JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1994, p. 2425–2433
0095-1137/94/$04.00+0

Vol. 32, No. 10

Use of Various Genetic Markers in Differentiation of *Mycobacterium bovis* Strains from Animals and Humans and for Studying Epidemiology of Bovine Tuberculosis

DICK VAN SOOLINGEN,[1] PETRA E. W. DE HAAS,[1] JAN HAAGSMA,[2] TONY EGER,[2] PETER W. M. HERMANS,[3] V. RITACCO,[4] A. ALITO,[5] AND JAN D. A. VAN EMBDEN[1]*

Laboratory for Bacteriology and Antimicrobial Agents, and Unit for Molecular Microbiology, National Institute of Public Health and Environmental Protection, 3720 BA Bilthoven[1]; Laboratory for Bacteriology, Central Veterinary Institute, 8200 AB Lelystad[2]; and Laboratory of Pediatrics, Sophia Children's Hospital, Erasmus University Rotterdam, 3000 DR Rotterdam,[3] The Netherlands; Laboratory for Bacteriology, CONICET, Pan American Institute for Food Protection and Zoonoses, Martinez (1640),[4] and Laboratory for Bacteriology, Institute for Bacteriology, CICV-INTA, Moron (1708),[5] Argentina

Received 25 March 1994/Returned for modification 2 June 1994/Accepted 5 July 1994

One hundred fifty-three *Mycobacterium bovis* strains from cattle, various animal species from zoos and wild parks, and humans were analyzed for three different genetic markers for use in the epidemiology of bovine tuberculosis. *M. bovis* strains isolated from cattle were found to carry a single IS*6110* element, whereas the majority of strains from other animals such as antelopes, monkeys, and seals harbored multiple IS*6110* elements, suggesting that the reservoirs in cattle and wild animals are separated. Because the single IS*6110* element in cattle strains is located at the same chromosomal position, strain differentiation by insertion sequence fingerprinting was hampered. Therefore, we investigated the usefulness of the direct repeat and polymorphic GC-rich repeat elements for strain differentiation. Both markers allowed sufficient strain discrimination for epidemiological purposes. Evidence is presented that in Argentina, most human *M. bovis* infections are due to transmission from cattle, whereas *M. bovis* infections among humans in the Netherlands are mainly contracted from animals other than cattle. Various outbreaks of *M. bovis* among animals and humans are described, including a small one which likely involved transmission from human to human.

In many countries, bovine tuberculosis is still a major infectious disease among cattle and other domesticated animals, and it causes great economic losses in agricultural areas. The causative agent, *Mycobacterium bovis*, is also found among a great variety of other animals, and transmission to humans constitutes a public health problem. The diagnosis of bovine tuberculosis is presently dependent mainly on clinical manifestation of the disease, skin testing, and culture of this slow-growing pathogen. The control of bovine tuberculosis and its dissemination to humans involves pasteurization of milk, destruction of skin test-positive animals, and systematic control of carcasses during meat inspection. In the Netherlands, this policy has led to an almost complete eradication of tuberculosis among cattle since 1958 (22). In countries where bovine tuberculosis is still endemic, the identification of sources of infection remains a major method of controlling this disease. In several countries, wild animals, like deer, badgers, bison, elk, and possums, constitute important potential reservoirs for transmission to cattle and humans. However, little is known of the extent of spillover from these natural reservoirs because of the lack of reliable tools for studying such transmission.

One of the most powerful recently developed tools for tracing the infection routes of tuberculosis is strain differentiation by DNA techniques. *M. bovis* belongs with *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and *Mycobacterium microti* to the genetically closely related group of the so-called *M. tuberculosis* complex bacteria. Collins and de Lisle (4) were the first to use restriction enzyme analysis of the genomic DNA of *M. tuberculosis* complex bacteria for strain differentiation. By improvement of the technique, *M. bovis* strains could also be differentiated (5–8, 13), but the differences in banding patterns are slight and comparison of the patterns is difficult. Recent studies have shown that *M. tuberculosis* complex bacteria harbor various polymorphic repetitive DNA sequences, which can be exploited for strain differentiation (9, 14, 15, 18, 21, 23, 25, 27, 32, 34).

The most widely used genetic marker for the epidemiology of tuberculosis is the insertion sequence (IS) element IS*6110* (21, 23, 27, 30, 33), which is usually present in multiple copies in *M. tuberculosis*. Because it has the ability to move within the genome to different locations, chromosomal restriction fragments carrying this element are highly polymorphic (33). Recent studies have shown that typing of *M. tuberculosis* strains by IS*6110* restriction fragment length polymorphism (RFLP) is an excellent epidemiological tool for studying outbreaks, nosocomial infections, human immunodeficiency virus-associated transmission, and the dissemination of multidrug-resistant strains (1, 3, 16, 17, 21, 26, 27, 33).

In contrast to *M. tuberculosis*, isolates of *M. bovis* have been found to harbor often only a single or a few copies of the IS*6110* element (8, 12, 32, 33), thus limiting the power to discriminate among different strains. Furthermore, one may expect that *M. bovis* strains harboring a single IS*6110* copy carry this element at a previously identified chromosomal locus, which is a hot spot for IS integration in *M. bovis* BCG and *M. tuberculosis* (22). This explains why Collins and coworkers found that, in New Zealand, 97% of 160 *M. bovis* strains investigated carried a single IS*6110* element and that these strains could be differentiated into only 11 different RFLP types, the vast majority belonging to two predominant types

* Corresponding author. Mailing address: National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

TABLE 1. IS*6110* fingerprint types of *M. bovis* strains from human and animal sources

