Mycobacterium bovis: Polymerase Chain Reaction Identification in Bovine Lymphonode Biopsies and Genotyping in Isolates from Southeast Brazil by Spolytotyping and Restriction Fragment Length Polymorphism

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Diagnosis of the Mycobacterium tuberculosis complex by direct PCR of mediastinal lymphnode DNA and microbiological tests were compared in cattle suspicious of bearing tuberculous-like lesions detected during slaughter.

The PCR procedure applied on DNA samples (n=54) obtained by adding α-casein into the thiocyanate extraction mix was positive in 70% of the samples. PCR confirmed the identification of 23 samples (100%) that grew in culture, 9 samples (60%) that failed to grow in culture, plus 6 (37.5%) samples that resulted in growth of bacterial contaminants. Genotyping by IS6110-RFLP and DR-spoligotyping analysis of seven samples revealed the presence of several polymorphisms. Seven of the isolates contained multiple copies of IS6110, thus defining the existence of five singular genotypes.

Key words: Mycobacterium bovis diagnosis - DNA polymorphism - thiocyanate guanidinium - Brazil

Bovine tuberculosis is an important zoonosis worldwide. Mycobacterium bovis, the causative agent of this disease in cattle, is also a pathogen for man and other economically important animals. M. bovis is a member of the M. tuberculosis complex, a group that includes also M. tuberculosis, M. africanum and M. microti.

In Brazil, the prevalence in animals is estimated between 0.9-2.9% (Kantor & Ritacco 1994). Therefore, between 1.35 and 4.35 million cattle might be contaminated with the microorganism. In humans, diagnosis relies on clinical manifestations, skin testing and subsequent identification of the bacteria by Ziehl-Neelsen (ZN) stain and microbiological tests. Unfortunately, culturing sample isolates requires 4-6 weeks to attain the desired cell growth needed for identification. Moreover, although the ZN procedure is fast, it lacks specificity and sensitivity. A similar situation is observed during the diagnosis of M. bovis in animals. McIlroy et al. (1986) reported that upon diagnosis of tuberculin-positive animals, tuberculous lung lesions were evident in 70% of reactive cattle, while M. bovis was isolated from nasal or tracheal mucus samples in just 19% of confirmed cases. These discrepancies illustrate the need for more sensitive and accurate methods to assist in the control of this zoonosis.

PCR has been successfully applied by us and other groups to detect members of M. tuberculosis complex. DNA amplification of specific sequences of DNA by the PCR technique has been reported to provide rapid diagnosis of many diseases and is especially useful for the direct detection of M. bovis in bovine tissue samples (Kolk et al. 1992, Kox et al. 1994, Liebana et al. 1995, Wards et al. 1995, Vitale et al. 1998, Zanini et al. 1998, Romero et al. 1999). By and large, the success of PCR depends on the availability of DNA, free of contaminants that impair the amplification process. The isola-
tion of DNA with thyocianate-diatomaceous silica is an efficient technique to release DNA from various sources. However, the presence of impurities in the DNA solution that interfere with the PCR remains as an obstacle for universal adoption of this technique (Kolk et al. 1992). A recent study shows that addition of α-casein can circumvent this problem (Boom et al. 1999). We adopted this procedure to identify in lymph node, from slaughtered animals bearing macroscopic lesions, the presence of *M. tuberculosis* complex, by PCR, and compare it with culturing techniques.

The most widely used genotyping method is DNA fingerprinting in which the insertion element is IS6110; it is recommended if the typing status of the population is unknown. IS6110 produces extensive fingerprint diversity among *M. tuberculosis* (Yang et al. 1998). Although the IS6110-RFLP method is not the best choice for typing *M. bovis* isolates that have a single copy of IS6110, especially, if compared with procedures that measure direct repeats (DR) or the polymorphic GC-rich sequence (PGRS), its use is recommended for the typing of isolates with multiple copies of IS6110 (Cousins et al. 1998). One of the limitations of RFLP-based typing systems is the requirement of a well grown culture for DNA extraction. The time-lag between isolation of *M. tuberculosis* complex from a clinical sample and the growth of a mycobacterial culture is often too long (Suffys et al. 1997).

Recently, an alternative typing technique has been developed, spoligotyping (Kamerbeek et al. 1997), based on the enzymatic amplification of the DR locus of *M. bovis*. This method detects the presence or absence of spacers within the DR locus. The resolving power of spoligotyping is preferred instead of IS6110-RFLP, when few IS6110 elements are present. This method can distinguish easily between *M. tuberculosis* and *M. bovis*, can be used with culture positive clinical samples, as well as directly from a specimen and has been used to identify the clonal nature of isolates (Roring et al. 2000). Along with spoligotyping, a new typing procedure involving detection of polymorphisms in *M. bovis* by hybridization with a pUCD probe of *Alu* I digested DNA is being reported (O’Brien et al. 2000)

Having shown the presence of *M. bovis* in various isolates from Southeast Brazil we further characterized the samples collected in different sites by IS6110-RFLP and DR-spacer spoligotyping, to determine their degree of relatedness.

