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Spacer Oligonucleotide Typing of *Mycobacterium bovis* Strains from Cattle and Other Animals: a Tool for Studying Epidemiology of Tuberculosis

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The spacer oligonucleotide typing (spoligotyping) method was evaluated for its ability to differentiate *Mycobacterium bovis* strains. This method detects the presence or absence of spacers of the direct repeat locus of the *M. bovis* genome. The spacers in the direct repeat locus are amplified by PCR and are detected by hybridization of the biotin-labelled PCR product with a membrane containing oligonucleotides derived from spacer sequences that have previously been bound to a membrane. One hundred eighty-two *M. bovis* isolates from domestic animals (cattle, goat, sheep, and cats) and wild animals (deer and wild boar) were spoligotyped, and the results were compared with those obtained by IS*6110* restriction fragment length polymorphism analysis. Two rather homogeneous clusters of isolates containing 20 and 4 types, respectively, were identified by spoligotyping. The first cluster included isolates from cattle, cats, and feral animals. By spoligotyping, isolates from the Spanish wild boar and deer had the same pattern as some bovine isolates, suggesting transmission between these animals and cattle and highlighting the importance of the study of these reservoirs. The second cluster included all the caprine and ovine isolates. Within each cluster, the patterns of the different strains differed only slightly, suggesting that the spoligotypes may be characteristic of strains from particular animal species. Spoligotyping proved to be useful for studying the epidemiology of bovine *M. bovis* isolates, especially of those isolates containing only a single copy of IS*6110*. In view of our results, we suggest fingerprinting all *M. bovis* strains by the spoligotyping method initially and then by IS*6110* restriction fragment length polymorphism typing of the strains belonging to the most common spoligotypes.

Bovine tuberculosis is an endemic infectious disease that causes significant economic losses in Spanish cattle. *Mycobacterium bovis*, the causative agent of bovine tuberculosis, can also cause disease in a variety of domestic and wild animals (35). *M. bovis* is a member of the *M. tuberculosis* complex, which includes *M. bovis* BCG, *M. tuberculosis*, *M. africanum*, and *M. microti*.

The diagnosis of tuberculosis in Spanish cattle relies on skin testing, and all positive animals are slaughtered. This policy started in 1965 and has led to a reduction in the prevalence of this disease. However, in recent years, the rate of the decrease in the prevalence has slowed. In 1994, 5.7% of the herds were positive and the eradication campaign cost $43.1 million. In other countries where control has proven to be difficult, the identification of alternative sources of infection in other farmed animals, such as goats and sheep (1, 2, 8, 9), and feral animals, such as deer (3, 15) and badgers (4, 24, 26), has been important in efforts to control the disease.

Strain differentiation by restriction fragment length polymorphism (RFLP) analysis has proven to be a very useful tool for epidemiologic studies of human tuberculosis. The insertion sequence (IS) IS*6110* (which is virtually identical to IS*986* and IS*987*) (19, 20, 26, 34) has been widely used as a genetic marker for the study of *M. tuberculosis* because of its variability in copy numbers and its ability to move within the genome of the different *M. tuberculosis* strains. Although strains of *M. tuberculosis* with a single copy of IS*6110* and strains lacking IS*6110* have been found (13, 30, 44), most strains harbor 6 to 20 copies of this element (33, 39). RFLP analysis with IS*6110* has been successfully applied to the study of human *M. tuberculosis* outbreaks, relapsed infections, human immunodeficiency virus-associated transmission, dissemination of multidrug-resistant strains, and nosocomial infections (11, 12, 14, 20, 25, 28, 32), and a standardized methodology has been proposed (36).

The differentiation of *M. bovis* isolates with IS*6110* depends on their origin (37). The majority of bovine isolates harbor only a single copy of this element (5, 10, 39, 40), located at a previously identified chromosomal locus, a 1.9-kb *Pvu*II restriction fragment (10, 37, 40). *M. bovis* strains isolated from animals other than cattle have been found to contain several copies of IS*6110* (37). This suggests that the various animal reservoirs may contain different *M. bovis* types (37). In a recent study, we found that a significant percentage (51%) of *M. bovis*

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isolated from Spanish cattle harbored multiple copies of IS*6110* (23).