Country of origin	Source	No. of strains	No. of strains of RFLP type[a]: 1A	1B	1C	1D	1E	1F	1G	1H	2A	2B	2C	2D	2E	2F	3A	3B	3C	3D	3E	3F	3G	3H	3I	4A	4C	4D	4E	5A	5B	5C	5D	5E	5F	5G	5H	6A	6B	6C	6D	20A	20B	20C
Argentina	Cattle	15	1			11	1		1		1						1[c,d]													2[c,d]								2[c,d]							
	Seals	5																																										
	Cats	2										1				1																													
	Humans	20				18						2																																
The Netherlands	Cattle	36		3[b]		11				3[b]																															19[b]			
	Humans	47	2	1	11		2	1	1				1	1	1				1	1	1	2	1	3	1		1	1	1		1	2	5[b]	2	1	1								
	Zoo	13				5[e]																							1[f]										1[g]		6[b,h]			
	Unknown	1																																									1[i]	
Saudi Arabia	Wild park	14																								5[b,j]			1[j]									7[b,j]			1[j]		
Total		153	1	5	1	56	1	2	2	4	1	3	1	1	1	1	1	1	1	1	1	2	1	3	1	5	1	1	1	2	2	1	2	5	2	1	1	7	2	19	1	1	6	1

[a] The number in the designation of the RFLP type represents the number of IS*6110*-hybridizing bands.
[b] Strains from suspected outbreaks.
[c] Types that are related and share at least three bands.
[d] Seals captured at the Argentinean Atlantic sea coast;
[e] A lion, an impala, two monkeys, and an African antelope.
[f] A monkey.
[g] A waterbuck.
[h] Two antelopes, two waterbucks, and two oryxes accommodated at four different zoos in the Netherlands.
[i] An unknown source.
[j] An oryx and different antelope species kept in a wild park in Saudi Arabia.

(8). Because of the poor IS*6110*-associated RFLP in *M. bovis*, other genetic markers are needed for practical use in the epidemiology of bovine tuberculosis.

Two other repetitive DNA elements have been used for strain differentiation of *M. tuberculosis*: (i) the polymorphic GC-rich repeat sequence (PGRS) (12, 24, 25, 30) and (ii) the direct repeat (DR) sequence (20). The nature and the driving force for PGRS-associated RFLP is presently not understood. In contrast, a recent study on the nature of the polymorphism of the DR region showed that homologous recombination between neighboring or distant DRs is likely to be involved in the DNA polymorphism of this chromosomal region of bacteria belonging to the *M. tuberculosis* complex (19).

Recently, Cousins and colleagues (12) showed that the polymorphism in the *M. bovis* genome of PGRS DNA-containing fragments allows significant improved strain differentiation compared with IS*6110* analysis. This study was undertaken to compare the usefulness of IS*6110*, DR, and PGRS for the differentiation of *M. bovis* strains from various sources.

We confirm that PGRS- and also DR-associated RFLP can be an excellent tool for the differentiation of *M. bovis* strains. However, in contrast with the data of Collins et al. (8) and Cousins et al. (12), we show that, depending on their origin, *M. bovis* strains can also be differentiated to a large extent by IS*6110* fingerprinting.

MATERIALS AND METHODS

Mycobacterial strains. The *M. bovis* strains isolated from humans in the Netherlands were obtained from Regional Health Laboratories. All *M. bovis* strains from animal sources in the Netherlands and Saudi Arabia were from the Central Veterinary Institute in the Netherlands. The *M. bovis* strains derived from human and bovine sources in Argentina were obtained from the Pan American Institute for Food Protection and Zoonosis and the Institute for Bacteriology in Argentina. The strains were identified as *M. bovis* by the following bacteriological criteria: growth stimulation by pyruvate, growth inhibition by thiophen, negative for nitrate reductase and pyrazinamidase activity, and smooth colony morphology.

DNA techniques. Genomic DNA extraction and Southern blotting were performed as described previously (28, 31). DNA fragments for Southern blot hybridization were labeled by using the enhanced chemiluminescence gene detection system (Amersham International plc, Amersham, United Kingdom).

The IS*6110* probe used in this study was a 245-bp DNA fragment amplified by PCR as described previously (33). The DR probe consisted of a 36-mer oligonucleotide (20). Plasmid pTBN12, containing the PGRS sequence (24), was a kind gift of B. Ross (Fairfield Infectious Disease Hospital, Fairfield, Australia) and was used in its entirety for hybridization experiments (30).

Computer analysis of DNA banding patterns was done with the BioImage Whole Band Analyzer, version 3.1 (Millipore Corporation, Ann Arbor, Mich.). Molecular weights were calculated by superimposing the autoradiograms obtained with the mycobacterial DNA probe and the autoradiogram of the internal markers of known size, as described before (28, 29). Patterns were compared by using a deviation value of 3%. The similarity value (S_{AB}) was calculated as described before (33).

RESULTS

DNA fingerprinting of *M. bovis* isolates with IS*6110* as a genetic marker. One hundred fifty-three *M. bovis* strains were subjected to Southern blot analysis with labeled IS*6110* DNA

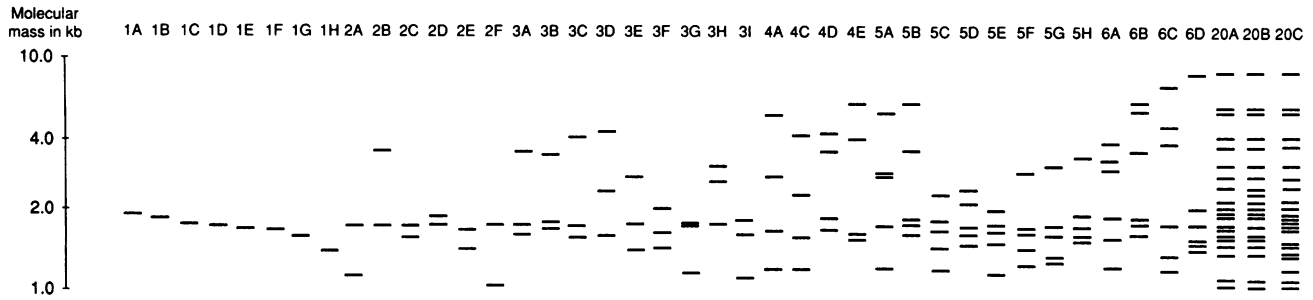

FIG. 1. Schematic representation of IS*6110* DNA fingerprints of all 153 *M. bovis* strains analyzed in this study.

as a probe. These isolates originated from cattle and humans in the Netherlands and Argentina, various animals in Dutch zoos and a wild park in Saudi Arabia, and from diseased seals and cats in Argentina. Forty-two different IS*6110* patterns were found, and the number of IS*6110*-hybridizing bands ranged generally from one to six. Unexpectedly, a few *M. bovis* strains with 20 IS copies were found (Table 1 and Fig. 1). Strains containing a single copy of IS*6110* were predominant, and in 56 of these 72 "single banders," the insertion sequence was located on a 1.9-kb *Pvu*II fragment (type 1D; see Table 1). The second most predominant RFLP type comprised 19 isolates containing six IS copies (type 6C). As will be discussed below, these latter strains originated from a single outbreak. The majority of the strains from cattle harbored a single IS*6110* element, whereas the vast majority (29 of 34) of the *M. bovis* isolates from a wide variety of other animals contained multiple IS*6110*-hybridizing *Pvu*II fragments. These data suggest that the presence of a single IS*6110* copy in *M. bovis* is characteristic for cattle strains.

