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Simple and Rapid Detection of *Mycobacterium tuberculosis* Complex Organisms in Bovine Tissue Samples by PCR

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***Mycobacterium bovis* is a slowly growing microorganism, and confirmation of the diagnosis by conventional culture is a lengthy process. A simple, rapid method for the extraction of DNA from bovine tissue samples was developed and used in a PCR designed for the diagnosis of tuberculosis. Tissues from 81 cattle from tuberculosis-infected herds (group 1) and 19 cattle from tuberculosis-free herds (group 2) were tested in this PCR, and the results were compared with those of conventional culture. The PCR assay detected 71.4% of the culture-positive animals from group 1. Tissues from all animals in group 2 were negative in the PCR assay and by culture. The described method could be used as a rapid screening technique which would be complementary to culture of tissue specimens for the routine diagnosis of bovine tuberculosis. The PCR technique is much faster than culture and reduces the time for diagnosis from several months to 2 days. It also provides for the detection of *M. bovis* when rapidly growing *Mycobacterium* spp. are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become nonviable.**

Bovine tuberculosis caused by *Mycobacterium bovis* is an infectious zoonotic disease that is a serious economic and health problem in cattle herds. It is an important disease in the Spanish cattle population, and a campaign to eradicate tuberculosis from Spanish cattle herds has been in progress for the last 15 years. The number of cattle with positive skin test reactions in many parts of Spain is still very high, with an average of 3.52% tuberculin reactors during 1991. During the same year the cost of compensation paid to farmers for the slaughter of reactor animals (animals with positive skin test reactions) was US$61.4 million.

The single intradermal tuberculin test (IDTT) is the official test used in the eradication campaign for the detection of infected animals in the field. A review of the available data suggested that the sensitivity of the IDTT is only moderate (average, 72%) (6), and in more recent work the IDTT was found to have a sensitivity of only 65.6% (21). Although the specificity of the IDTT is generally high (98.8%) (6), it does have other disadvantages. The ability to predict the presence or absence of disease from test results is dependent on the prevalence of the disease in the population tested as well as on the sensitivity and specificity of the test (9). The higher the prevalence the more likely it is that a positive test is predictive of the disease, but as the level of infection drops the proportion of test-positive cattle showing no evidence of tuberculosis increases (10). One reason for this could be the false-positive reactions caused by antigenic cross-reactivity with atypical mycobacteria. In addition, the skin test site must be read 72 h after injection of the tuberculin, and because of the alteration of the subsequent immune status of the individual animal tested, cattle cannot be retested for 60 to 90 days (17). Because of these problems it becomes necessary to confirm the results obtained from the IDTT by other diagnostic procedures.

The interferon gamma (IFN-γ) assay is a recently developed whole-blood in vitro assay (18). In Australian field trials the sensitivity and specificity of this test were shown to be 93.6 and 96.2%, respectively (21). The main disadvantages of this test are the logistical problems of performing the test under the tight time constraints required (blood samples need to be incubated with tuberculin within 8 h of collection), problems with antigenic cross-reactivity, and cost. Although benefits are expected from this test, doubts remain as to whether the IFN-γ assay will come to be recognized as an alternative to IDTT. The IFN-γ test is currently being evaluated in several countries.

Culture is considered to be the "gold standard" and the definitive test for the confirmation of bovine tuberculosis. However, microbiological diagnosis of *M. bovis* is an extremely slow procedure which may take as long as 2 to 3 months. An additional 2 to 3 weeks is required for the biochemical identification of isolates. It is also known that the sensitivity of culture is not 100%, and false-negative culture results may occur (5). Several rapid diagnostic methods have been developed. The use of DNA probes has been reported for the rapid identification of the *M. tuberculosis* and *M. avium-M. intracellulare* complex (4, 7, 12), and the development of radiometric methods (15) has reduced the time taken for the detection of *M. tuberculosis*. However, these techniques are neither as fast nor as sensitive as required. 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 rapid detection of fastidious or slowly growing pathogens (2, 11, 14). The use of PCR for the detection of *M. tuberculosis* in human specimens such as sputum has been reported previously (3, 13), but to our knowledge there have

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not been any studies on the use of PCR for the direct detection of *M. bovis* in bovine tissue specimens.

