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Differentiation by Molecular Typing of *Mycobacterium bovis* Strains Causing Tuberculosis in Cattle and Goats

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Forty *Mycobacterium bovis* isolates from cattle and goats were analyzed by using different repetitive genetic markers. The 23 *M. bovis* strains from goats were found to carry six to eight copies of the insertion sequence IS*6110*. In contrast, most of the bovine isolates contained only a single copy of this element. The standardized IS*6110* fingerprinting by restriction fragment length polymorphism (RFLP), described for *Mycobacterium tuberculosis* strains, allowed the differentiation of caprine strains. Although this method was not useful for typing bovine isolates, the repetitive elements pTBN12 and DR proved to be suitable for this purpose. A procedure using PCR which amplifies IS*6110* in the outward direction was found to be as sensitive as RFLP for typing *M. bovis* strains from goats. The use of PCR and RFLP methods based on the IS*6110* polymorphism would be useful for epidemiological studies of caprine tuberculosis. The results are consistent with different strains of *M. bovis* being implicated in bovine and caprine tuberculosis.

Bovine tuberculosis remains an important disease in many countries of the world, causing significant economic losses and proving difficult to control. The causative agent, *Mycobacterium bovis*, is also responsible for tuberculosis in goats and other animals of agricultural importance (9, 17, 19). While eradication schemes in developed countries have helped limit the infection in cattle, tuberculosis in goats is widespread in Mediterranean countries (1, 4, 12) and often is a very severe disease (4, 6). Although mixed farming of cattle and goats is not common practice, close contact of these species does sometimes occur during grazing. In spite of this reservoir of infection, the nature of caprine strains of *M. bovis* as well as the extent of transmission between cattle and goats remains unstudied. In addition, histopathological characteristics of tuberculous tissue samples from cattle and goats were shown to have some differences which could be attributed to different host immune responses or to different *M. bovis* strains infecting cattle and goats (6).

A great effort is being made towards the development of tools for differentiating strains that belong to the *M. tuberculosis* complex, and molecular techniques have proven to be very useful. Different polymorphic repetitive DNA sequences permit the analysis and comparison of strains in epidemiological studies. The insertion sequence IS*6110* is currently being highlighted as the most useful marker for typing *M. tuberculosis* strains, as this element is usually present in multiple copies and in different locations, resulting in restriction fragment length polymorphisms (RFLP) (10). IS*6110* fingerprinting by RFLP has been standardized through the use of *Pvu*II as the restriction enzyme of choice to digest mycobacterial genomic DNA and the use of an IS*6110* sequence 3′ from the *Pvu*II site as the probe (21). Newly developed PCR typing methods, some of which are based on the IS*6110* polymorphism (7, 13, 14), aim to decrease the time necessary for identification of *M. tuberculosis* complex strains (5, 11).

Although IS*6110* is present in most of the *M. tuberculosis* complex strains, the number of copies of this element seems to differ among them. *M. bovis*, including *M. bovis* BCG, is usually found to have only one copy of IS*6110* (2), in most cases at the same location, which limits its usefulness as a genetic marker. The best results have been obtained by using probes of IS*6110* containing the sequences which flank the internal *Pvu*II site on either side (2, 18). However, strains of *M. bovis* with multiple copies of IS*6110* have been isolated from wild animals, and these strains have an associated polymorphism that allows for epidemiological studies (22). Furthermore, other genetic markers, such as the repetitive elements pTBN12 (15) and DR (8), have been used with success in epidemiological studies of *M. bovis* strains with only a single copy of IS*6110* (3, 18, 22).

In the present study, caprine and bovine strains of *M. bovis* isolated in Spain have been typed and differentiated by using IS*6110*, pTBN12, and DR. We can also demonstrate the presence of multiple copies of IS*6110* in *M. bovis* strains isolated from goats. Our results indicate that the use of PCR and RFLP methods based on the IS*6110* polymorphism will be useful for epidemiological studies of caprine tuberculosis.