Strains from cattle. *M. bovis* strains from cattle originating from the Netherlands and Argentina predominantly contained a single IS*6110* copy (31 of 51 strains); however, if the 19 outbreak strains containing six copies of IS*6110* are excluded, 31 of the 32 remaining *M. bovis* strains belong to a single band type. Irrespective of whether the country of origin was Argentina or the Netherlands, the vast majority (22 of 31 strains) contained IS*6110* DNA on a 1.9-kb *Pvu*II restriction fragment (type 1D, Table 1), whereas the fragment sizes of the other strains differed only slightly from the sizes of the predominant type (Fig. 1). The remaining isolate contained two IS copies (type 2A; see Table 1).

The largest cluster of strains with a particular, multibanded pattern comprised 19 *M. bovis* strains of RFLP type 6C (see Table 1), isolated in 1992, when an outbreak of tuberculosis was observed among cattle housed at three farms in the Netherlands. Intensive investigations indicated that the suspected common source of infection was a young, diseased bull which was imported in 1991 from Austria and which was accommodated at all three farms implicated in the unusual outbreak. Isolates from nine cows from the three farms exhibited an identical DNA pattern, composed of six IS*6110*-containing *Pvu*II restriction fragments (type 6C, see Table 1), and this pattern was identical to those of two *M. bovis* isolates from the diseased bull, confirming that this animal indeed was the source of infection among the various cows.

Strains from humans. Eighteen of 20 human strains isolated in Argentina contained a single IS*6110* copy and belonged to type 1D, the predominant type among cattle isolates (Table 1). In contrast, the *M. bovis* IS*6110* fingerprints of strains from humans in the Netherlands were very diverse. Twenty-nine strains contained two or more IS*6110* copies, and these displayed 20 different RFLP patterns. None of these patterns was found among *M. bovis* strains of animal origin.

Surprisingly, five human isolates from the Netherlands showed identical multiband patterns (type 5E; Table 1). These strains were isolated by a public health laboratory in the region of Amsterdam, and three strains were isolated from members of a single family, whereas one strain was isolated from an individual living in the same apartment building. The remaining strain originated from a patient from the same province, but no epidemiological relationship could be established. Apparently, at least four strains of this cluster were from a small *M. bovis* outbreak among humans, although these individuals had no known frequent contact with domesticated or other animals.

Strains from various animal species. We investigated 13 *M. bovis* isolates from four different zoos in the Netherlands. The isolates were from a lion, impala, oryx, antelopes, waterbucks, and primates. Only four RFLP patterns were found among these 13 strains (Fig. 1 and Table 1). Six strains were of pattern 20B, with 20 IS*6110* bands. These six strains were isolated during the period from 1987 to 1988 from various hoofed animals (waterbuck, oryx, and antelope) in a single zoo, suggesting that this particular *M. bovis* strain caused an outbreak. The other most prevalent type of *M. bovis* strain, found among animals in four different zoos, was the cattle fingerprint type 1D, with a single 1.9-kb IS*6110* *Pvu*II fragment (Table 1). The remaining two zoo strains were of RFLP patterns 5A and 6D; the latter was a unique type, whereas type 5A was also found once among isolates from animals in a wild park in Saudi Arabia (discussed below).

Furthermore, we investigated 14 *M. bovis* strains isolated from various antelope species from two wild parks in Saudi Arabia. Most of the oryxes were suspected to have been infected during their transport in a container from one wild park to another in Saudi Arabia by contact with a diseased oryx. Table 1 and Fig. 2 show that DNA patterns 4A and 6A were predominant among the isolates from these animals, including the oryx. These data suggest that indeed the suspected transmission from the oryx to other animals might have taken place during transport.

One strain isolated from an oryx was unusual in that it harbored 20 IS copies (type 20A). As mentioned previously, strains with such high numbers of IS*6110* copies are exceptional among *M. bovis*. Therefore, we compared the IS*6110* pattern of this oryx strain with the IS*6110* patterns of two other *M. bovis* strains that also carry an exceptionally high number of IS*6110* copies. One of these strains was isolated in a zoo in the Netherlands, and the other was a laboratory strain isolated at least 20 years ago and used in an international study. Figure 3

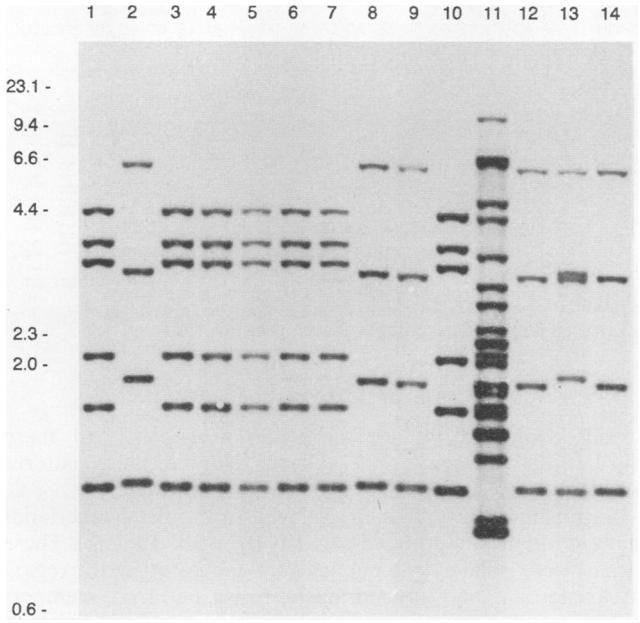

FIG. 2. IS*6110* DNA fingerprints of *M. bovis* isolates from animals of different oryx and gazelle species kept in a wild park in Saudi Arabia. Numbers at left indicate sizes of standard DNA fragments (in kilobase pairs).

