate reductase negative, they belong to *M. africanum* African type I. *M. africanum* is primarily a human pathogen and rarely causes animal disease. A recent study documenting the cause of TB in pigs and cattle by *M. africanum* was reported in Norway in 1992 (1). Our result showing that 2 of 89 animal isolates are *M. africanum* suggests that animal TB caused by *M. africanum* is rare. On the other hand, variants of *M. tuberculosis* that differ in biochemical parameters can cause human TB, and such variants have been found in different geographic regions of the world (7, 10, 20). Some variants are given a name, such as *M. africanum*, while others are simply called variants or biovars without a proper name, such as those found in Guinea-Bissau (10). While the biological meaning of such variations in biochemical parameters among variants of *M. tuberculosis* or *M. bovis* is not clear, the existence of a spectrum of variants that differ in biochemical parameters is probably due to selection by subtle differences in environmental and host niches where these organisms reside. It is tempting to speculate whether the differences in these biochemical parameters relate to the pathogenicity and host specificity of these mycobacteria.

The current definitive method for distinguishing *M. bovis* from *M. tuberculosis* still depends on testing of several biochemical parameters, which is tedious and time-consuming (several weeks). We have in the present study developed a rapid PCR-SSCP test based on detection of a characteristic stable point mutation in the *M. bovis pncA* gene to differentiate *M. bovis* from *M. tuberculosis*. The characteristic mutation in the *pncA* gene has been found in 87 of 89 animal TB isolates, although 2 "*M. bovis*" strains which had a wild-type *pncA* sequence have been found to be *M. africanum*. Because *M. africanum* very rarely causes animal disease and because *M. bovis* strains are known to have defective PZase indicative of the *pncA* mutation, the SSCP test developed in this study should be useful for rapid differentiation of *M. bovis* from *M. tuberculosis* strains based on detection of this mutation. Such a rapid test will be particularly useful in identifying human TB caused by *M. bovis*. This distinction is important because the *M. bovis* disease should not be treated with PZA, to which the bovine bacilli are naturally resistant. In areas of endemicity where bovine and human TB coexist, the rapid SSCP test developed in this study can monitor the spread of bovine TB to humans and should be a valuable epidemiological tool.

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ADDENDUM

After the manuscript was written, we learned that a study on detecting an *oxyR* mutation in *M. bovis* for differentiation of bovine and human tubercle bacilli was in press (19).

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