M. tuberculosis complex isolates also contain five or six copies of a second transposable element, IS*1081* (7). Between 91 and 98% of *M. bovis* isolates tested displayed the same IS*1081*-associated fragment pattern, identical to that of most *M. tuberculosis* strains tested (5, 40). The differences between patterns depended only on the size of one of the six IS*1081*-containing fragments, indicating that this element transposes rarely, and little heterogeneity in the sites of insertion in the genome (5) can be expected, hampering its usefulness for typing *M. tuberculosis* isolates.

Other repetitive elements have been used in the study of the epidemiology of tuberculosis. These include the polymorphic GC-rich repeat sequence (PGRS), found in the *M. tuberculosis* complex and some other mycobacteria (29), and the direct repeat (DR) sequence (16, 19). These elements have been used to probe *Alu*I-digested *M. tuberculosis* (11, 30, 38, 44) and *M. bovis* (10, 37) genomic DNAs.

The DR element was identified by Hermans and coworkers (19) in 1991 when characterizing the insertion element IS*986* of *M. bovis* BCG and the chromosomal DNA flanking this IS element. They were looking for the possible differences between IS*986* and IS*6110* which may explain the apparent transposition incapacity of the former IS. This chromosomal region was found to contain a large number of DRs of 36 bp interspersed by spacer DNA 35 to 41 bp in length. The total number of DR copies in *M. bovis* BCG was determined to be 49, clustered at a unique position on the chromosome. The 30th DR was split by the insertion of IS*987*. DRs are invariably and exclusively present in isolates of the *M. tuberculosis* complex. Further analysis of the DR-containing region in different strains revealed that it is polymorphic in size and composition (19). One DR plus the adjacent unique spacer sequence is termed a "direct variable repeat" (DVR) and is numbered according to the archetype DVRs in *M. bovis* BCG P3 (16). When the DR regions of several isolates were compared, it was observed that the order of the spacers was about the same in all isolates, but deletions and insertions of DVRs occurred. The polymorphism in various isolates comprises the absence or presence of one or more DVRs.

The spacer oligonucleotide typing (spoligotyping) method described by Kamerbeek et al. (21) detects the presence or absence of spacers of known sequence in an isolate in two steps. PCR is used to amplify the spacers between the DRs. The reverse primer used in the PCR is biotin labelled, so that all reverse strands synthesized are labelled. Individual spacers are then detected by hybridization of the biotin-labelled PCR product containing spacer DNA to a membrane to which 37 oligonucleotides derived from spacers in *M. tuberculosis* H37Rv and six spacers from *M. bovis* BCG have been covalently linked.

The aim of the present work was to evaluate this new method for the differentiation of isolates of *M. bovis* and its usefulness as a tool for studying the epidemiology of bovine and caprine tuberculosis. *M. bovis* isolates from various animal sources, both domestic (cattle, goats, sheep, and cats) and wild (deer and wild boars) animals, were typed by spoligotyping. The results indicate that this typing method is a reliable tool that can be applied in epidemiologic studies of animal tuberculosis.

MATERIALS AND METHODS

Mycobacterial strains. One hundred eighty-two *M. bovis* strains isolated from samples received at the Departamento de Patología Animal I (Facultad de Veterinaria, Madrid, Spain) were studied. These samples came from cattle (n = 129), goats (n = 44), a sheep, wild boars (*Sus scrofa*) ($n \times 4$), deer (*Cervus elaphus*) (n = 2), and cats (n = 2) from several regions of Spain.

The 129 bovine strains originated from 34 herds from different geographical areas of Spain. Farms b1 to b13 were located in various areas in León (northwest), farm b14 was located in Cantabria (north), and farms b15 and b16 were situated in Madrid and Guadalajara, respectively (central Spain). Herds b17 to b34 were from several villages in Catalunya (northeastern Spain).

The 44 caprine strains originated from eight herds. Herds c1 to c6 were located in different villages in Catalunya. Farms c1 and c2 were very close. Herds c7 and c8 originated from Madrid. The sheep came from herd o1, in the same area where property c6 is situated.