The aim of the study described here was to develop a DNA amplification system which could be used for the direct detection of *M. bovis* in animal tissue samples collected at slaughter to provide a more rapid confirmation of bovine tuberculosis. A simple DNA extraction method was developed and used in the PCR system. The results of the direct PCR were compared with the results obtained by conventional culture procedures.

MATERIALS AND METHODS

Sample collection. For the present study 100 animals, 81 cattle from *M. bovis*-infected herds and 19 cattle from noninfected herds, were tested as part of the eradication campaign during 1991 and 1992. Tissue samples were obtained at a slaughterhouse, where the animals were necropsied by a routine technique (1). In animals with macroscopic lesions, the lesioned material was collected. If no lesions were detected, samples were taken from mediastinal, retropharyngeal, and bronchial lymph nodes and from the lung. These samples were placed in sterile containers and were kept at −80°C until they were processed. All samples collected were tested by mycobacterial culture, auramine staining, and PCR.

Group 1 animals. Group 1 consisted of 81 cattle which originated from herds infected with bovine tuberculosis and were positive when tested by IDTT or the IFN-γ assay.

Group 2 animals. Group 2 consisted of 19 animals that originated from herds known to be free of bovine tuberculosis. All of these animals were negative for bovine tuberculosis by the IFN-γ assay and IDTT and had no macroscopic lesions at necropsy.

IFN-γ assay. Aliquots of heparinized whole blood were incubated with purified protein derivative (PPD) antigen for 16 h, and the plasma was removed and assayed for bovine IFN-γ in a sensitive enzyme immunoassay (18) following the manufacturer's instructions (CSL Limited, Parkville, Australia). A comparative assay in which each animal's IFN-γ responses to bovine PPD and avian PPD were compared was performed.

Bacteriology. Tissues were divided into equal portions, when possible, and half was kept frozen for a confirmatory analysis in case the cultures needed to be repeated. All samples were thawed at 4°C and were processed as follows. Fat and fibrous tissue were removed by aseptic technique, and the surface of the sample was examined for gross lesions. Approximately 5 g of the remaining tissue (from areas with lesions if they had them or randomly if they did not) were cut into small pieces with a sterile scalpel blade. These pieces of tissue were homogenized with 3 ml of sterile distilled water during 15 min in a stomacher (FSE Ltd.). Two milliliters of the homogenate was separated for decontamination by a modification of the method proposed by Tacquet and Tison (19) by the addition of 3 ml of a solution of 1% sodium hydroxide–3% lauryl sulfate, after which they were incubated for 30 min. The mixture was neutralized with 8.75% orthophosphoric acid by using bromocresol blue as an indicator, and the neutralized suspension was centrifuged at 3,500 rpm for 30 min (Sigma 3-10 centrifuge). The supernatant was discarded, and 0.25 ml of the pellet was inoculated onto Coletsos medium (Biomedics; Biomérieux), Coletsos medium with lyncomycin, nalidixic acid, and actidione (Biomedics), and Löwenstein-Jensen medium without glycerol (Biomedics). Tubes were checked for growth at 5, 7, and 10 days and then weekly. After 5 months, tubes with no evidence of growth were recorded as negative and were discarded. The original samples were recultured if the culture medium became contaminated during the incubation time.

Colonies suspected of being mycobacteria were examined for the presence of acid-fast bacilli by the Ziehl-Neelsen technique. All acid-fast bacilli detected were subcultured onto Coletsos and Löwenstein-Jensen media at 37°C in order to get enough growth to perform identification procedures. Identification of the isolates was based on macroscopic features such as growth rate, pigment production, colony morphology, and results of standard biochemical tests (20) such as niacin production, nitrate reduction, Tween 80 hydrolysis, growth in the presence of 2-thiophene carboxylic acid hydrazide, arylsulfatase activity, and urease. Isolates were also tested by using the Accuprobe DNA probe (Gen Probe) for the detection of *M. tuberculosis* and *M. avium-M. intracellulare* complexes according to the manufacturer's instructions.