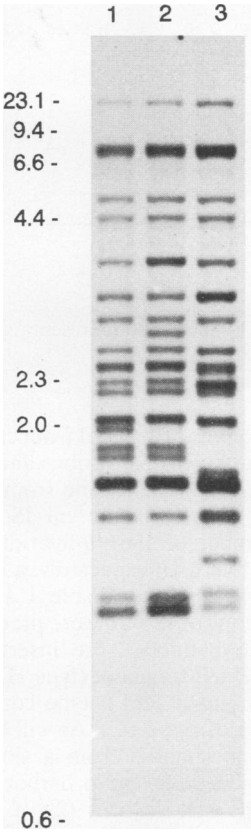

FIG. 3. IS*6110* DNA fingerprints of three *M. bovis* strains containing 18 to 20 copies of the element. Isolate sources: lane 1, oryx in a wild park in Saudi Arabia; lane 2, oryx in a zoo in the Netherlands; lane 3, *M. bovis* isolate of unknown source used in an international study at least 20 years ago. Numbers at left indicate sizes of standard DNA fragments (in kilobase pairs).

shows that all three strains share the majority of their IS*6110* *Pvu*II fragments, indicating that the strains constitute a group of genetically closely related *M. bovis* strains carrying about 20 IS*6110* copies.

Five *M. bovis* strains were isolated between July and December 1992 from four diseased seals captured at the Argentinean Atlantic sea coast. These isolates contained three, five, or six IS*6110* copies, and the RFLP types were not shared by strains from any of the other sources. Remarkably, all five of the IS*6110* banding patterns (types 3A, 5B, and 6B) were highly related (see Table 1 and Fig. 4), suggesting relatively recent acquisition of a particular strain.

Two *M. bovis* isolates were from urban domestic cats from the city of Buenos Aires. The IS fingerprints were distinct, and both consisted of two bands (types 2B and 2F; Table 1 and Fig. 1). RFLP type 2F was unique, whereas type 2B was found twice among human isolates from Argentina. These data are suggestive of transmission between cats and humans.

Differentiation of *M. bovis* strains containing a single IS*6110* copy by using PGRS and DR DNA. Because a large proportion of the *M. bovis* strains carried a single IS*6110* element on a similarly sized *Pvu*II fragment, we tried to further differentiate such strains by using the additional genetic markers PGRS and DR. For this purpose, we selected 12 human and 2 cattle *M. bovis* strains, of which 13 contained a single IS*6110* copy, and in 10 of these single-copy strains, the element was located on a 1.9-kb fragment (predominant type 1D). All strains were isolated in the Netherlands. We used labeled PGRS and DR DNA to probe *Alu*I-digested genomic DNA from these 14 strains, and the results are shown in Fig. 5. The three strains with a unique IS*6110* pattern also displayed a unique PGRS and DR banding pattern (Fig. 5, lanes 7, 12, and 13). The remaining 10 single-copy strains (IS*6110* pattern 1D) were differentiated into seven PGRS banding patterns and five DR banding patterns. Two clusters of strains with identical PGRS banding patterns were found, comprising three strains (Fig. 5B, lanes 1 to 3, cluster 1) and two strains (Fig. 5B, lanes 5 and 10, cluster 2), respectively. The first three strains were also identical in DR typing (Fig. 5C, lanes 1 to 3), but two more strains showed the same DR pattern (Fig. 5C, lanes 6 and 9). Another pair of strains had identical DR patterns (Fig. 5C, lanes 5 and 10). Although we have no direct evidence for the epidemiological relatedness of the isolates in cluster 1, identical by all three typing methods, a closer inspection of the sources of the strains revealed that transmission by a common source was not unlikely, because two strains were isolated from cattle and one from a human source in a small farming region in the southern part of the Netherlands.

In addition, we typed a human isolate containing two IS*6110* copies, one of which was located on a 1.9-kb *Pvu*II fragment. Both the PGRS and DR banding patterns were different from those of the other 13 strains investigated (Fig. 5A, B, and C, lanes 14).

Twenty-six additional *M. bovis* strains, all of IS*6110* RFLP type 1D, were investigated by PGRS fingerprinting. Nine isolates were from cattle from Dutch farms, and these strains clustered into three different PGRS types, three strains of each type (data not shown). All animal strains within a cluster originated from the same farm. Thus, clustering was likely to be associated with transmission. The remaining 17 strains were from Argentina; 11 were from humans, and 6 were from cattle. Twelve different PGRS patterns were obtained (Fig. 6). Five

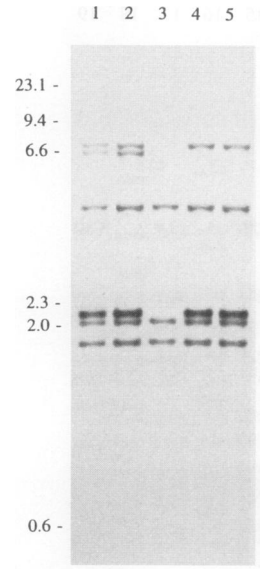

FIG. 4. IS*6110* DNA fingerprints of five *M. bovis* isolates from four diseased seals captured at the Atlantic coast in Argentina. The strains in lanes 1 and 2 were isolated from a single seal. Numbers at left indicate sizes of standard DNA fragments (in kilobase pairs).

strains displayed an identical PGRS pattern, three isolated from bovine and two from human sources (Fig. 6, lanes 7, 8, 13, and 14). Among the human as well as among the bovine isolates, two other clusters of two strains with identical PGRS patterns were found (Fig. 6, lanes 4 and 6 and lanes 15 and 17, respectively).