The four wild boars were hunted in a natural reserve in Extremadura, in the southwest of the country. Three of them had granulomatous lesions in several lymph nodes, lung, and liver. The fourth boar had lesions in the elbow joint. The two deer, with pulmonary disease, were captured in Catalunya. The two pet cats belonged to private owners from the city of Madrid. The first cat was a female with generalized disease. The second one was a male with lesions involving a lung and associated lymph nodes.

The strains were identified as *M. bovis* by slow growth, colony morphology, acid-fast staining, standard biochemical tests (42), and PCR amplification of the IS*6110* (20), 16S rRNA-MPB70 (43), and IS*1081-mtp40* (22) sequences.

Bacterial growth and chromosomal DNA isolation. The mycobacterial strains were grown in 15 ml of Middlebrook 7H9 medium supplemented with OADC (oleic acid, albumin, dextrose, citric acid) enrichment w/WR 1339 (Difco, Detroit, Mich.), 1 g of pancreatic digest of casein (Difco) per liter, and 4 g of sodium pyruvate per liter. The cells were harvested by centrifugation at 3,700 $\times$ *g* in a Sigma 3-10 centrifuge, and chromosomal DNA was isolated as described previously (10).

In vitro amplification of spacer DNA by PCR. PCR amplification and detection of DNA were performed by the method of Kamerbeek et al. (21). DNA amplifications were performed in 50-μl reaction volumes containing 10 μl of 5× reaction buffer [335 mM Tris-HCl (pH 8.8 at 25°C), 83 mM $(NH_4)_2SO_4$, 1 mM (each) deoxynucleoside triphosphates, 2.25% Triton X-100, 1 mg of gelatin per ml], 2 mM $MgCl_2$, 120 ng of each oligonucleotide primer (primers DRa and DRb; Oswel DNA Services, Southampton, United Kingdom), 1.1 U of *Tth* plus enzyme (Biotech International, Bentley, Western Australia, Australia), and approximately 25 ng of template DNA. The oligonucleotide primers DRa (5′-CCG AGA GGG GAC GGA AAC-3′) and DRb (5′-GGT TTT GGG TCT GAC GAC-3′), described by Kamerbeek et al. (21), amplify the spacer regions between the DRs. The reverse primer (DRb) is biotin labelled at the 5′ end.

The amplifications were carried out in a Corbett FTS-1 thermal cycler (Biotech International) by using 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 30 s at 72°C. The first denaturation and the final extension steps were held for 10 min.

The amplified DNAs were visualized after electrophoresis at 100 V for 0.6 h in a 2% agarose gel stained with ethidium bromide (0.5 μg/ml). No discrete bands were observed; rather, smears of staining representing the small fragments of different lengths, which were not well resolved, were observed.

M. tuberculosis H37Rv, *M. bovis* BCG P3, and purified water were included as controls with every batch of tests. Forty-two test organisms and the three controls could be run in each batch of tests.

Hybridization with the PCR product and detection. Membranes containing the bound spacer oligonucleotides were prepared in Bilthoven, The Netherlands, and spoligotyping was performed in Perth, Australia. The membrane was washed in prewarmed 2× SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 7.2]) supplemented with 0.1% sodium dodecyl sulfate (SDS) for 5 min at 60°C. The membrane was placed in a miniblotter (MN45; Immunetics, Cambridge, Mass.) in such a way that its slots were perpendicular to the line pattern of the applied oligonucleotides. Twenty microliters of PCR product was added to 150 μl of 2× SSPE–0.1% SDS and was heat denatured at 99°C for 10 min. Each slot was filled with 150 μl of the diluted PCR product, and the hybridization was performed at 60°C for 1 h. Subsequently, the slots were emptied by aspiration, and the filter was removed and washed twice in 2× SSPE–0.5% SDS for 10 min at 60°C to remove the unhybridized products. The membrane was incubated in 10 ml of 2× SSPE–0.5% SDS plus 5 μl of streptavidin-peroxidase conjugate (Boehringer, Mannheim, Germany) for 45 min at 42°C. The membrane was washed twice in 2× SSPE–5% SDS for 10 min at 42°C and was then rinsed in 2× SSPE for 5 min at room temperature. Hybridized DNA was detected with the ECL detection liquid (Amersham, Buckinghamshire, United Kingdom) and by exposing ECL-Hyperfilm (Amersham) to the membrane for 12 min.

