Different Strategies for Molecular Differentiation of
Mycobacterium bovis Strains Isolated
in Sardinia, Italy

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Different genetic markers were used to analyze 22 Mycobacterium bovis strains isolated from cattle in Sar-
dinia and one human isolate. IS6110 DNA fingerprinting differentiated the strains into six patterns, whereas
with enterobacterial repetitive consensus sequence primers produced seven clusters. PCR ribotyping followed
by digestion with HaeIII and PvuII produced five and seven patterns, respectively. PCR with the (GTG)5 oligo-
nucleotide primer showed the best discriminatory power, generating eight clusters among the strains analyzed.

Bovine tuberculosis remains a major infectious disease among cattle worldwide, with a particularly high incidence in devel-
oping countries, causing great economic losses (1, 4, 5, 10, 20). In Sardinia (Italy), after an eradication program was launched in the late 1950s, the number of cases of the disease progressively decreased until 4 years ago, when an increase in bovine tuberculosis was noticed. This rise was probably due to impor-
tation of infected animals. In recent years, Mycobacterium bovis has been isolated in cases of human tuberculosis, mostly of no-
osomial origin and in immunocompromised patients (3, 7, 17).

M. bovis belongs to the homogeneous Mycobacterium tuberculosis complex group, and identification of these microorganisms was possible on the basis of the use of amino acids, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mycobacterial proteins, and determination of phage susceptibility (2, 4). Re-
cently, new DNA techniques were developed in order to iden-
tify and differentiate these bacteria (1, 6, 8, 9, 12, 21). The identification of different insertion elements, such as IS1081 and IS6110, specific for the M. tuberculosis complex, led to the application of these sequences in Southern blot experiments (4). Unfortunately, IS6110 is present in only one or a few copies within the genome of M. bovis. In strains that carry one copy of IS6110, it is usually found at one chromosomal loca-
tion, a “hot spot” between a cluster of direct repeats (4, 19). Other repetitive DNA elements that have been used for strain differen-
tiation are the polymorphic GC-rich repeat sequence (5, 12, 13) and the direct repeat sequences (5, 6, 12, 13), and the spo-
ligotyping method has recently been described (1, 2). The polymorphism generated from the polymorphic GC-rich repeat sequence probe allowed significant improvement of strain differentiation compared with IS6110 analysis (1, 7, 8). Previously, we have reported the usefulness of other genetic
markers, such as enterobacterial repetitive consensus sequences (ERIC) and the (GTG)5 oligonucleotide, in PCR ex-
periments to differentiate M. tuberculosis strains (14, 15). Here, we evaluated the use of these methods to differentiate M. bovis

strains and compared the results with those of IS6110 DNA fingerprinting analysis.

We analyzed 129 specimens from different cattle suspected of having bovine tuberculosis in different parts of Sardinia. Twenty-three strains of M. bovis were isolated from different specimens (Table 1). The samples were collected from June 1996 to September 1998. Sixteen were from three different herds and one human isolate in Cagliari (South Sardinia), 13 were collected from 13 different animals of herd 621, 1 was isolated from herd 692 in Cagliari, 1 was isolated from herd 1322 in Cagliari, and the last strain from South Sardinia was isolated from a patient in the Cagliari Hospital. Five other strains were collected from two different herds in Nuoro (East Sardinia). Two strains were isolated from two different herds in Oristano (West Sardinia). M. bovis ATCC 27290 and M. tuberculosis H37Rv, purchased from the American Type Culture Collection, were used as standard strains. All of the strains used in this study were identified as M. bovis by bio-
chemical methods, the niacin test, susceptibility to thiophene carboxyl hydradize (Sigma Chemical Co.), and a species-
specific PCR described by Rodriguez et al. (11).

Mycobacterial strains were grown in 10 ml of 7H9 medium supplemented with oleic acid at 0.6 g/liter, bovine albumin at 50 g/liter, dextrose at 20 g/liter, and catalase at 0.03 g/liter, and genomic DNA was extracted and analyzed as described previously (14). For IS6110 fingerprinting, the DNA was cut with restriction endonuclease PvuII, subjected to electrophoresis in 0.8% agarose gel, blotted on nylon membranes, and hybridized with plasmid pBK831, containing the 0.45-kb BamHI-SpiI frag-
ment of IS6110 (14), previously labelled with the enhanced-
chemiluminescence gene labelling kit (Amersham Interna-
tional, Amersham, United Kingdom).

