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Use of Highly Specific DNA Probes and the Polymerase Chain Reaction To Detect *Mycobacterium paratuberculosis* in Johne's Disease

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DNA probes that hybridize to a mycobacterial insertion sequence, IS*900*, present in multiple copies in the genome of *Mycobacterium paratuberculosis* were found to be highly specific for *M. paratuberculosis*. DNA sequences derived from IS*900* were used to prepare DNA primers for detection and identification of *M. paratuberculosis* by the polymerase chain reaction. Highly specific direct detection of *M. paratuberculosis* DNA in feces from cattle with Johne's disease was obtained. The polymerase chain reaction test had a sensitivity equal to or greater than that obtained by standard culture techniques and was much more rapid, taking only hours compared with 6 to 12 weeks for culture.

Paratuberculosis, or Johne's disease, is a chronic enteritis of ruminants which causes diarrhea and emaciation (4, 16, 19). Caused by *M. paratuberculosis*, this disease infects 2.9% of dairy and 0.8% of beef cattle populations in the United States (17). Prevalence in cattle worldwide ranges from 0 to 15% (4, 16, 19). The disease also affects other ruminants, such as goats and sheep. *M. paratuberculosis* has also been implicated in Crohn's disease (regional enteritis) of humans (5, 15, 14).

Attempts to control Johne's disease are severely hampered by inadequate diagnostic procedures. *M. paratuberculosis* is an intracellular pathogen which replicates in macrophages in the lamina propria and is shed into the feces during disease. At present, diagnosis involves culture of the organism from feces. However, *M. paratuberculosis* requires the iron-chelating growth factor, mycobactin (1), for growth in vitro and is extremely slow growing. Primary isolation of the organism from feces takes 6 to 12 weeks (4, 16). Despite decontamination steps, cultures are often lost because of contamination. In addition, clinically infected animals may shed organisms sporadically during disease and therefore, false-negative results may occur. Diagnosis based on immunological techniques such as skin testing with Johnin, enzyme-linked immunosorbent assay, and complement fixation tests is hampered by false-negative results due to anergy during clinical disease (2), false-positive results due to prior immunological exposure, and interference due to reactivity of the tests to related environmental mycobacteria.

DNA probes would offer many advantages in Johne's disease diagnosis. The recently developed polymerase chain reaction (PCR) (18) should allow sensitive detection of DNA from *M. paratuberculosis* and has already been applied to the detection of other mycobacteria (9, 10). However, *M. paratuberculosis* is very closely related to common environmental organisms of the *Mycobacterium avium* complex, particularly *M. avium* (11, 13–15, 21). (The terminology of the *M. avium* complex is still somewhat confused. However, we will refer to those strains that we have examined with DNA probes, which demonstrated restriction fragment length polymorphism banding patterns that were closely related to those obtained for the classical *M. avium* serotypes 1, 2 or 3, as *M. avium* [14]. Those strains which gave restriction fragment length polymorphism banding patterns that did not resemble those for *M. avium* or that have not been examined are referred to as *M. avium* complex.) Only a 1 to 2% base substitution separates *M. avium* and *M. paratuberculosis* (13, 14), and although restriction fragment length polymorphisms differentiate between the two species (13, 14), most randomly isolated DNA probes hybridize equally well to DNA from both species. A test based on DNA probes has been described for Johne's disease (12); however, the probe used hybridized equally well to both *M. paratuberculosis* and *M. avium* DNAs, limiting its possible diagnostic value. Additionally, no differences were detected in the DNA sequence of a 65,000-molecular-weight gene (65K gene) of *M. avium* and *M. paratuberculosis*, precluding development of an *M. paratuberculosis*-specific PCR based on this gene (9).

We have, however, identified a clone, pMB22, which has been used to differentiate *M. paratuberculosis* and *M. avium* (13–15). pMB22 hybridizes to multiple restriction fragments of *M. paratuberculosis* DNA but to a much smaller number of *M. avium* DNA fragments (13–15). pMB22 has been shown to contain a single copy of a mycobacterial insertion sequence, designated IS*900*, and the sequence of IS*900* has been determined (7). The element is 1.45 kilobases and is related to the 1.49-kilobase insertion sequence IS*110* from *Streptomyces coelicolor* (3). The overall structure of IS*900* contained in pMB22, showing the location of a large open reading frame, is shown in Fig. 1. IS*900* is present in multiple (10 to 20) genomic copies in all *M. paratuberculosis* strains examined but absent from *M. avium* DNA, although sequences related to IS*900* are found in some mycobactin-dependent *M. avium* strains (J. J. McFadden, E. P. Green, M. Moss, M. Tizard, F. Portaels, Z. Kunze, and J. Hermon-Taylor, submitted). We have therefore evaluated the use of DNA sequences from IS*900* for specific identification of *M. paratuberculosis* and for direct detection by PCR of *M. paratuberculosis* in fecal samples from cattle.