Smear examination. Smears were made from the fresh material at the time of culture. They were air dried, heat fixed, and stained with phenolated auramine by a modification of the Smithwick method in which Ziehl fuchsine replaced acridine orange as the counterstain. The stained smears were examined at a magnification of ×400 by transmission fluorescence microscopy.

DNA amplification. (i) Inactivation of pathogens. A 0.5-ml aliquot of the decontaminated sample was incubated at 75°C for 1 h to inactivate the mycobacteria and the other pathogens (3).

(ii) Bacterial lysis and DNA extraction. The inactivated samples were centrifuged in a microcentrifuge for 5 min at 13,000 rpm (Eppendorf 5415-C centrifuge), and the supernatant was discarded. The pellet was washed with 1 ml of sterile distilled water and was recentrifuged for 5 min. The pellet was resuspended in 100 ml of a proteinase K (0.1 mg/ml) solution, and the mixture was incubated for 1 h at 56°C. After incubation, the samples were boiled for 5 min, and after they had equilibrated to room temperature they were centrifuged for an additional 5 min. Finally, the supernatant was transferred to a new 1.5-ml microcentrifuge tube. The DNA was recovered by using the Magic Minipreps DNA purification system (Promega).

(iii) DNA amplification by PCR. The PCR amplification was run under standard conditions (nucleotide concentration, 0.5 mM; Mg^{2+} concentration, 1.5 mM; *Taq* polymerase [Promega], 2.5 U) with 5 ml of extracted DNA in a final volume of 50 ml. The oligonucleotide primers used were described by Plikaytis and others (16). These primers (IS41, 5′ CCT GCG AGC GTA GGC GTC GG 3′; IS43, 5′ TCA GCC GCG TCC ACG CCG CCA 3′) anneal to a sequence in the repetitive DNA element IS*6110*. The expected size of the amplified DNA fragment is 317 bp, and the bands were visualized after electrophoresis of 1/10 of the reaction mixture in an agarose gel stained with ethidium bromide (0.5 μg/ml). PCR was performed in a Pharmacia Thermal Sequencer by using 1 cycle at 94°C for 10 min, 30 cycles of denaturation for 2 min at 94°C, annealing for 2 min at 68°C, and extension for 2 min at 72°C and 1 cycle at 72°C for 10 min.

(iv) PCR internal control. A recombinant plasmid was constructed by site-directed oligonucleotide mutagenesis by using the Muta-Gene In Vitro Mutagenesis Kit, version 2 (Bio-Rad), as an internal control for the PCR. This plasmid, pPG10, contains the sequences of oligonucleotides IS41 and IS43 on opposite strands and separated by 1,250 bp. The mutant plasmid pPG10 was analyzed by PCR, giving a 1.25-kb amplification fragment, as expected. Amplification by PCR of *M. tuberculosis* or *M. bovis* DNA in the presence of pPG10 produces two amplified fragments, one from the genomic DNA of mycobacteria of 317 bp and another from plasmid pPG10 of 1,250 bp. When the two bands of 317 and 1,250 bp are the amplification products of a clinical sample, this confirms that the sample contains *M. tuberculosis* complex. If the result is a single band of 1,250 bp, then no mycobacteria are present in the sample. If the 1,250-bp band fails to appear, the result indicates that the PCR has been inhibited. The positive control plasmid was introduced so that we could detect false-negative results. Positive and negative controls were run with each batch of tests.