After detection, the membrane was stripped by using four 20-min washes in 1% SDS at 80°C; this was followed by washing for 15 min in 20 mM EDTA (pH 8) at room temperature. The membrane was stored damp in a sealed plastic bag at 4°C until it was reused.

Analysis of the hybridization patterns was performed with the aid of a computer program (GelCompar, version 3.1; Applied Maths, Kortrijk, Belgium).

IS*6110* RFLP. The IS*6110* fingerprints of 120 of the strains were known. Digestion of genomic DNA with the restriction enzyme *Pvu*II, Southern blot hybridization with two digoxigenin-labelled IS*6110* probes (IS*6110*-R was directed at the right-hand side of the *Pvu*II site in IS*6110*, and IS*6110*-L was directed at the left-hand side of the same *Pvu*II site), and detection with the

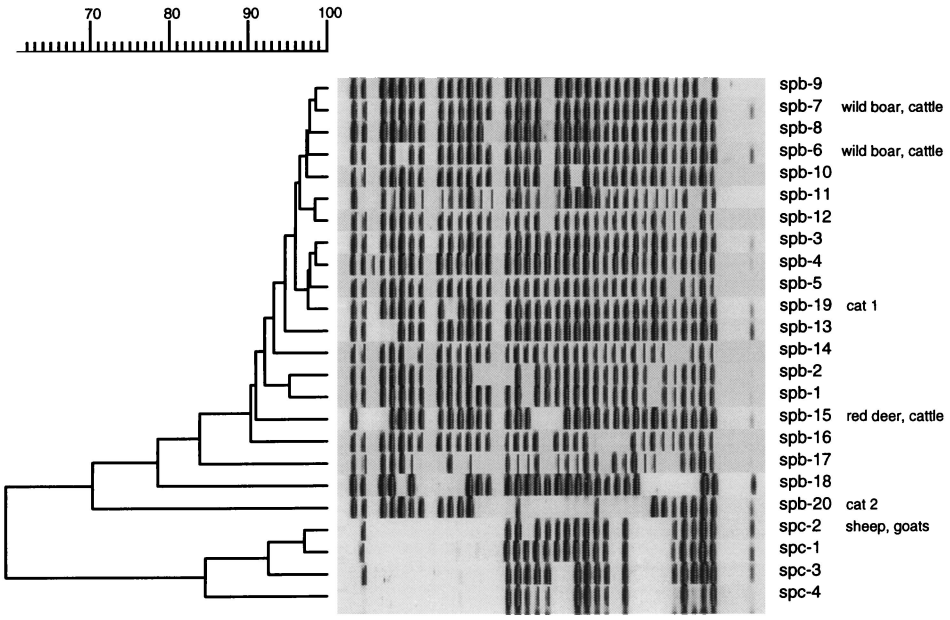

FIG. 1. Dendrogram showing the relationship of 24 representative spoligotypes identified from 182 *M. bovis* isolates from cattle, deer, wild boar, goats, cats, and a sheep. spb and spc are the two clusters identified in mostly bovine isolates and caprine isolates, respectively. When spb and spc strains were identified in other animal species, it is noted on the dendrogram.

alkaline phosphatase-conjugated antibody DNA detection kit (Boehringer) have been described previously (23).

RESULTS

Spoligotyping of *M. bovis* isolates. Twenty-four different spoligotypes were identified among the 182 *M. bovis* isolates investigated. All isolates tested lacked the spacers 39 to 43, a characteristic property that distinguishes *M. bovis* from *M. tuberculosis* (21). Occasionally, spacer 42 showed a weak reaction, but since this reaction gave an inconsistent result (sometimes negative and sometimes weakly positive), it was finally considered a negative result. The control strains *M. tuberculosis* H37Rv and *M. bovis* BCG P3 yielded consistent patterns in all tests.