Computer analysis of the PGRS fingerprints of type 1D strains showed that none of the 10 *M. bovis* isolates from the Netherlands was identical to any of the 17 strains from Argentina. The extent of relatedness of the PGRS banding patterns between the Dutch and Argentinean *M. bovis* strains having IS*6110* pattern 1D is depicted in a dendrogram (Fig. 7).

Previously, we showed that *M. tuberculosis* strains from India harboring a single IS*6110* element on a similarly sized restriction fragment could be differentiated by DR and PGRS fingerprinting (30). For comparison, we included these Indian strains in the dendrogram. The strains are divided into three main clusters, one containing only Indian strains and two containing either Argentinean or Dutch *M. bovis* strains. Therefore, it seems that an association exists between the PGRS RFLP patterns and the geographic origin of the isolates.

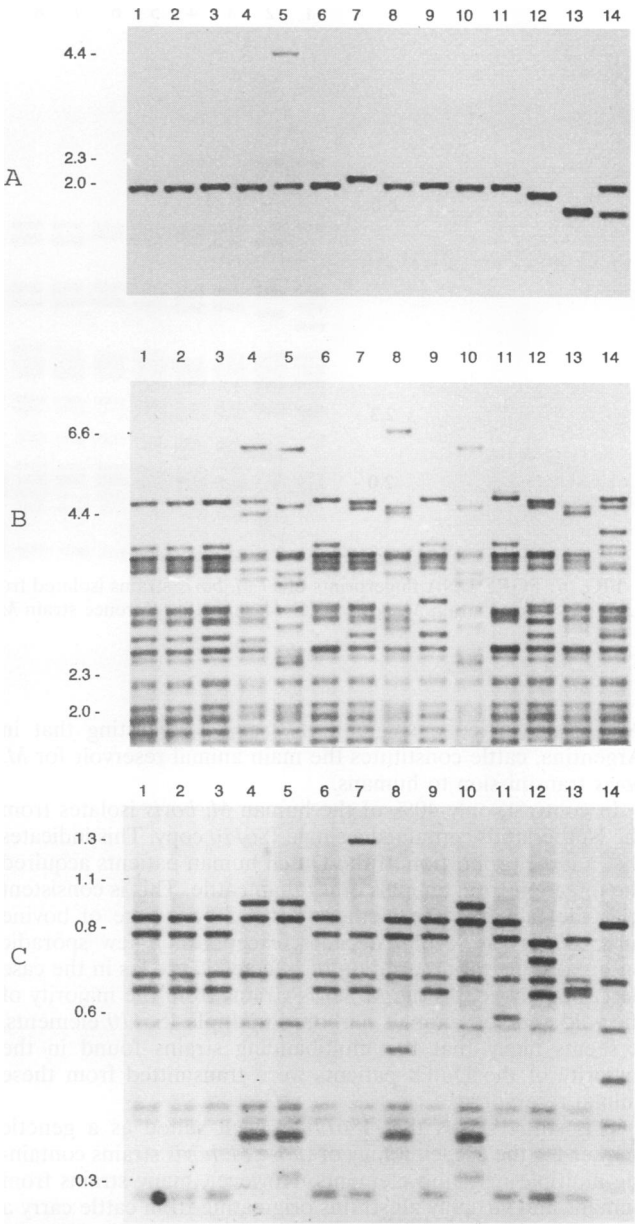

FIG. 5. RFLP analysis of *M. bovis* DNA with three different probes: (A) IS*6110*, (B) PGRS, and (C) DR. Panel A contains fingerprints from *Pvu*II-digested DNAs, while those in panels B and C are from *Alu*I-digested DNAs. Lanes 1 and 2 contain DNA from isolates of cattle; other isolates were of human origin. Two clusters with identical IS*6110*/PGRS/DR patterns are in lanes 1 to 3 and in lanes 5 and 10, respectively. Numbers at left indicate sizes of standard DNA fragments (in kilobase pairs).

DISCUSSION

Previous studies have shown that the vast majority of *M. bovis* strains contain a single IS*6110* element, often at the same chromosomal locus, thus hampering strain differentiation with IS*6110* as a probe (12, 20, 32). Although the animal species from which the *M. bovis* strains originated were not specified in the largest study (8, 12), it is likely that the majority were strains isolated from cattle, as they were of veterinary origin.

Our results on IS*6110* typing of *M. bovis* strains differ from previous results in that we encountered a larger proportion of strains carrying multiple IS*6110* copies. Multiple-banded strains originated mainly from animals other than cattle in zoos and wild parks and from Dutch human patients. This suggests that the various animal reservoirs of *M. bovis* may contain different *M. bovis* types, depending on the host. In this study, virtually all cattle strains from Argentina and the Netherlands, except 19 from a single large outbreak in the Netherlands and a two-copy strain from Argentina, were found to contain a single IS*6110* element. These results indicate that the presence of a single IS*6110* element is very characteristic of *M. bovis* in cattle.

Furthermore, 90% of the human *M. bovis* isolates from

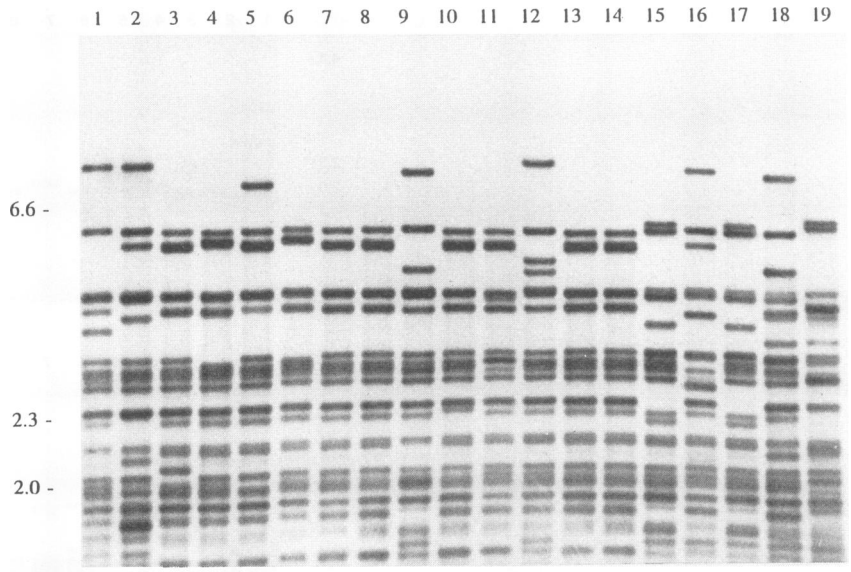

FIG. 6. PGRS DNA fingerprints of 17 *M. bovis* strains isolated from human (lanes 1 to 11) and bovine (lanes 12 to 17) sources in Argentina. Lane 18, vaccine strain *M. bovis* BCG 44; lane 19, reference strain *M. tuberculosis* Mt 14323.