**MATERIALS AND METHODS**

The samples used in this study from 54 slaughtered bovines showing tuberculous-like lesions in mediastinal and retropharyngeal lymph nodes were collected at four slaughterhouses located in Southeast Brazil (Ituiutaba, Divinópolis and Sabará, State of Minas Gerais; Colatina, State of Espírito Santo) during years 1998-1999. No more than one sample was selected per animal. The samples were (2-5 g) decontaminated by rinsing with a 6% H2SO4 solution during 30 min at room temperature, as previously described (Marks et al. 1972). A fraction of each sample used for culture was seeded in Lowenstein-Jensen (L-J) medium, without glycerol, or in Stonebrink medium and incubated at 37°C for 45 days. At this time, positive identification of *M. bovis* was confirmed by ZN stain and biochemical tests [pyruvate, niacin, nitrate reduction, urease, pyrazinamidase and catalase activities and inhibition by thiofeno-2 carboxylic acid and p-amine salicylic acid (PAS) in glutamate-added egg solid medium (without glycerol) (Romano et al. 1996)]. *M. tuberculosis* H37Rv, was used as the reference strain.

**PCR** - The PCR and DNA extraction of each specimen was done as follows: 100 mg of tissue was homogenized in 1.2 ml of buffer 20 mM Tris-HCl pH 7.0, containing 1.2 mg α-casein, 12 mg proteinase K, 0.5% Tween 20 and incubated for 12 h at 60°C, followed by 15 min at 100°C. To this suspension was added 1,10 v/v of lysis buffer containing 5.25 M thiocyanate guanidinium (GuSCN), 50 mM Tris-HCl pH 6.4, 20 mM EDTA, 1.3% Triton X-100, 0.1% α-casein and 1.2% of diatomaceous silica (Boom et al. 1990, 1999, Kolk et al. 1992, Kox et al. 1994). Following mixing, the diatom-DNA suspension was rinsed three times with buffer 5.25 M GuSCN, 50 mM Tris-HCl, pH 6.4 and decanted by centrifugation (12,000 x g, 15 s) as described by Boom et al. (1990), followed by three rinses with 10 mM Tris, 1mM EDTA pH 8.0 (TE) at room temperature. Elution of DNA was done with 0.3 ml TE, following incubation at 56°C for 10 min. The DNA recovered was subjected to PCR in the presence of primers INS-1 and INS-2 from *M. tuberculosis* complex insertion sequence IS6110, as previously described (Hermans et al. 1990). A positive PCR reaction is scored when a 245 bp fragment is detected, following 6% PAGE and silver stain.

**IS6110-RFLP polymorphism** - Genomic digestion of *M. bovis* DNA with *Pvu* II and RFLP analysis was carried as described by Van Soolingen et al. (1991).

The 245 bp fragment used to probe the IS6110 sequence, was obtained by PCR with the INS1 and INS2 primers (Hermans et al. 1990). Genomic DNA of *M. bovis* was digested with restriction endonuclease *Pvu* II, electrophoresed through 0.8% agarose gel, and transferred to a positively charged
nylon membrane (Amersham Pharmacia) by Southern blotting technique (Southern 1975). Briefly, DNA is denatured by 2 x 15 min incubations in 0.5 M NaOH, 1.5 M NaCl and neutralized by 2 x 15 min incubations in 0.5 M Tris-HCl, pH 7.5; 3 M NaCl before overnight capillary transfer in 20 x SSC (3 M NaCl, 300 mM sodium citrate; pH 7.0). After transfer, the membranes were rinsed in 2 x SSC and allowed to air dry. DNA was fixed to the membrane in a hot air oven at 120°C for 20-30 min. Membranes are stored in a sealed plastic bag between 3MM Whatman filter paper at -20°C until used.

**Spoligotyping analysis** - The spoligotyping was performed according to Kamerbeek et al. (1997) and Zumarraga et al. (1999). Amplification of the DR region from *M. bovis* was done with two primers specific for the DR locus. The PCR products were hybridized to a spoligo-membrane to which synthetic oligonucleotides of the DR regions of *M. tuberculosis* H37Rv and *M. bovis* BCG were covalently bound and detected using a streptavidin-peroxidase conjugate (Roche Diagnostics Ltd, UK) and the ECL (Amersham Pharmacia Biotech, UK) detection system as described (Kamerbeek et al. 1997). The membranes were kindly provided by The National Institute of Public Health and the Environment, RIVM, The Netherlands.

**RESULTS AND DISCUSSION**

Fifty-four biopsy samples from lymph nodes were dissected from slaughtered animals bearing tuberculous-like lesions. Following culture for 45 days (23) 42.6 % grew isolates identified by biochemical testing and ZN, as *M. bovis*. A fraction of the samples (15) failed to grow in culture representing 27.7%, or contained bacterial contaminants (16) 29.6%, as verified by ZN and biochemical reactions.