By using GelCompar, the spoligotypes were clustered into two main groups, containing 20 and 4 types, respectively (Fig. 1). Within each group, the patterns of the different spoligotypes differed only slightly. The results suggest that these spoligotyping patterns are characteristic of animal species.

***M. bovis* from cattle.** All strains from cattle lacked spacers 3, 9, and 16 and harbored spacers 1, 13, 18, 23, 25, 30, and 38 (Fig. 1). This method classified the 129 isolates into 18 spoligotypes (Table 1). The most common type was spb-7; it was found in 59 isolates (46%) belonging to 15 cattle herds (44%) scattered throughout Spain. This type could be further differentiated into 11 IS*6110* types by using the results obtained with both IS*6110*-R and IS*6110*-L probes. Types spb-3 and spb-13 were the second and third most frequent types, respectively, and both appeared in isolates from 14 animals from five herds in different geographical areas. They could be distinguished into two IS*6110* types [types 1A(1A) and 12A (the types in parentheses are those obtained by RFLP analysis with the IS*6110*-L probe] and three IS*6110* types [1A(1C), 1A(1D), and 7B], respectively. The control strain *M. bovis* BCG was also type spb-3. Type spb-8 was found in isolates from four herds. Other spoligotypes were found in isolates from only one or two farms. On some farms all the isolates were the same type, whereas on other farms several spoligotypes were found. Follow-up investigations on some farms provided details about possible reasons for this. Herds b5, b9, b11, and b13 had had several tuberculin-positive animals during the last few years, and farmers were known to buy and sell cattle frequently. Herd b2 had been tuberculosis-free before an outbreak was reported, and the 12 strains that originated from cows housed on this farm shared the same pattern.

Forty nine (51%) of the 97 isolates with a known IS*6110*-R RFLP type harbored a single copy of this element located on a 1.9-kb *Pvu*II fragment (type 1A), and isolates of this type were present on 19 (71%) of 27 farms. These isolates were grouped into four types (types were 1A, 1B, 1C, and 1D) by IS*6110*-L RFLP. Spoligotyping identified nine different types in this group (types spb-3, spb-4, spb-5, spb-6, spb-7, spb-8, spb-9, spb-13, and spb-17).

If the results of the spoligotyping method and the IS*6110*-associated RFLP analysis were combined, 30 different types could be identified in the bovine isolates.

***M. bovis* from goats and a sheep.** The spoligotypes obtained from the *M. bovis* organisms isolated from goats and sheep were very similar and were clearly different from those obtained from cattle (Fig. 1). The main characteristics of their patterns were the absence of spacers 1, 3 to 16, 28, and 30 to 33.

Spoligotyping grouped the 45 goat and sheep isolates into four patterns (patterns spc-1 to spc-4); IS*6110*-R RFLP analysis grouped the 31 isolates into five types (Table 2). With the exception of two individual animals, animals on a property were infected with *M. bovis* strains of the same spoligotype. The largest cluster of strains, type spc-3, comprised isolates from 26 animals from farms c1, c2, and c3; all these strains were IS*6110*-R RFLP type 6B. The distribution of this pattern on several farms might be because of the exchange of animals between neighboring farmers. Two IS*6110*-R RFLP types were identified within each of spoligotypes spc-1 and spc-2.

TABLE 1. Spoligotyping and IS*6110* RFLP results for *M. bovis* isolates from bovine herds