Argentina carried a single IS*6110* copy, suggesting that in Argentina, cattle constitutes the main animal reservoir for *M. bovis* transmission to humans.

In contrast, only 40% of the human *M. bovis* isolates from the Netherlands contained a single IS*6110* copy. This indicates that a large proportion of the Dutch human patients acquired the infection from animals other than cattle. This is consistent with the fact that the Netherlands has been free of bovine tuberculosis for several decades, except for a few sporadic outbreaks due to importation of infected cattle, as in the case described above. As the *M. bovis* strains from the majority of the wild and zoo animals harbored multiple IS*6110* elements, it seems likely that the multibanding strains found in the majority of the Dutch patients were transmitted from these animal reservoirs.

This study shows that IS*6110* is well suited as a genetic marker for the epidemiology of those *M. bovis* strains containing multiple insertion elements. However, many strains from humans and virtually all strains originating from cattle carry a single IS*6110* element and cannot be differentiated satisfactorily by IS*6110* fingerprinting. Because of the poor power to differentiate *M. bovis* strains carrying a single IS*6110* copy by IS typing, we tested the polymorphic repetitive sequences DR and PGRS for better strain discrimination. The chromosomal DR cluster in *M. bovis* BCG contains 49 DNA direct repeats of 36 bp, interspersed by unique spacer sequences of 35 to 41 bp (20). Groenen et al. (22) recently showed that the polymorphism in the DR region among *M. tuberculosis* isolates is likely caused by homologous recombination between neighboring or distant DRs, leading to varying DR numbers. Because IS*6110* is located within the DR region, the IS*6110*-containing restriction fragment in single-IS-copy-containing *M. bovis* strains also carries DR sequences. Therefore, the length polymorphism of such fragments observed in this study and that of Collins et al. (8) is likely caused by variation in the number of DRs flanking the IS*6110* DNA.

The PGRS repeats consist of multiple clusters of short, nonidentical 24-bp repeats, separated by varying sequences 1 to 30 bp in length (24). The genetic mechanism leading to PGRS-associated polymorphism is presently unknown. This study shows that both the DR- and PGRS-associated DNA polymorphism allows the differentiation of *M. bovis* strains which cannot be distinguished by IS*6110* because of its presence at a unique chromosomal location.

Both sequences seem to be suited for this purpose, although the PGRS probe allowed a somewhat better differentiation than DR. Cousins et al. (12) recently subjected 26 *M. bovis* strains from human, cattle, and six wild animals to PGRS typing, and 20 different PGRS patterns were found. This level of discrimination is comparable to that observed in our study. Although no data about the stability of the PGRS banding patterns are available, population-based studies of *M. tuberculosis* in Australia suggest that the extent of polymorphism associated with PGRS DNA is similar to that of IS*6110* in multi-IS*6110*-copy-containing strains (15).

While we did not have direct evidence on epidemiological relationships between single-IS-banded *M. bovis* strains, the finding that strains within a small cluster of strains with identical PGRS banding patterns were isolated from a very restricted geographic area in the Netherlands is highly suggestive of such a relationship. The time of isolation of the strains in this cluster varied over 5 years, reinforcing the idea that the PGRS RFLP types are rather stable.

Various previously suspected *M. bovis* outbreaks among humans and cattle were confirmed by DNA typing. In addition, a few unsuspected epidemiological relationships were disclosed, indicating that DNA typing is a powerful tool with which to study the epidemiology of bovine tuberculosis.

In 1990, Cousins et al. (10) isolated *M. bovis* for the first time from seals and sea lions in New Zealand and Australia, and this group showed transmission from a captive seal to a seal trainer by showing an identical PGRS banding pattern with *M. bovis* isolated from a seal. The seal and sea lion strains harbored a single IS*6110* copy (10). This is in contrast to the five seal isolates from Argentina analyzed in this study. Interestingly, the IS*6110* banding patterns of the latter five strains had significant similarity, indicating infection with a genetically