(v) Southern blot hybridization. Southern blot hibridization was performed on all samples which gave a positive result after ethidium bromide staining so that the specificity of the PCR could be confirmed. After electrophoresis, the amplified DNAs were transferred onto nylon membranes (Promega) by the Southern blot method (8). After a 3-h prehybridization in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.5% sodium dodecyl sulfate (SDS)–100 mg of denaturated salmon sperm DNA per ml at 60°C, the membranes were hybridized with 1.57×10^6 cpm of ^{32}P-labelled probes in the same buffer for at least 3 h and 30 min at 60°C. Two different probes were used, one for an internal fragment (positions 816 to 836) of the IS*6110* repetitive element (5′ CTC ACC TAT GTG TCG ACC TG 3′) and the other for the internal positive control (5′ CGA CTC ACT ATA GGG CGA AT 3′). Subsequently, the filters were then washed once with 2.5× SSC–0.5% SDS at 60°C for 20 min and once with 0.1% SSC–0.1% SDS at 60°C for 10 min. The filters were briefly air dried and were exposed to radiographic film in cassettes containing intensifying screens at −70°C for various lengths of time.

For the purpose of the analysis, animals from which *M. bovis* was cultured were considered to have tuberculosis.

TABLE 1. Results from culture of *M. bovis* and PCR of animals in groups 1 and 2

Culture result	No. of animals			
	PCR positive[a]		PCR negative	
	Group 1	Group 2	Group 1	Group 2
Positive	35	0	14	0
Negative	2	0	30	19
Total	37	0	44	19

[a] Product of DNA amplification visualized in agarose gel stained with ethidium bromide.

RESULTS

Forty-nine of the 81 animals which were selected from *M. bovis*-infected herds by virtue of their positive results in the skin test or IFN-γ assay were culture positive for *M. bovis* (Table 1). Of the 49 culture-positive animals, 35 (71.4%) were positive for *M. tuberculosis* complex when their tissues were tested by PCR. Two of the remaining 32 animals which were culture negative in this group were PCR positive. Of the 30 culture- and PCR-negative animals in group 1, 21 (70%) were

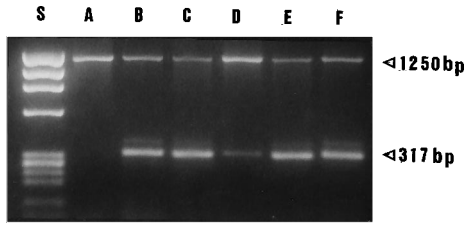

FIG. 1. Determination of mycobacterial DNAs in clinical samples by PCR. Lane S, restriction endonuclease *Hae*III-digested phage ϕX174 DNA used as DNA size standard, (the sizes were 1,358, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp); lane A, a negative control with amplification of 10 ng of bovine DNA; lane B, amplification of DNA prepared from American Type Culture Collection reference strain *M. tuberculosis* ATCC 25177; lanes C to F, amplification of DNA prepared from clinical tissue samples. Procedures for DNA preparation and PCR amplification and the sequences of the primers are given in the text; DNA was visually inspected through UV light after agarose gel electrophoresis. A DNA band of 317 bp indicated the presence of *M. tuberculosis* complex organism DNA. A band of 1,250 bp indicated that the PCR was not inhibited.

animals without macroscopic lesions and their tissues were auramine stain negative. The remaining nine animals had macroscopic lesions or their tissues were auramine stain positive.

All of the samples from the 19 animals from group 2, which were from tuberculosis-free herds and IDTT and IFN-γ assay negative, were culture negative and negative by PCR.

A typical agarose gel stained with ethidium bromide to detect the 317-bp diagnostic PCR products after amplification of extracted DNA from clinical samples is shown in Fig. 1. The specificity was confirmed by Southern blot analysis with the internal oligonucleotide probe for IS*6110* and the probe for the internal positive control for the PCR (Fig. 2).