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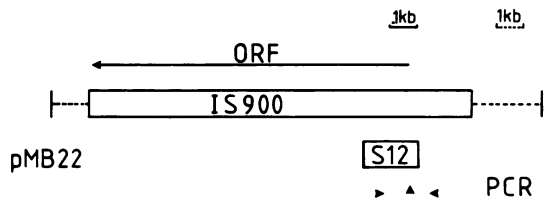

FIG. 1. Structure of IS*900* contained in clone pMB22. Locations of the single open reading frame (ORF), subclone pMB22/S12, PCR primers IS*900*/115C (▶) and IS*900*/921 (◀), and the PCR probe (▲) are shown. kb, Kilobase.

MATERIALS AND METHODS

Mycobacterial strains. Mycobacterial strains were *M. paratuberculosis* ATCC 19698 and *M. paratuberculosis* Ben ATCC 43544; *M. avium* serotype 1 ATCC 15769, serotype 2 Caddigg 16741, serotype 5 25546-759 (20), serotype 6 (20), serotype 8 (14), complex serotype 11 (20), and complex serotype 27 (20); *M. kansasii* TMC 1201; *M. phlei* NTCC 8573; *M. tuberculosis* H37RV; *M. bovis* BCG (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom); and *M. smegmatis* NTCC 333. Additional *M. avium* and *M. avium* complex strains examined were isolated from acquired immune deficiency syndrome patients (8).

Fecal samples. Fecal samples of known culture status, which had been taken from cattle suspected of being infected with *M. paratuberculosis*, were obtained from the Wisconsin Animal Health Laboratories, Madison.

Preparation of mycobacterial DNA. Mycobacteria were grown either in liquid culture or on slopes. Cells were harvested in TEN buffer (50 mM Tris hydrochloride, pH 8; 100 mM EDTA; 150 mM NaCl), washed, digested with 10 mg of subtilisin (Carlsburg, Sigma type VIII, Sigma Chemical Co., St. Louis, Mo.) ml^{-1} for 3 h at 37°C, and lysed with 5 mg of lysozyme (Sigma) ml^{-1} for 3 h at 50°C. Sodium dodecyl sulfate and pronase (Calbiochem-Behring, La Jolla, Calif.) were added to 1% and 3 mg ml^{-1}, respectively, followed by incubation for 24 h with an additional pronase treatment after 18 h. DNA was phenol-chloroform extracted, RNase A digested, and ethanol precipitated.

Sample preparation. Fecal material was suspended in sufficient 0.2 N NaOH to produce a 10% (wt/vol) suspension. The samples were allowed to settle for 20 min at room temperature. A portion of fecal supernatant (0.5 ml) was applied to a 4-ml column containing a layer of Whatman cellulose P-11 (Whatman, Inc., Clifton, N.J.) (1 ml) over which was layered 3 ml of Whatman DE-52 cellulose. After application of the sample suspension, the column was washed with 15 ml of 0.2 N NaOH. Following centrifuging of the eluate and removal of the supernatant, the pellet was resuspended in 0.2 ml of 0.2 N NaOH and transferred to a 1.5-ml screw-cap Eppendorf tube, and the cells were lysed at 120°C for 10 min. Samples were allowed to cool to room temperature prior to being analyzed by PCR.

DNA probes and manipulations. Clone pMB22 was derived from a genomic library prepared from a strain of *M. paratuberculosis* isolated from a Crohn's disease patient. DNA was cloned into the plasmid vector pGEM-1 (Promega Biotec, Madison, Wis.). Subclones of pMB22 were prepared by standard techniques. Synthetic oligonucleotide PCR primers and probes were synthesized on an Applied Biosystems 381A DNA synthesizer operated in the trityl-on mode. Oligonucleotides were purified by reverse-phase chromatography on a NovaPak C18 radial-pack column (Waters Chromatography, Div. Millipore Corp., Milford, Mass.). Following detritylation with 80% acetic acid, the oligonucleotides were lyophilized and rechromatographed as described above. The oligonucleotide fractions were collected, pooled, and lyophilized. The sequences of the oligonucleotides used in this study are as follows:

PCR probe: AGGTTGTGCCACAACCACCTCCGTA
IS*900*/150C: CCGCTAATTGAGAGATGCGATTGG
IS*900*/921: AATCAACTCCAGCAGCGCGGCCTCG

The PCR primers, IS*900*/150C and IS*900*/921, are designed to amplify a 229-base-pair target sequence that can be detected with the PCR probe sequence (Fig. 1).