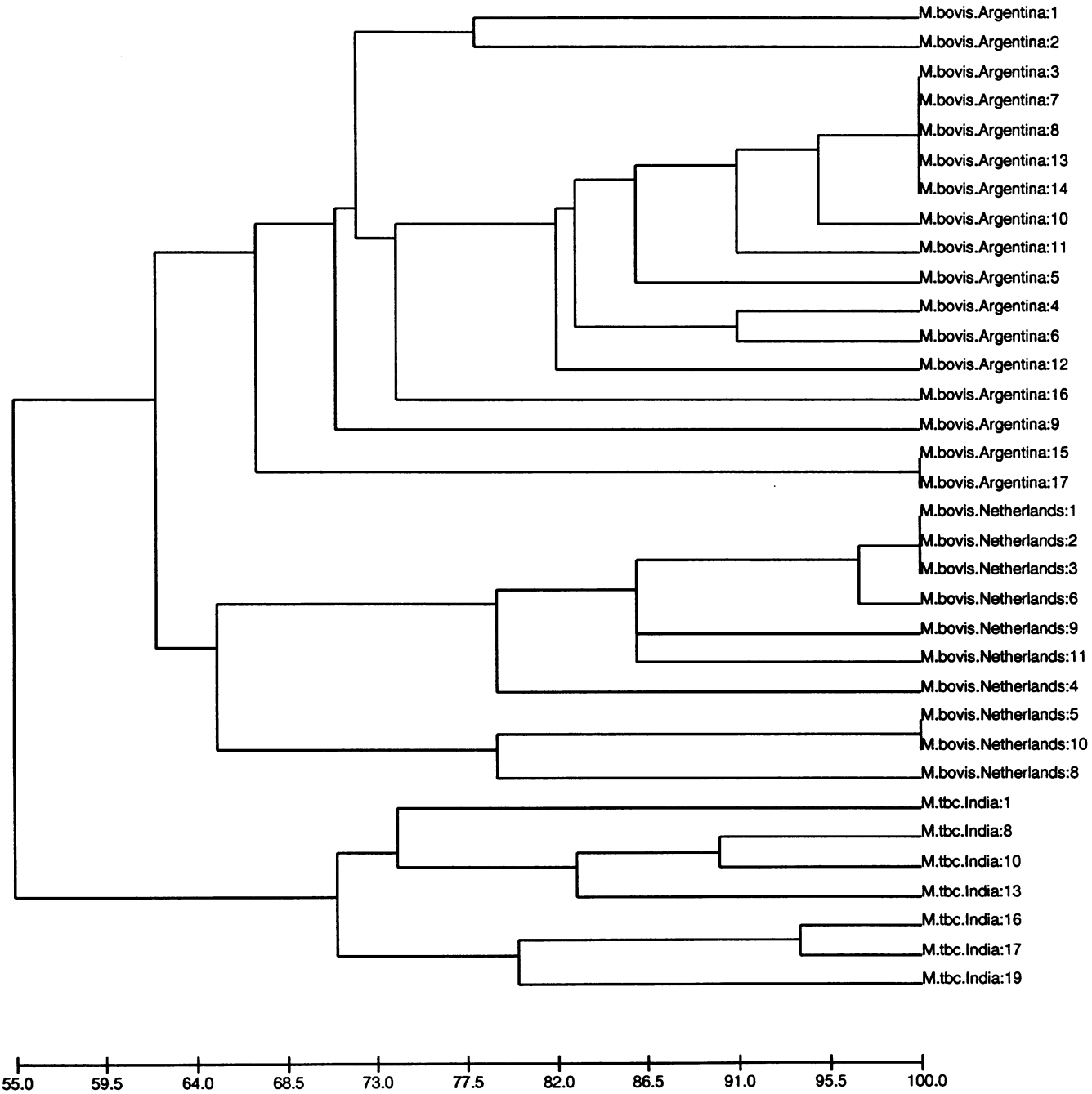

FIG. 7. Dendrogram based on computer-assisted comparison of PGRS DNA fingerprints of *M. bovis* strains isolated in Argentina and the Netherlands and *M. tuberculosis* strains isolated in India. All *M. bovis* strains invariantly contained a single IS*6110* band of 1.9 kb, and all *M. tuberculosis* strains carried a single IS*6110* element on a restriction fragment of 1.5 kb. Dutch strains correspond to those in Fig. 5B, Argentinian strains correspond to those in Fig. 6, and Indian strains correspond to those in Fig. 3D of reference 30.

rather homogeneous group of *M. bovis*. The genetic relationship between mycobacterial seal isolates from Australia (10, 11) and Argentina is yet to be examined.

A previous study on the DNA polymorphism of IS*6110*-containing restriction fragments of *M. tuberculosis* strains isolated from central Africa suggested an association between IS*6110* fingerprint patterns and geographic origin (33). The computer analysis on the relatedness of PGRS fingerprints of *M. bovis* strains containing a single IS*6110* copy demonstrates that *M. bovis* isolates from Argentina and the Netherlands belong to genetically distinct groups and that grouping was according to geographic origin. This indicates that within these countries, a particular group of genetically related strains is endemic for an extended time, probably many decades or centuries, long enough for diversification of clones, which may have been disseminated intercontinentally in ancient times.

ACKNOWLEDGMENTS

The technical assistance of K. Kremer, J. E. M. Pijnenburg, T. van der Laan, and Li Qing is gratefully acknowledged. We thank E. H. Rozendal for photographic reproduction.

This study was financially supported by the World Health Organization Programme for Vaccine Development and the European Community Program for Science Technology and Development.