On the other hand, direct PCR from lymph nodes was positive for *M. bovis* in (38) 70.3% of the assayed samples. Each of the positive culture specimens yielded positive PCR as well (42.6%), while (9) 60% of samples negative for culture yielded positive PCR and (6) 37.5% of contaminated cultures afforded positive PCR as well. The high levels of microbial contaminants observed is probably a consequence of the poor sanitary conditions at the slaughterhouse, since handling of specimens following collection was aseptically done. It should be mentioned that PCR was sensitive enough to detect *M. bovis* in a large proportion (60%) of those samples that failed to grow in culture; this fact was emphasized by Liebana et al. (1995).

In a similar study, the efficiency of three methods to diagnose *M. bovis* in bovine clinical samples was compared. Again, PCR showed the highest efficiency (11.4%), compared to bacteriological culture (0.4%) and microscopic examination (0.4%). Based on these figures PCR is 28.5 times more efficient than culturing and direct microscopy (Romero et al. 1999). Their data confirm the advantage of PCR for diagnosis of *M. tuberculosis* complex over other procedures. The effectiveness of PCR reported here is superior because we used animal tissue with visible tuberculous-like lesions, while Romero et al. (1999) samples were mostly derived from cattle with positive intradermal tuberculin reaction. Also, their sampling was done on blood, nasal secretion and milk.

On the other hand, our PCR was 1.6 fold more efficient than culturing, while their efficiency was 28.5 fold higher when comparing similar procedures. Again, this difference could be attributed to the different source of samples used in both studies.

The improved identification obtained in this study is attributed to removal of unwanted inhibitors of unknown nature by α-casein, as our early attempts to extract DNA with GUSCN-diatomeaceous without casein reduced the efficiency of this diagnosis by 20-30% (data not shown), as previously reported (Boom et al. 1999). According to Kodavanti et al. (1996) the extraction of nucleic acids from inflamed tissue having a high influx of eosinophils by the conventional acid guanidinium thiocyanate phenol-chloroform (AGPC) procedure fails to yield intact DNA suitable for PCR or Northern blot analysis. Addition of undegraded nucleic acids samples from an inflamed lung to a total nucleic acids solution from saline control rat lung yielded electrophoretic profiles of degraded material, indicating the presence of nucleases-like activity in the nucleic acids extract from lung tissues having an eosinophil influx. The data is interpreted as if the conventional AGPC procedure apparently fails to completely remove nucleases associated with induced pulmonary eosinophil influx.

Genotyping of the IS6110 locus revealed two (n=2), three (n=2) or four (n=3) inserts, thus evidencing the presence of multiple copies of IS6110 in seven of these isolates (Fig. 1). The 1.9 kb fragment found in most *M. bovis* samples was detected in 16 isolates (not shown). Based on these profiles, the seven samples were grouped into five different genotypes. Three of them (A65/A66/A68) isolated in different areas showed similar multicycle profile composed of four IS6110 copies, while isolates A74 and A82 with three copies, and finally A19 and A69 with two insertion sequences.

Spoligotyping experiments also identified five different patterns among the isolates, three of them...
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DNA from 65 Argentinean *M. bovis* samples hybridized with the 245-bp probe. Gutierrez et al. (1995) also observed four different patterns of *M. bovis* in cattle and goats from Spain using *Pvu*II-digested DNA+RFLP with IS6110 probe. By contrary, spoligotyping of four Brazilian clusters of *M. bovis* showed a higher degree (83%) of homology (Zumarraga et al. 1999) and a predominant single copy IS6110 genotype has been reported in bovine isolates from Argentina. Moreover, the study with Argentinean isolates, suggested that strains with multiple copies of IS6110 were originated from wild or zoo animals rather than from cattle (Zumarraga et al. 1999).

On the other hand, a comparison of the spoloigotypes shown here with those reported by Zumarraga et al. (1999) on a Latin American population show that both groups lack spacers 3, 9, 16, 39-43. Also, two of the spoloigotypes reported here A74 and A82, were previously described as 12S or 12A and 20A, respectively, by Zumarraga et al. (1999). The spoloigotype A74, which is identical to spoloigotype for *M. bovis* BCG was described by Zumarraga et al. (1999), in seven Argentinean bovines, eight animals other than bovine and a human isolate from Spain.

Finally, the data suggest a diversity of genotypes containing multiple copies of IS6110 in *M. bovis* from Southeast, Brazil. The state harbors near 22 million cattle heads that represent 15% of the Brazilian population. A more comprehensive analysis is under way to determine the dominant genotype in this area and also to establish whether a different genotype frequency is found in other regions of the country. It is also concluded that the PCR procedure presented can be completed within 24 h, therefore, it can be of valuable help during sanitary inspection at slaughterhouses for condemnation of sacrificed animals suspected of having the disease.
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REFERENCES