PCR. PCRs were performed as described previously (18). Briefly, 1- to 5-μl samples were added to 95 to 100 μl of amplification master mix containing 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleoside triphosphates 1 μM each primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a 0.5-ml Eppendorf tube. PCR amplifications were performed with 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min in a Thermal Cycler (Perkin-Elmer Cetus).

PCR product hybridization analysis. Samples of PCR amplification products (10 μl) were subjected to electrophoresis on a 1.6% agarose gel according to standard protocols. Southern hybridization analysis was conducted following alkaline transfer of separated DNA fragments from the agarose gel to a sheet of Zetabind nylon membrane (Bio-Rad Laboratories, Richmond, Calif.). The nylon membrane was neutralized with 1× SSC (0.15 M NaCl plus 0.015 sodium citrate), and blocked with 5× SSC containing 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% sodium dodecyl sulfate. Blots were hybridized with approximately 10^5 cpm of PCR probe 5′ end labeled with ^{32}P ml^{-1} in fresh blocking solution. Hybridization was conducted for 2 h at 65°C. Following two 1× SSC–1% sodium dodecyl sulfate washes and one 0.1× SSC–1% sodium dodecyl sulfate wash at 65°C, the membrane was subjected to autoradiography for 3 h at −80°C with intensifying screens (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

To develop an *M. paratuberculosis*-specific test, we investigated the use of a *Sau*3A-generated subclone of pMB22, pMB22/S12 (Fig. 1), as a hybridization probe. Radiolabeled pMB22/S12 was hybridized to DNA extracted from *M. paratuberculosis*, mycobacteria of the closely related *M. avium* complex, and a number of additional mycobacterial species. We examined 12 typed *M. avium* complex strains (see above) plus all 45 *M. avium* complex strains isolated from acquired immune deficiency syndrome patients, as described previously (8), including serotypes 1, 2, 3, 4, 5, 6, 8, 10, 11, 16, and 27. Representative results are shown in Fig. 2. None of the mycobacterial DNA samples except *M. paratuberculosis* hybridized to pMB22/S12. Therefore, we investigated the use of DNA sequences derived from the pMB22/S12 portion of IS*900* as DNA primers for enzymic amplification of *M. paratuberculosis* DNA by PCR.

Regions of the sequence corresponding to the location of pMB22/S12 were used to synthesize PCR primers in order to amplify a 229-base-pair product and hybridization probe internal to the PCR primers. The specificity of the IS*900*-based PCR was examined with various mycobacterial DNA

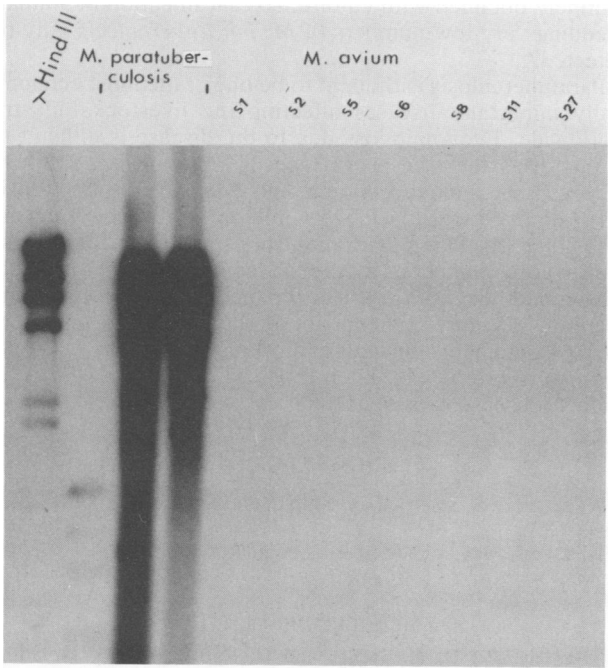

FIG. 2. Southern hybridization analysis of genomic DNA extracted from *M. paratuberculosis* and *M. avium* complex strains probed with pMB22/S12. DNA samples were digested with the restriction endonuclease *Pvu*II, electrophoresed through 1% agarose, blotted, probed with ^{32}P-radiolabeled probe pMB22/S12, and autoradiographed. The serotyped *M. avium* complex strains are as described in Materials and Methods. The two *M. paratuberculosis* strains are American neotype ATCC 19698 (left) and Ben ATCC 43544 (isolated from Crohn's disease).