Spoligotype	IS*6110* type[a]	Farm	No. of isolates
spb-1	7A	b2	12
spb-2	5B	b1	1
spb-3	1A(1A)	b23	1
	1A(1A)	**b29**[b]	1
	2A	**b29**	1
	12A	b4	3
	—[c]	b17	4
	—	b23	3
	—	**b33**	1
spb-4	1A(1C)	**b5**	1
spb-5	1A(1A)	**b13**	3
	—	**b13**	1
spb-6	1A(1B)	**b9**	1
spb-7	1A(1A)	b7	2
	1A(1A)	b8	1
	1A(1A)	**b12**	1
	1A(1A)	b32	19
	1A(1B)	b20	1
	1A(1B)	b26	1
	1A(1D)	b31	3
	1D	**b11**	1
	2A	**b29**	13
	2B	b32	1
	3A	**b29**	3
	3B	**b29**	1
	3C	**b29**	1
	4A	b24	1
	5C	b30	1
	5D	b24	1
	—	b24	1
	—	b25	1
	—	b26	2
	—	**b27**	1
	—	b32	1
	—	**b33**	1
	—	b34	1
spb-8	1A(1B)	b6	1
	1A(1B)	**b9**	3
	1A(1B)	**b11**	1
	—	b28	2
spb-9	1A(1A)	b16	1
	1A(1A)	**b33**	1
spb-10	6D	b15	1
spb-11	—	**b27**	1
spb-12	1C	b3	1
spb-13	1A(1C)	**b5**	1
	1A(1C)	b10	3
	1A(1D)	b14	1
	1A(—)	b19	1
	7B	b18	1
	—	b18	4
	—	b19	3
spb-14	11A	**b12**	1
spb-15	—	b21	4
		b22	1
spb-16	4B	**b11**	1
spb-17	1A(1B)	**b13**	1
spb-18	1B	**b33**	3

[a] The numeral in the IS*6110* type represents the number of bands hybridizing with the IS*6110*-R probe. The type identified by IS*6110*-L RFLP analysis is indicated in parentheses.
[b] Farms in which several spoligotypes were found are indicated in boldface type.
[c] —, not available.

The pattern spc-I/5A (indicating the spoligotype/IS*6110*-R RFLP type) was observed in isolates from herd c5, and the pattern spc-I/6C was observed in the only isolate from farm c7 and all strains from herd c8. After some inquiries, we found out that the goat from farm c7 was bought from farm c8 1 year before the diagnosis of disease. Spoligotype spc-2/8A was found in the isolates from the animals in herd c4, and spoligotype spc-2/6A was found in the isolates from the animals in herd c6. Pattern spc-4 was observed in only one isolate, from an animal on farm c1. The ovine isolate showed the same pattern as isolates from animals in herd c6.

By combining the results of the spoligotyping method with the IS*6110*-associated RFLP analysis, the caprine *M. bovis* strains could be classified into six patterns.

***M. bovis* from various animal species.** The spoligotyping results of the isolates from the wild boar, deer, and pet cats are presented in Table 3. Pattern spb-6, found in three of the four isolates from wild boars, was also found in one strain from property b9. The IS*6110*-R RFLP type of these three isolates was the cattle fingerprint type, type 1A. The isolate from the

TABLE 2. Spoligotyping and IS*6110* RFLP results for caprine and ovine *M. bovis* isolates

Spoligotype	IS*6110* type[a]	Farm	No. of isolates
spc-1	5A	c5	2
	6C	c7	1
	6C	c8	5
	—[b]	**c2**[c]	1
spc-2	6A	c6	1
	6A	o1	1
	8A	c4	2
	—	c4	1
	—	c6	4
spc-3	6B	**c1**	6
	6B	**c2**	12
	6B	c3	1
	—	**c1**	4
	—	**c2**	2
	—	c3	1
spc-4	—	**c1**	1

[a] The numeral in the IS*6110* type represents the number of bands hybridizing with the IS*6110*-R probe.
[b] —, not available.
[c] Farms in which two spoligotypes were found are indicated in boldface type.

TABLE 3. Spoligotyping and IS*6110* RFLP results for *M. bovis* isolates from diverse animal sources

Spoligotype	IS*6110* type[a]	Animal	No. of isolates
spb-6	1A(1B)	Wild boar	3
spb-7	—[b]	Wild boar	1
spb-15	—	Deer	2
spb-19	1A(1A)	Cow no. 1	1
spb-20	—	Cow no. 2	1

[a] The numeral in the IS*6110* type represents the number of bands hybridizing with the IS*6110*-R probe. The RFLP type identified by IS*6110*-L RFLP analysis is indicated in parentheses.
[b] —, not available.

fourth animal was type spb-7. The isolates from the two deer were both type spb-15; remarkably, this type was also found in isolates from the cattle from farms b21 and b22. Although a definitive epidemiological relationship could not be established, the transmission from the wild animals to cattle (or vice versa) might have taken place when the animals shared the same pasture.