REFERENCES

1. **Beck-Sagué, C., S. W. Dooley, M. D. Hutton, J. Otten, A. Breeden, J. T. Crawford, A. E. Pichenik, C. Woodley, G. Cauthen, and W. R. Jarvis.** 1992. Hospital outbreak of multidrug resistant *Mycobacterium tuberculosis* infections. JAMA **268:**1280–1286.
2. **Brian, R. E., J. I. Tokars, M. H. Grieco, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, W. R. Jarvis, and S. D. Holmberg.** 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. **23:**1514–1521.
3. **Cave, M. D., K. D. Eisenach, P. F. McDermott, J. H. Bates, and J. T. Crawford.** 1991. IS*6110*: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Mol. Cell. Probes **5:**73–80.
4. **Collins, D. M., and G. W. de Lisle.** 1984. DNA restriction endonuclease analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. J. Gen. Microbiol. **130:**1019–1021.
5. **Collins, D. M., and G. W. de Lisle.** 1985. DNA restriction endonuclease analysis of *Mycobacterium bovis* and other members of the tuberculosis complex. J. Clin. Microbiol. **21:**562–564.
6. **Collins, D. M., and G. W. de Lisle.** 1987. BCG identification by DNA restriction fragment patterns. J. Gen. Microbiol. **133:**1431–1434.
7. **Collins, D. M., G. W. de Lisle, and D. M. Gabric.** 1986. Geographic distribution of restriction types of *Mycobacterium bovis* isolates from brush-tailed possums (Trichosurus vulpecula) in New Zealand. J. Hyg. **96:**431–438.
8. **Collins, D. M., S. K. Erasmuson, D. M. Stephens, G. F. Yates, and G. W. de Lisle.** 1993. DNA fingerprinting of *Mycobacterium bovis* strains by restriction fragment analysis and hybridization with insertion elements IS*1081* and IS*6110*. J. Clin. Microbiol. **31:**1143–1147.
9. **Collins, D. M., and D. M. Stephens.** 1991. Identification of insertion sequence, IS*1081*, in *Mycobacterium bovis*. FEMS Lett. **83:**11–16.
10. **Cousins, D. V., B. R. Francis, B. L. Gow, D. M. Collins, C. H. McGlashan, A. Gregory, and R. M. MacKenzie.** 1990. Tuberculosis in captive seals: bacteriological studies on an isolate belonging to the *Mycobacterium tuberculosis* complex. Res. Vet. Sci. **48:**196–200.
11. **Cousins, D. V., S. N. Williams, R. Reuter, D. Forshaw, B. Chadwick, D. Coughran, P. Collins, and N. Gales.** 1993. Tuberculosis in wild seals and characterisation of the seal bacillus. Aust. Vet. J. **70:**92–97.
12. **Cousins, D. V., S. N. Williams, B. C. Ross, and T. M. Ellis.** 1993. Use of a repetitive element isolated from *Mycobacterium tuberculosis* in hybridization studies with *Mycobacterium bovis*: a new tool for epidemiological studies of bovine tuberculosis. Vet. Microbiol. **37:**1–17.
13. **de Lisle, G. W., D. M. Collins, A. S. Loveday, W. A. Young, and A. F. Julian.** 1990. A report of tuberculosis in cats in New Zealand, and the examination of strains of *Mycobacterium bovis* by restriction endonuclease analysis. N. Z. Vet. J. **38:**10–13.
14. **Doran, T. J., A. L. M. Hodgson, J. K. Davies, and A. J. Radford.** 1992. Characterisation of a novel repetitive DNA sequence from *Mycobacterium bovis*. FEMS Lett. **96:**179–186.
15. **Dwyer, B., K. Jackson, K. Raios, A. Sievers, E. Wilshire, and B. Ross.** 1993. DNA restriction analysis to define an extended cluster of tuberculosis in homeless men and their associates. J. Infect. Dis. **167:**490–494.
16. **Edlin, B. R., J. I. Tokars, M. H. Grieco, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, W. R. Jarvis, and S. D. Holmberg.** 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. **326:**1514–1521.
17. **Fischl, M. A., R. B. Uttamchandani, G. L. Daikos, et al.** 1992. An outbreak of tuberculosis caused by multiple-drug resistant tubercle bacilli among patients with HIV infection. Ann. Intern. Med. **117:**177–183.
18. **Fomukong, N. G., J. W. Dale, T. W. Osborn, and J. M. Grange.** 1992. Use of gene probes based on the insertion sequence IS*986* to differentiate between BCG vaccine strains. J. Appl. Bacteriol. **72:**126–133.
19. **Groenen, P. M. A., A. E. Bunschoten, D. van Soolingen, and J. D. A. van Embden.** 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*: application for strain differentiation by a novel typing method. Mol. Microbiol. **10:**1057–1065.
20. **Hermans, P. W. M., D. van Soolingen, E. M. Bik, P. E. W. de Haas, J. W. Dale, and J. D. A. van Embden.** 1991. The insertion element IS*987* from *Mycobacterium bovis* BCG is located in a hot spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infect. Immun. **59:**2695–2705.
21. **Hermans, P. W. M., D. van Soolingen, J. W. Dale, A. R. Schuitema, R. A. McAdam, D. Catty, and J. D. A. van Embden.** 1990. Insertion element IS*986* from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. J. Clin. Microbiol. **28:**2051–2058.
22. **Huitema, H.** 1988. Tuberculosis in animals and man with the attention to reciprocal transmission of mycobacterial infections and the successful eridication of bovine tuberculosis in cattle in the Netherlands. Royal Netherlands Tuberculosis Association, The Hague.
23. **McAdam, R. A., P. W. M. Hermans, D. van Soolingen, Z. F. Zainuddin, D. Catty, J. D. A. van Embden, and J. W. Dale.** 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS*3* family. Mol. Microbiol. **4:**1607–1613.
24. **Ross, B. C., K. Raios, K. Jackson, and B. Dwyer.** 1992. Molecular cloning of a highly repeated element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. J. Clin. Microbiol. **30:**942–946.
25. **Ross, B. C., K. Raios, K. Jackson, A. Sievers, and B. Dwyer.** 1991. Differentiation of *Mycobacterium tuberculosis* strains by use of a nonradioactive Southern blot hybridization method. J. Infect. Dis. **163:**904–907.
26. **Small, P. M., and J. D. A. van Embden.** 1994. Molecular epidemiology of tuberculosis. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
27. **Thierry, D., A. Brisson-Noël, V. Vincent-Lévy-Frébault, S. Nguyen, J. Guesdon, and B. Gicquel.** 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. J. Clin. Microbiol. **28:**2668–2673.
28. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. W. M. Hermans, C. Martin, R. Mcadam, T. M. Shinnick, and P. M. Small.** 1992. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
29. **van Embden, J. D. A., D. van Soolingen, P. M. Small, and P. W. M. Hermans.** 1992. Genetic markers for the epidemiology of tuberculosis. Res. Microbiol. **143:**385–391.
30. **van Soolingen, D., P. E. W. de Haas, P. W. M. Hermans, P. M. A. Groenen, and J. D. A. van Embden.** 1993. Comparison of various repetitive elements as genetic markers for srain differentiation and epidemiology of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **31:**1987–1995.
31. **van Soolingen, D., P. E. W. de Haas, P. W. M. Hermans, and J. D. A. van Embden.** 1993. DNA fingerprinting of *Mycobacterium tuberculosis*. Methods Enzymol. **235:**196–205.
32. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, and J. D. A. van Embden.** 1992. Insertion element IS*1081*-associated restriction fragment length polymorphism in *Mycobacterium tuber-*

culosis complex species: a reliable tool for recognizing *Mycobacterium bovis* BCG. J. Clin. Microbiol. **30:**1772–1777.

33. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
34. **Zainuddin, Z. F., and J. W. Dale.** 1989. Polymorphic repetitive DNA sequences in *Mycobacterium tuberculosis* detected with a gene probe from a *Mycobacterium fortuitum* plasmid. J. Gen. Microbiol. **135:**2347–2355.