samples (Table 1), including DNA from mycobactin-dependent *M. avium* complex strains FP157R, WP11/79, and FP7941, which contained elements related to IS*900* (McFadden et al., submitted). Also examined was DNA from a strain of *S. coelicolor* A3 (2) which was harboring IS*110* (3). DNA (1 ng) from each strain was subjected to PCR. The detection of any product by Southern blotting was recorded as a positive result (Table 1). Only *M. paratuberculosis* strains gave a positive result.

To investigate the use of IS*900* sequences and the PCR for diagnosis of Johne's disease, DNA was extracted from fecal samples obtained from cattle with Johne's disease.

We were unable to obtain reproducible results by PCR analysis on crude fecal suspensions. Because standard DNA preparation procedures are time-consuming and labor intensive, a rapid sample preparation protocol with ion-exchange chromatography was developed. Fecal samples with known culture status were processed as described above, while target DNA sequences were amplified by the PCR technique and amplification products were analyzed by Southern hybridization. The results (Fig. 3) demonstrated that all eight culture-positive samples exhibited the expected IS*900*-specific 229-base-pair PCR fragment. However, two of six culture-negative fecal samples tested also tested positive by PCR. Repeated sample preparation and PCR testing of these fecal samples (three times) gave identical results, indicating that contamination was not occurring during the preparation of samples for PCR. A reagent-negative control was used to document the absence of contamination of the PCR reagent (Fig. 3A, lane 6). A dilution series of cultured *M. paratuberculosis* organisms (counted by plate dilution) was added to a PCR-negative fecal sample and indicated a detection limit of approximately 10^2 organisms spiked into the fecal sample (Fig. 3A, lanes 7 through 12).

TABLE 1. Results of PCR testing

Strain	PCR result[a]
M. paratuberculosis ATCC 19698	+
M. paratuberculosis Ben ATCC 43544	+
M. avium serotype 1 ATCC 15769	−
M. avium serotype 2 Caddigg 16741	−
M. avium serotype 5 25546-759 (19)	−
M. avium WP11/79 (6, 15)	−
M. avium FP7941[b]	−
M. avium complex FP157R[b]	−
S. coelicolor A3 (2)	−

[a] +, Positive; −, negative.
[b] Obtained from the collection of F. Portaels, Institute of Tropical Medicine, Antwerp.

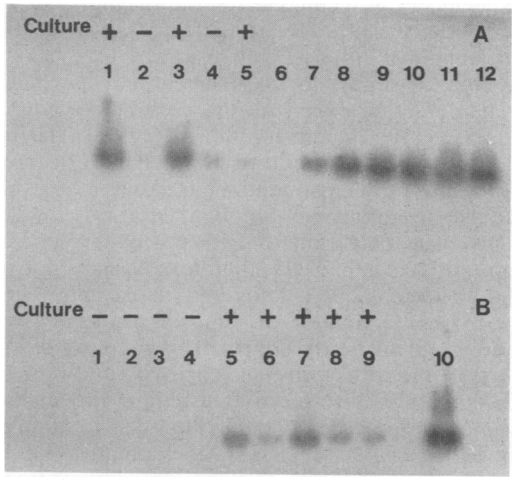

FIG. 3. PCR detection of IS*900*-specific sequences in culture-positive (+) and -negative (−) samples obtained at 12 weeks. Following preparation and amplification, samples were Southern blotted and probed for the expected 229-base-pair, *M. paratuberculosis*-specific amplification product. (A) Lanes 1 through 5, Cultured field samples; lanes 6 through 11, 0, 100, 500, 1,000, 5,000, and 10,000 organisms, respectively, added to culture-negative DNA samples; lane 12, 1 ng of *M. paratuberculosis* genomic DNA. (B) Lanes 1 through 9, Cultured field samples; lane 10, 1 ng of *M. paratuberculosis* genomic DNA.