MATERIALS AND METHODS

Mycobacterial isolates. Forty isolates identified as *M. bovis* by biochemical tests were examined. Seventeen were bovine strains from six farms (b-I to b-VI), and 23 were caprine strains from five different herds (g-I to g-V). Bovine isolates came from herds for which the last tuberculin test showed reactor levels higher than 50%. Caprine herds had high tuberculosis prevalence rates, and the goats were chosen by the manifestation of clinical symptoms of the disease. These herds were located in different villages from Aragon in the northeast and Valencia in the east of Spain, and some of the villages were very close together. Herds g-I, b-I, and b-II were located in the same village; herds g-IV, g-V, b-IV, and b-V were located in four different villages, the longest distance between them being 40 km; and herds g-II, b-II, and b-IV were from farms separated by less than 10 km. Samples taken for culturing included lymph nodes or lung tissue with lesions typical of tuberculosis. Bovine samples were collected in the abattoir, and caprine samples were collected during necropsy. The isolates were initially grown in Coletsos medium and subcultured in 7H9 Middlebrook broth. *M. bovis* BCG Pasteur was used as a reference strain.

DNA techniques. Following extraction of mycobacterial DNA as previously

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TABLE 1. Distribution of strains among caprine herds and RFLP patterns determined by using the standard IS*6110* probe and out-PCR[a]

Herd	Strain no.	RFLP pattern with:	
		IS*6110*	Out-PCR
g-I	1–7	A	A
g-II	8	B	B
g-III	9	C	C
g-IV	10–13	D	D
	14	E	E
g-V	15	D	D
	16–19	E	E
	20–23	F	F

[a] Out-PCR amplifies from the extremities of IS*6110* in the outward direction.

described (21), 2 to 4 μg of each sample was digested overnight with *Pvu*II for analysis with the IS*6110* probe or with *Alu*I for pTBN12 and DR analysis. Restriction enzymes were purchased from Boehringer or Promega and were used according to the manufacturer's instructions. Digests were electrophoresed at 37 V for 14 h in 20-cm gels of 0.8% agarose for IS*6110*, 0.7% agarose for pTBN12, and 1.2% agarose for DR. Southern blotting and hybridization studies were performed as previously described (16). Four different probes were used, and two of them were based on the insertion sequence IS*6110*; one of the two was an 867-bp PCR-amplified fragment 3′ from the *Pvu*II site and was used for the study of the caprine strains, and the other probe was the entire 1,358-bp IS*6110*, which was obtained by PCR with a single primer, IS*6110* IR, to the terminal inverted-repeat sequence (20). The plasmid pTBN12 was kindly provided by Bruce Ross, Fairfield Hospital, Fairfield, Victoria, Australia. The direct-repeat probe consisted of a 36-mer oligonucleotide (8). Probes were labelled by the ECL system (Amersham International).

PCR typing method (out-PCR). The typing method used is based on the method of Ross and Dwyer (14), which consists of the amplification of DNA from the extremities of IS*6110* in the outward direction. The main modification of the original protocol was the use of a single primer based on the invert-repeat fragments located at the ends of IS*6110* whose sequence is GACIIICCGGG GCGGTTCA, where I is inosine. The 3′ end of the primer is directed outwardly from both sides of the inverted repeats present in IS*6110*, amplifying the flanking sequences between two copies of IS*6110*. The PCR products were analyzed by electrophoresis through a 1.4% agarose gel and stained with ethidium bromide. PCR was carried out with approximately 10 ng of genomic DNA in a final volume of 100 μl by using buffer supplied by Perkin-Elmer, 1 μM primers, 3 μM $MgCl_2$, 1 U of *Taq* polymerase, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, and 200 μM dTTP. The reactions were performed in an automated thermal cycler (Perkin-Elmer). DNA samples were denatured by incubation for 3 min at 94°C before amplification for 40 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. Positive and negative controls were used for each reaction. The positive control was the reference strain *M. tuberculosis* 14323, and the negative control consisted of a sample of the PCR mix without the addition of DNA. Each sample was tested at least three times in order to verify the reproducibility of the results.

RESULTS

RFLP analysis of caprine strains. The intradermal tuberculin test was performed for more than 60 goats from five different herds. Positive reactions were observed with 42 goats, and cultures were available from 23 of these animals. With the 867-bp IS*6110* probe, six different types could be observed among the 23 *M. bovis* isolates (Table 1 and Fig. 1a). All the isolates from herd g-I showed the same pattern (pattern A). The single strain from herd g-II showed pattern B. Strain 9 from herd g-III showed pattern C. The isolates from herd g-IV were analyzed, and patterns D and E were observed in four and one isolates, respectively. Nine isolates were analyzed from herd g-V and patterns D, E, and F were observed. Pattern D was obtained with one isolate, pattern E was obtained with four isolates, and a new pattern, F, was observed with four isolates (Table 1). Patterns D, E, and F, shared by herds g-IV and g-V, were very similar. The patterns had six bands in common, and each pattern was characterized by the presence of one or two bands in a different location (Fig. 1a).