The spoligotypes found in the isolates from urban domestic cats, types spb-19 and spb-20, were not found in isolates from other animals. The strain isolated from the first cat was spoligotype spb-19, quite similar to those obtained from cattle; however, the spoligotype of the isolates from the second cat showed significant differences from that of the strain isolated from the second cat (Fig. 1).

DISCUSSION

The DR-containing region was characterized by Hermans and coworkers (19). This region was found to contain multiple DRs of 36 bp, interspersed by spacer DNA of 35 to 41 bp. Polymorphism in various strains comprises the absence or presence of one or more DVRs, discrete blocks of DR plus a spacer. Two types of rearrangements might contribute to the degree of polymorphism observed in this chromosomal region: homologous recombination and IS-mediated rearrangements (16). Spoligotyping is a method used to detect the spacers present in a strain and has been proposed as a method for detecting and typing *M. tuberculosis* strains (21).

After typing 182 *M. bovis* strains by the spoligotyping method, 24 different types were identified. The spoligotyping method had a high level of discriminatory ability and allowed us to disclose several epidemiologic relationships. Some spoligotypes were restricted to small areas, forming an endemic group of related strains, but others were well distributed in the country, probably because of the trade of cattle, mainly dairy cows. It allowed the establishment of types from a determined animal species, the source of some newly detected cases of infection, and the transmission of infection between animals and between herds.

Several investigators have suggested that the various animal reservoirs of *M. bovis* may contain different *M. bovis* types, depending on the host (37). We have used the spoligotyping method to gather information about *M. bovis* isolates involved in bovine and caprine tuberculosis. Interestingly, the spoligotypes of the *M. bovis* isolates clustered into two main groups, the first one with isolates from cattle, cats, and feral animals and a second one with isolates from goats and sheep. The spoligotyping patterns found in each group had significant similarities, indicating that infection was with a genetically homogeneous group of *M. bovis* isolates or that the spacer regions identified by this method are very stable.

We have evaluated the usefulness of the spoligotyping method for further differentiating bovine *M. bovis* strains. The 129 bovine strains were grouped into 18 types. In the present study, we found that this new typing method is well suited for the epidemiologic study of bovine *M. bovis* strains, especially those strains containing a single IS*6110* element. A single IS*6110* element is characteristic of *M. bovis* strains from cattle, and the fact that a large proportion of these bovine isolates carry the IS*6110* element at the same chromosomal locus restricts strain differentiation with this marker. Ninety-seven percent of 160 *M. bovis* strains from New Zealand carried a single copy of IS*6110* (5), 31 of 51 strains from Argentina and The Netherlands carried single copies of this element (31 of 32 if 19 outbreak strains with six copies of this element are excluded) (37), and 16 of 17 bovine isolates in Spain harbored a single copy of this element (17). Virtually all these isolates that originated from cattle could not be satisfactorily typed by IS*6110* fingerprinting. In the present study, the spoligotyping technique with a 1.9-kb *Pvu*II fragment successfully classified the isolates with a single copy into nine different groups.

The most prevalent type was the cattle spb-7; it was found in 59 isolates (46%) from 15 herds (44%) scattered throughout the country. This type could be further differentiated into 9 types with the IS*6110*-R probe and into 11 types by using the results obtained with both IS*6110*-R and IS*6110*-L probes. The combined use of both typing methods further classified all the bovine *M. bovis* isolates into 30 types. In view of our results and in order to obtain good differentiation of the isolates, we suggest initial fingerprinting of all *M. bovis* strains by the spoligotyping method and then IS*6110* typing of the isolates belonging to the most prevalent spoligotypes.

Our results obtained by spoligotyping of caprine *M. bovis* isolates agreed with those of RFLP typing published recently (17, 23). In both studies, the caprine isolates shared several copies of the IS*6110* element, suggesting that they formed a particular group of genetically similar *M. bovis* isolates responsible for caprine tuberculosis in Spain. Spoligotyping of the caprine *M. bovis* isolates from diverse geographical areas also clustered these isolates into a very homogeneous group. The fact that the ovine isolate clustered with the caprine isolates is not surprising, because mixed farming with herds of goats and sheep is a common practice in some areas of Spain. We could not attribute this strain variation to the lack of transmission between cattle and goats or to host preference by a particular strain. Further investigations of isolates that originated from farms where both animal species are housed would be necessary.