PCR was performed by using primers ERIC1R (5′-ATGT AAAGTC CCGGGT ACCATCAC) and ERIC2 (5′-AAATAGT GACTGGGGT GAGGC), each at a concentration of 1.0 μM as previously described (15). Amplification reactions were per-
fomed in a 50-μl final volume containing 1 U of Taq poly-
merase, 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 200 μM each deoxynucleoside triphosphate (Gibco BRL Life Technology, Paisley, United Kingdom). Reaction mixtures were overlaid with 1 drop of paraffin oil and then incubated for

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2 min at 94°C, followed by 35 cycles of 94°C for 45 s, 52°C for 1 min, and 70°C for 10 min and a final extension at 70°C for 20 min as previously described (15). The amplification products were visualized by electrophoresis at 90 V for 90 min in 1.8% Methaphor agarose gel (FMC Bioproducts, Rockland, Maine) and staining of the gel with ethidium bromide.

PCR with the (GTG)₅ oligonucleotide was performed as previously described (14). Briefly, after the lysis of mycobacteria, primers IS₂A and GTG₁ were used to amplify chromosomal DNA. Amplification products were then visualized after electrophoresis on agarose gel.

PCR ribotyping was performed by using two primers complementary to conserved regions near the 3’ end of the 16S RNA and the 5’ end of the 23S RNA of rnr as previously reported (15). The sequences of the primers are as follows: R₁, 5’-TTGTACACACGGCCGGTCA; R₂, 5’-GAAACATCTAATCCT. Amplifications were carried out in a final volume of 25 µl. Thirty cycles of amplification were performed, and each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min at 72°C. The last cycle consisted of a 10-min extension at 72°C. The amplification products were digested with endonucleases HaeIII and PvuII (Amersham International), and the digests were visualized by 2% agarose gel electrophoresis and staining with ethidium bromide.

All DNA amplifications were performed in a Hybaid TR3CM220 (Omnigene) DNA thermal cycler. The restriction fragment length polymorphisms obtained by PCRs with ERIC and the (GTG)₅ oligonucleotide and IS₆₁₁₀ fingerprinting were scanned with the Screen Machine II software (Fast Multimedia AG, Munich, Germany) and evaluated with the Image Master software (Pharmacia Biotech, Uppsala, Sweden).

![FIG. 1. Southern blot of chromosomal DNAs of M. bovis strains showing the representative patterns observed after hybridization with the 0.45-kb BamHI-SalI fragment of IS₆₁₁₀ used as a probe and HinfIII-digested λ DNA. Lanes: M, HinfIII-digested λ DNA; 1, strain 3; 2, strain 30; 3, strain 92; 4, strain 94; 5, strain 95; 6, strain 0.](http://aem.asm.org/
IS6110 fingerprinting. Pattern analysis with IS6110 used as a probe against PvuII-digested mycobacterial DNAs revealed six different patterns among the 23 M. bovis strains analyzed (Table 1 and Fig. 1). The largest group of isolates (n = 11) showed a typical hybridization pattern of one band at 1.9 kb (Fig. 1, lane 1), like control strain M. bovis ATCC 27290 (data not shown), and another group of three isolates presented one band at 5.5 kb (Fig. 1, lane 6). Four isolates revealed the presence of multiple bands; one had five bands, two had six bands, and one had nine bands (Table 1 and Fig. 1). The remaining five isolates did not present hybridization bands (Fig. 1, lane 2). In particular, in the Cagliari region, we isolated M. bovis strains from three herds. In one herd, 621 (Table 1), M. bovis infection was widespread among the cattle, and we recovered 13 isolates. Seven of them revealed the presence of a 1.9-kb hybridizing band (Fig. 1, lane 1), two showed the 5.5-kb pattern band (Figure 1, lane 6), one isolate generated nine bands, and the other three strains did not show hybridization with the IS6110 probe. The second herd (1322) presented only one isolate with no hybridization band, and the isolate from herd 692 showed the 1.9-kb band pattern. In the Nuoro region, we studied two different herds. In the first (1345), we isolated three strains; two produced the same pattern of six bands (Fig. 1, lanes 3 and 5), while the third generated the same pattern without the top band (Fig. 1, lane 4). In the other herd (1513), we isolated two strains presenting the classical 1.9-kb band pattern. The remaining two herds belonged to the western region of Sardinia (Oristano). Both of them presented one M. bovis isolate. The strain isolated in herd 416 showed a 5.5-kb band, whereas the other herd (868) presented a strain with no hybridizing band.
The only strain from a patient (a woman in her 60s) was isolated in Cagliari and produced the classical 1.9-kb band (strain S1 in Table 1).