All the different patterns obtained from goats had a high degree of similarity; three bands with molecular sizes of 1.35, 1.80, and 2.11 kb, respectively, were common to all the patterns; and a fourth band of 1.45 kb was common to all patterns, with the exception of pattern C (Fig. 1a).

When the caprine strains were analyzed by the out-PCR technique, the isolates from herd g-I showed pattern A whereas the strains from herds g-II and g-III each showed a different pattern, patterns B and C. Strains from herds g-IV and g-V were analyzed by out-PCR, and three different patterns, D, E, and F, were obtained. These results were in agreement with those obtained by hybridization with IS*6110* (Table 1 and Fig. 1b).

Ten strains from the five herds which had six different IS*6110* patterns were probed with pTBN12 and DR. With pTBN12, only three types could be detected, whereas the DR probe distinguished five different types. The patterns obtained with the pTBN12 and DR probes were less polymorphic than were those obtained with IS*6110* for goat isolates (data not shown).

RFLP analysis of bovine strains. The intradermal tuberculin test was performed for more than 300 cattle from six different

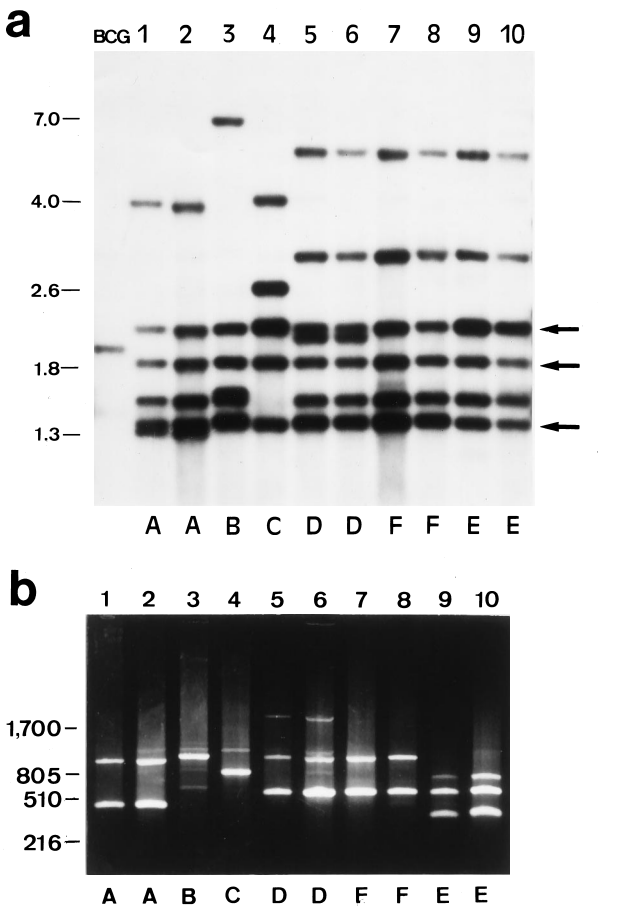

FIG. 1. Restriction enzyme digests of *M. bovis* BCG and 10 *M. bovis* field isolates from goats, digested with *Pvu*II and probed with the right arm of IS*6110* (a), and the amplification products of the same isolates following out-PCR (b). Lanes 1 and 2, strains 1 and 3 from herd g-I; lanes 3, strain 8 from g-II; lanes 4, strain 9 from g-III; lanes 5 and 10, strains 10 and 14, respectively, from g-IV; lanes 6, 7, 8, and 9, strains 15, 20, 23, and 18, respectively, from g-V. The respective patterns (see Table 1) are indicated below the lanes, and the sizes of standard DNA fragments, in kilobase pairs for panel a and in base pairs for panel b, are indicated on the left. Arrows on the right indicate the bands shared.