DISCUSSION

Sequences contained in the insertion element IS*900* are clearly highly specific for *M. paratuberculosis*. Despite the very high degree of DNA homology between *M. paratuberculosis* and *M. avium*, none of the strains examined hybridized to pMB22/S12 or gave PCR products with IS*900* primers. Even those mycobactin-dependent *M. avium* complex strains that contain sequences related to IS*900* failed to give PCR products. Presumably, differences in DNA sequence between IS*900* and the element present in these strains preclude PCR amplification. IS*900* therefore represents a source of highly specific DNA sequences that may be used as DNA probes for detection of *M. paratuberculosis* without interference from other mycobacteria, including *M. avium*.

The data presented here document the utility of the

IS*900*-specific PCR technique for the diagnosis of Johne's disease in dairy cattle by using fecal sampling. For the limited number of culture-positive samples we have examined so far, a sensitivity of 100% detection was obtained by PCR. The detection limit with the present procedures was approximately 10^2 organisms ml of feces^{-1}. The limit of detection reported for culture is similar (17); however, because of the problems mentioned above, a large proportion of false-negatives may be obtained at this low level of organisms. The detection of PCR-positive results among culture-negative samples was therefore perhaps not surprising. The PCR technique is known to be extremely susceptible to cross-contamination of samples, especially by those from prior amplification products. This property of the PCR technique is due to its extreme sensitivity and represents a potential source of false-positive results. However, extreme care was taken to avoid contamination during sample preparation. PCR-negative fecal samples were always extracted and tested in parallel with test samples. Target-free, PCR reagent negative controls were always performed. Sample and PCR reagent manipulations were executed with utmost care. The entire procedure, from sample preparation to PCR, was performed three times, and identical results were obtained in each case. We therefore believe that the positive PCR results in culture-negative samples reflect a substantial false-negative rate associated with fecal culture. Although culture is said to be capable of detecting 10^2 organisms ml^{-1} (17), it is likely that because of the problems mentioned above, practical detection limits may often be substantially higher. Alternatively, it may be that the PCR was, in fact, more sensitive than was estimated from the data shown in Fig. 2. Accurate counting of mycobacteria is notoriously difficult, and it is possible that fewer than 10^2 organisms ml^{-1} were detected. Using purified DNA as target, we routinely detect amounts of DNA equivalent to a single genome.

Unfortunately, it is not possible to exhaustively attempt to culture low levels of *M. paratuberculosis* from PCR-positive, culture-negative samples, as the samples are several months old by the time culture results are obtained. The culture-negative fecal samples all came from cattle suspected of being infected with *M. paratuberculosis*, and some of those cattle had clinical symptoms of Johne's disease. We therefore believe that the positive results by PCR are detecting false-culture-negative samples. Some of these may be from cattle shedding low numbers of organisms but with clinical disease, whereas in other cases, we may be detecting subclinical infections.

A possible source of error that could give rise to false-negative PCR results can arise from PCR inhibition by fecal-sample constituents. However, verification of a negative result can easily be achieved through detection of *M. paratuberculosis* DNA spiked into the negative sample. When this verification method was applied to the negative PCR samples described in this study, no evidence of inhibition of PCR by any of the samples was found (data not shown). Other experiments (to be presented elsewhere) document the presence of PCR inhibitors in field fecal samples and their removal or inactivation by the sample preparatory techniques used here.

The diagnosis of paratuberculosis is hampered by lack of rapid, accurate, and sensitive tests. Here, we describe a test, based on a DNA probe that is extremely specific for *M. paratuberculosis*, for the detection of *M. paratuberculosis* in fecal samples. The test is very rapid, with results in less than 24 h from receipt of the sample compared with 8 to 12 weeks for culture. The sensitivity obtained is greater than that routinely obtained with culture, so that infection in animals shedding very low numbers of *M. paratuberculosis* may be detected.

Paratuberculosis is thought to be one of the most economically important diseases affecting the livestock industry worldwide. Economic loss due to the disease is difficult to estimate, as the true prevalence of infection is unknown; however, the annual economic loss due to paratuberculosis was estimated to exceed $15.4 million in the New England area alone (4). Attempts to control or eradicate the disease are severely hampered by the inadequacies of present diagnostic methods. We describe, for the first time, a rapid and accurate diagnosis scheme for paratuberculosis infection, which would allow efficient control of the disease. This may become a priority if the role of *M. paratuberculosis* in Crohn's disease is established.

ACKNOWLEDGMENTS

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