The potential role of wild animals in the maintenance of *M. bovis* infection in cattle is of particular importance in countries where eradication programs had substantially reduced the incidence of bovine tuberculosis but where sporadic outbreaks still occur (3, 24, 27, 31). Collins and coworkers (6) found the same restriction endonuclease type in *M. bovis* strains from deer, possums, and cattle from the same areas in New Zealand. Spoligotyping identicated that *M. bovis* isolates from the Spanish wild boars and deer had the same pattern as some bovine isolates. The isolates from three wild boar had single copies of IS*6110* (type 1A), which is typical of cattle isolates. Although we have no direct evidence of the epidemiologic relatedness of the isolates, transmission by a common source or between different animal species was not unlikely.

The spoligotyping pattern of the strain from the first cat was quite similar to those of the strains obtained from cattle, which suggests that infection resulted from contact with infected cattle. Possible routes of transmission could have been through ingestion of offal from infected cattle or through aerosol

spread if a cat had close contact with infected cattle in a barn. The spoligotype of the isolate from the cat with lesions in the respiratory tract differed substantially from the spoligotypes of the cattle isolates; it is not possible at this stage to identify the possible cause of infection in this cat. Examination of more isolates from Spain may provide information on the source of infection in this animal.

A major advantage of spoligotyping over RFLP analysis is its simplicity and speed and the requirement for only a small amount of DNA. RFLP is time-consuming and highly labor-intensive. To obtain the same level of differentiation among the bovine strains by RFLP analysis, it was necessary to compare the results with those obtained by hybridization with two IS*6110* probes. To be able to use the computer analysis system, it was necessary to perform a hybridization with the internal markers of known molecular weights. Similar strain characterization was achieved with just one hybridization when working with the spoligotyping method. PCR amplification of the spacers could also be performed with lysed bacteria, with the same result (data not shown), therefore avoiding the need for subculturing, and may also be performed with DNA extracted from clinical samples. Forty-two samples can be tested in every batch, and the results were reproducible. The control strains *M. tuberculosis* H37Rv and *M. bovis* BCG P3 always yielded the same results, and the reproducibilities of the patterns were confirmed by testing 20 of the samples in duplicate. Computer analysis of the spoligotypes was far easier than analysis of RFLP types, and both the methodology and analysis can be standardized in order to permit interlaboratory comparisons. International consensus on a standardized method of typing *M. tuberculosis* isolates has been achieved (36), and this has allowed for the comparison of isolates from different laboratories and the development of an international database of IS*6110* RFLP types, which now comprises the fingerprints of about 5,500 isolates. This database permits the tracing of the transmission of tuberculosis across borders (18, 41). Similarly, a database of spoligotypes of *M. bovis* isolates could be developed. Such a database would be easier to handle because it would need only the data on the presence or absence of any of the 43 spacers used. This could be done by defining a spoligotype as a 43-letter word and using a 3-letter code (a, present; b, absent; or 3, indeterminate, in the case of weak reactions). Comparison of spoligotypes between laboratories would not be dependent on sophisticated software, as is required for RFLP analysis (18), and a word processor could match patterns by using the sort command.

In summary, spoligotyping is a promising tool for the genetic typing of *M. bovis* isolates, especially those with a single IS*6110* element. The testing of many additional strains will be required for the complete evaluation of the technique for epidemiologic investigations. In our experience, analysis by spoligotyping was a useful technique for typing *M. bovis* strains from various animal sources. Using the spoligotyping method, we identified two rather homogeneous clusters of *M. bovis* strains. Our results suggest that it is unlikely that goats act as a reservoir for bovine tuberculosis in Spain, but this possibility cannot be ruled out entirely. *M. bovis* spoligotypes found in Spanish feral animals (deer and wild boars) were also found in bovine isolates, suggesting transmission between these animals and cattle. This finding highlights the need to study the prevalence of disease in these reservoirs, since control of disease in these animal species may play an important part in the eradication of *M. bovis* from cattle.

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