TABLE 2. Distribution of strains among bovine herds and RFLP patterns determined by using as probes the entire IS*6110*, pTBN12, and DR

Herd	Strain no.	RFLP pattern with:		
		IS*6110*	pTBN12	DR
b-I	24–26	A	A	A
b-II	27	B	B	B
b-III	28–30	C	C	C
b-IV	31	B	D	D
b-V	32–37	B	E	E
b-VI	38	C	C	F
	39	B	D	D
	40	D	F	G

herds. Positive reactions were observed with 180 cattle, and cultures were available from 17 of these animals. The DNA from these 17 *M. bovis* isolates was *Pvu*II digested and probed with the entire IS*6110*. Four different patterns, A to D, were observed (Table 2). Patterns A, B, and C showed two bands, indicating a single copy of IS*6110* (Fig. 2a). Pattern A was observed only in the three isolates from herd b-I. Pattern B was present in isolates analyzed from all the herds, with the exception of b-I and b-III. Pattern C was observed with isolates from herds b-III and b-VI. The three isolates from herd b-VI showed different patterns, B, C, and D. Pattern D, obtained only with strain 40, showed four copies of this insertion sequence.

Further discrimination among strains from unrelated herds could be obtained by using pTBN12 and DR. Six different patterns (A, B, C, D, E, and F) were detected with the pTBN12 probe (five patterns are shown in Fig. 2b), and seven different patterns (A, B, C, D, E, F, and G) were detected with the DR probe (six patterns are shown in Fig. 2c). All the herds tested, with the exception of b-VI, were found to have different RFLP types by using either of the two probes (Table 2). Three strains of different types (patterns C, D, and F with pTBN12 and F, D, and G with DR) were found in herd b-VI. Strains 28 to 30 from herd b-III and strain 38 from herd b-VI gave the same pattern when pTBN12 was used (C), whereas with DR the patterns were similar but not identical because of the presence of an additional band in pattern F from strain 38 (Fig. 2b and c).

DISCUSSION

Tuberculosis caused by *M. bovis* is a major economic and agricultural problem for farmers in Spain. Caprine tuberculosis is traditionally believed to be caused by the same strains that infect cattle; however, no molecular or epidemiological studies have been carried out to test this view. In this study, we have used a variety of DNA probes to gather information on the number and types of *M. bovis* strains involved in bovine and caprine tuberculosis.

The 23 caprine *M. bovis* isolates studied harbored between six and eight copies of IS*6110*, allowing the use of the standardized IS*6110* RFLP method to detect polymorphism among *M. bovis* strains isolated from goats. Recently, *M. bovis* strains harboring multiple copies of IS*6110* have been isolated from animals such as antelopes, monkeys, and seals (22), validating the use of IS*6110* as a genetic marker.

The six different RFLP patterns obtained are important from an epidemiological point of view. Patterns A, B, and C permit differentiation of strains from unrelated herds (g-I, g-II, and g-III). Patterns D, E, and F, although different, show a high degree of similarity that might be explained by the selective advantage for persistence of particular strain types in the area of origin. The presence of IS*6110* RFLP patterns D and E shared by herds g-IV and g-V could be explained by the exchange of animals between neighboring herds, which is common practice in this region.

All the strains from goats analyzed in the present study share at least three copies of IS*6110*, suggesting that a particular group of genetically similar *M. bovis* strains is responsible for caprine tuberculosis in Spain. This would seem to suggest that the strains affecting goats have a recent common ancestral origin or that these RFLP strain types are better adapted for infecting and surviving in goats. Further investigation of a greater number of strains isolated from goats from diverse geographical areas would be necessary to establish a general association between a particular strain type and caprine tuberculosis and to verify if the severe pathology observed to occur in goats (4, 6) is related to the strains, the host, or the epidemiological factors.

Analysis by out-PCR based on IS*6110* polymorphism proved to be a useful technique for typing caprine strains. The advantage of PCR over RFLP is that it is simple and quick and requires only a small amount of DNA. In order to decrease the