**PCR ribotyping.** Using two primers, R1 and R2, we amplified the intergenic spacer of the *M. bovis* isolates. The size of the amplified product was 520 bp in all of the strains tested except strain 94, which had a band of 540 bp (data not shown). In order to differentiate the isolates, we digested the PCR products with the endonucleases *Hae*III and *Pvu*II, since their restriction sites are well conserved among ribosomal operons. The results are shown in Fig. 2a and b for *Hae*III and *Pvu*II, respectively. We obtained five different patterns after digestion with *Hae*III, and the different profiles ranged from two to five bands, whereas after *Pvu*II digestion of PCR products, we obtained seven different patterns. Sixteen isolates, as well as *M. bovis* ATCC 27290, showed a good correlation with the two restriction patterns, while discrepancies were observed in seven strains.

**PCR with ERIC primers.** Using the ERIC primers, we amplified the DNAs of the 23 *M. bovis* isolates studied. After visualization of the PCR products, we identified seven different patterns among the 23 strains. The number of bands in the different profiles ranged from 2 to 10, and their molecular sizes ranged from 150 bp to more than 1,200 bp (Table 1 and Fig. 2c). Different profiles ranged from 2 to 10, and their molecular sizes ranged from three to eight, and their molecular masses ranged from 150 bp to more than 1,200 bp (Table 1 and Fig. 2c).

**PCR with the (GTG)₅ oligonucleotide.** In this work, in order to differentiate *M. bovis* strains, we applied the method that we previously proposed for typing of *M. tuberculosis* (14). Although most *M. bovis* strains have only one IS6110 insertion in their genome, the use of the (GTG)₅ oligonucleotide as a primer allowed the amplification of the *M. bovis* DNA analyzed. After PCR amplification, we observed eight different profiles among the 23 strains investigated (Table 1 and Fig. 2d). The patterns obtained were clear, the number of amplified bands ranged from three to eight, and their molecular masses ranged from 160 bp to more than 1,200 bp. Only 14 profiles (*M. bovis* ATCC 27290 included) could be correlated with those obtained by PCR with ERIC primers (Table 1).

This is the first epidemiological study on *M. bovis* strains isolated in Sardinia. The first notification of bovine tuberculosis was from Cagliari (herd 621, summer 1996); in this herd, we isolated 13 strains which produced 10 different patterns with all of the methods used. Strains 3 and 4, as well as strains 14 and 13, were not differentiated by the techniques used in this study. They were isolated from different cows at different times, and we probably isolated the same bacterial strain from different animals. The same strain (114) was isolated in another Cagliari herd (692) and in a cow from Nuoro (strain 97, herd 1513) (Table 1). One possibility is that the first strain isolated from an imported cow (from northern Italy) in Cagliari (herd 621, strain 4) spread in some way to herds 692 (Cagliari) and 1513 (Nuoro), since the last two strains were isolated 1 year later. It is interesting that 55% of *M. bovis* ATCC 2790 generated the same pattern with the three methods used (Fig. 2 and Table 1). This strain was isolated in 1971 in Copenhagen.

Three other isolates from Nuoro (herd 1345) showed a very similar pattern with IS6110 fingerprinting. Two strains (92 and 95) had the same fingerprint, with six hybridizing bands. In the third strain (94), the band with the highest molecular weight was missing, indicating a probably common origin; a similar result was obtained with an *M. tuberculosis* isolate from a patient (16). Curiously, only PCR ribotyping using *Pvu*II as a restriction enzyme was able to detect this difference among the three isolates (Table 1). Amplification of the ribosomal intergenic spacer is not a good method to detect differences among *M. bovis* isolates (due to the presence of only one ribosomal operon), but if it is combined with digestion of the amplification products, it can help in distinguishing mycobacterial strains. PCR with ERIC primers generated seven different families among the strains analyzed, but the patterns generated were difficult to interpret, whereas the profiles generated by PCR with the (GTG)₅ oligonucleotide were easier to read. This study revealed the superior discriminative power of the PCR with the (GTG)₅ oligonucleotide for differentiation of related *M. bovis* strains and the potential use of the techniques described to understand the epidemiology of infections due to *M. bovis*.

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