DISCUSSION

The results of the study described here suggest that the DNA amplification method tested is a useful technique for the detection of *M. bovis* and other pathogenic *M. tuberculosis* complex microorganisms directly from tissue samples. By this PCR method, 71.4% of the samples from animals that were confirmed to be infected with *M. bovis* by culture were found to be infected by PCR within 2 days. The technique has a distinct time advantage over the traditional methods of identification, because by this technique specimens could be screened in 1 to 2 days. In samples which were positive by PCR, these results were obtained on the second day, which is up to 8 to 20 weeks before confirmation could be obtained by traditional methods (4).

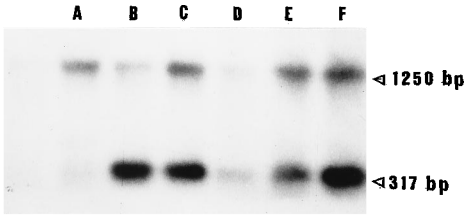

FIG. 2. Southern blot hybridization of amplified products with the internal oligonucleotide probe for IS*6110* and the probe for the internal positive control of the PCR. The sequences of the probes were described in the text. Lane A, bovine DNA which served as a negative control; lane B, amplification of DNA prepared from *M. tuberculosis* ATCC 25177; lanes C to F, DNA extracted from clinical samples. Procedures for DNA preparation, PCR amplification, and the sequences of the primers are given in the text.

The test is specific for the identification of *M. tuberculosis* complex organisms. Negative controls run with batches of samples failed to identify any contaminating DNA. Although the test was not able to distinguish between different members of the *M. tuberculosis* complex, all of the positive samples from bovine sources could be assumed to be *M. bovis*, since other members of the *M. tuberculosis* complex have never been isolated from cattle specimens in our laboratory.

Samples from two animals belonging to group 1 were negative when they were cultured and positive when they were tested by PCR. The samples from these two animals were positive in both IDTT and the IFN-γ assay, macroscopic lesions were found in the necropsy examination, and auramine-stained smears made from these lesions were positive for acid-fast bacilli. One of these samples yielded a fast-growing mycobacterium other than the *M. tuberculosis* complex on culture. PCR performed from a colony of the mycobacterium other than the *M. tuberculosis* complex gave a negative result, providing confirmation that it did not belong to the *M. tuberculosis* complex. It is possible that the *M. bovis* organisms present in the sample may have been overgrown by these other mycobacteria. *M. bovis* has a distinctive dysgonic type of growth and grows poorly compared with the growth of most other mycobacteria (including *M. tuberculosis*). It is quite possible for it to be overgrown in mixed cultures and not detected by conventional culture methods. The harsh decontaminants which are used to destroy contaminating bacteria in a sample can also have a harmful effect on *M. bovis*. This may explain the result for the other animal in group 1 which was culture negative but PCR positive, since PCR detects both viable and nonviable bacteria.

The PCR test that we applied to the direct detection of *M. bovis* in tissue samples was not as sensitive as culture and did not always detect those samples that contained small numbers of organisms. This may be because of the difficulty of extracting mycobacterial DNA from the samples without extracting eukaryotic DNA. Although the internal positive control did not detect any inhibition of the PCR, the presence of foreign DNA could interfere with the performance of the PCR, causing a drop in the sensitivity of the test when it is applied to samples with few bacilli present. It is envisaged that the sensitivity of this test could be improved if the mycobacteria could be selectively concentrated from the tissue before the beginning of the DNA extraction procedure.

The culture and PCR results for 21 of the 30 animals in group 1, which had no macroscopic lesions and whose samples were negative in the auramine staining test, suggested that these animals were not infected. The remaining nine animals may have had false-positive macroscopic lesions or false-positive results by the auramine staining test.

In summary, the results presented here indicate that the PCR technique can be successful for the direct detection of *M. bovis* organisms in bovine tissue samples. Although PCR detected only 71.4% of the *M. bovis*-infected animals in group 1, the PCR technique is much faster than culture, reducing the time for diagnosis to 2 days and providing the ability to detect the presence of *M. bovis* in samples even when organisms have become nonviable for culture or when there is an overgrowth by other mycobacteria.

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