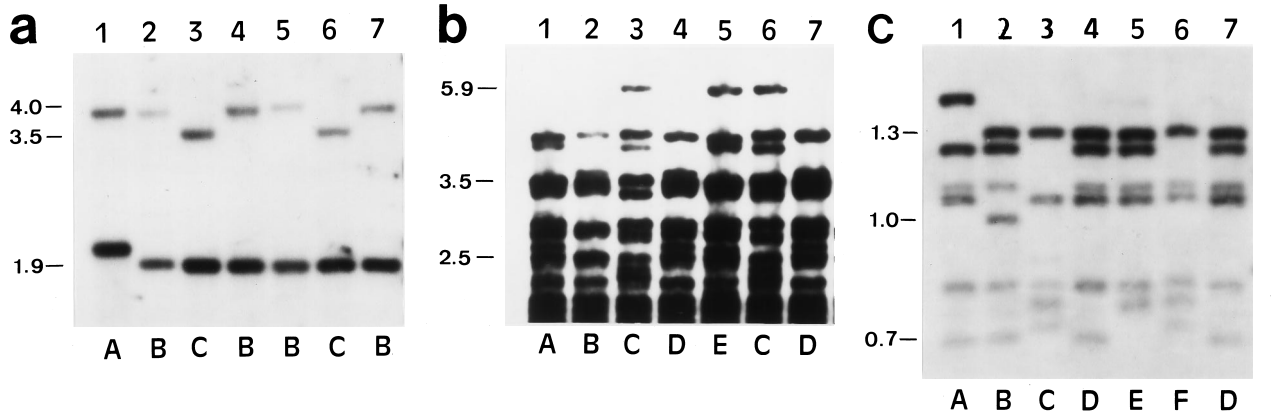

FIG. 2. RFLP of *M. bovis* DNA from seven bovine isolates probed with the entire IS*6110* (a), pTBN12 (b), and DR (c). DNA was digested with *Pvu*II (a) and *Alu*I (b and c). Lanes 1, strain 24 from herd b-I; lanes 2, strain 27 from b-II; lanes 3, strain 29 from b-III; lanes 4, strain 31 from b-IV; lanes 5, strain 34 from b-V; lanes 6 and 7, strains 38 and 39, respectively, from b-VI. Letters below the lanes indicate the respective patterns (see Table 2). The sizes of standard DNA fragments (in kilobase pairs) are indicated on the left.

time necessary for typing strains, we are now trying to apply this technique directly to clinical samples, which would permit a preliminary screening of the strains prior to cultivation and further analysis by RFLP. The PCR method allows the same level of discrimination between caprine isolates as does RFLP hybridization with IS*6110*, but the fact that fewer bands are detected restricts the comparison of results between laboratories where the standardized RFLP typing method is commonly used.

The standardized RFLP typing method, which uses IS*6110* from *M. bovis*, showed that 16 of the 17 cattle isolates harbored only a single copy of IS*6110*, with 3 strains showing a band of 2 kb and 13 strains showing a band of 1.9 kb. Even when the entire insertion sequence IS*6110* was used as a probe, only small differences among the bovine strains were observed. The two bands obtained with this probe for the 16 strains with a single copy of IS*6110* allowed the strains to be divided into three patterns, which was not sufficient to allow differentiation of strains with respect to herds. These results support those of Collins et al. (2) and Skuce et al. (18), who reported a small number of pattern types for *M. bovis* isolates. The most common pattern, present in 46 of 160 and 57 of 109 isolates, respectively, tested in the studies of Collins et al. and Skuce et al. is the same as our pattern B found in 8 of 17 bovine strains. *M. bovis* strains with multiple IS*6110* copies, as those with our pattern D, have been reported to occur mainly in wild animals (22) and only sporadically in cattle (18).

When pTBN12 and DR were used to type *M. bovis* strains from cattle, several polymorphic fragment sizes were detected, allowing discrimination among strains from neighboring or separated herds (Fig. 1). In other reports, it was claimed that pTBN12 was superior to DR for strain differentiation (22). However, our results are contrary to these findings.

The RFLP study, using either DR or pTBN12, of isolates from herd b-VI showed the presence of three different types of strains. The explanation for this is that cattle from different localities were incorporated into the herd in the last 12 months, prior to the time that the reported incidence of bovine tuberculosis suddenly increased.

In summary, by using several molecular typing methods, we find that the strains causing tuberculosis in Spanish cattle and goats are not the same. This result indicates that reservoirs for caprine and bovine tuberculosis are different. We cannot distinguish whether the host preference for particular strains is due to lack of transmission between animals or due to variation in virulence properties in the different hosts. The results do, however, address the need to reevaluate the evidence of *M. bovis* transmission among cattle and goats. While it is likely that such transmission does occur, its significance with regard to the overall incidence of bovine tuberculosis needs to be carefully assessed. Furthermore, as goats are very susceptible to tuberculosis, they have been proposed as good animal models for the study of the pathogenesis and pathology of tuberculosis and the evaluation of new tuberculosis vaccines. Molecular studies of tuberculosis and development of new vaccines are imperative for the eradication of caprine tuberculosis.

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