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Detection of Bovine Trichomoniasis with a Specific DNA Probe and PCR Amplification System

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Trichomoniasis is a widespread, economically important venereal disease of cattle which causes infertility and abortion. Effective control of trichomoniasis has been impeded by the insensitivity of traditional diagnostic procedures, which require the isolation and cultivation of the parasite, *Tritrichomonas foetus*, from infected cattle. We developed a 0.85-kb *T. foetus* DNA probe by identifying conserved sequences in DNAs from *T. foetus* that were isolated from cattle in California, Idaho, Nevada, and Costa Rica. The probe hybridized specifically to DNAs of *T. foetus* isolates from different geographic areas but not to DNA preparations of *Trichomonas vaginalis*, bovine cells, or a variety of bacteria from cattle. The probe detected DNA from a minimum of 10^5 *T. foetus* organisms. To improve sensitivity, a partial sequence of the probe was used to identify oligonucleotide primers (TF1 and TF2) which could be used to amplify a 162-bp product from *T. foetus* DNAs by PCR. A chemiluminescent internal *T. foetus* sequence probe was hybridized to Southern blots of the amplification product. This system detected as few as one *T. foetus* organism in culture media or 10 parasites in samples containing bovine preputial smegma. Analysis of 52 clinical samples showed that 47 (90.4%) of the 52 samples were correctly identified, with no false-positive reactions. In comparison, the traditional cultivation method detected 44 (84.6%) of the 52 samples from *T. foetus*-infected and uninfected bulls. These results indicate that the PCR-based amplification system could be a useful alternative method for the diagnosis of bovine trichomoniasis.

Trichomoniasis has a worldwide distribution and is a major cause of infertility in naturally bred cattle in many countries (1, 11, 21). The causative agent, *Tritrichomonas foetus*, is a flagellated protozoan parasite that is transmitted from infected, asymptomatic bulls to heifers or cows at the time of coitus. The parasite then invades the vagina, uterus, and oviduct, causing embryonic death and infertility which may last for 2 to 6 months (4, 10, 14). In the western United States, the prevalence of trichomoniasis among bulls is estimated to be at least 5 to 8% (14, 22). A recent U.S. Department of Agriculture National Animal Health Monitoring System survey of California beef herds showed that 15.8% of randomly sampled herds had *T. foetus* infections, with a 27% mean within-herd prevalence of infected bulls (5).

A major limitation to more accurately determining the prevalence and economic impact of bovine trichomoniasis, as well as controlling the disease, is the insensitivity of current diagnostic procedures. At present, the diagnosis of trichomoniasis is based primarily on the identification of flagellated *T. foetus* parasites, by microscopic examination, in preputial scrapings or washings from bulls or cervicovaginal secretions from female cattle. Often, the number of trichomonads in these samples from infected cattle is relatively low and the parasites generally must be grown in culture before they reach detectable numbers (7, 22, 46). Diagnostic sensitivity can be improved 10 to 48% if the samples are placed in special medium and cultured in the laboratory for 2 to 5 days (15, 27, 41, 42). However, despite the additional time and expense, this method of cultivation detects only 81 to 90% of naturally infected bulls and only 58 to 78% of infected female cattle (15, 40, 42).

The objective of the project described here was to obviate the need for cultivation and to increase the accuracy of diagnosis by developing a highly sensitive diagnostic DNA probe that specifically detects *T. foetus* in samples from infected cattle. In this report, we describe the evaluation of a 0.85-kb *T. foetus* DNA probe which was developed by identifying conserved sequences in DNAs from different *T. foetus* isolates and cloned into a plasmid vector. In addition, a more sensitive method of parasite detection with specific primers selected from the probe sequence to amplify *T. foetus* DNA by PCR is described.

MATERIALS AND METHODS

Parasite cultivation. *T. foetus* isolates from California (isolates D1, RE2, and NA4) (6), Idaho (isolates RE20, RE23, and RE24), Nevada (isolates RE19), and Costa Rica (isolates CR1, CR2, CR3, CR4, CR5, and CR6) (31) were cultivated in Diamond's medium (12) supplemented with 5% heat-inactivated bovine serum at 37°C. *Trichomonas vaginalis* isolates TO15 and TO48 (kindly provided by John Alderete, Health Science Center, University of Texas, San Antonio) were grown in Diamond's medium with 8% heat-inactivated horse serum at 37°C. Test samples were taken during the logarithmic phase of growth, and the number of

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parasites was determined by diluting aliquots with phosphate-buffered saline (PBS; pH 7) and counting in a hemocytometer (×100 magnification) by using a light microscope.

Specimen collection. Smegma samples from 10 infected and 2 uninfected bulls were collected by insertion of a sterile 24-in. (61-cm) uterine infusion pipette through the preputial orifice to the fornix of the prepuce. The prepuce was scraped while applying vacuum with a disposable syringe. Smegma samples were mixed with PBS (pH 7) to a total volume of 1.5 ml. One milliliter of the diluted smegma sample was inoculated into culture medium, and the mixture was incubated at 37°C. Parasite growth was determined by observing the culture with an inverted microscope every 24 h for 7 days. Another 200-μl aliquot of each sample was used for DNA isolation and slot blot analyses. Bulls were classified as infected if any samples were culture positive, because *T. foetus* causes persistent infections in bulls (4). Smegma samples collected from healthy bulls that were determined to be uninfected on the basis of at least three consecutive negative culture samples were used as diluent in sensitivity tests and as negative controls.

Preparation of DNA for Southern and slot blot analyses. *T. foetus* and *T. vaginalis* DNAs were prepared as described previously (36), with slight modifications. Briefly, 1×10^8 cells in the logarithmic phase of growth (1×10^6 to 2×10^6/ml) were harvested by centrifugation at 4,000 × *g* for 10 min. Cells were washed twice with PBS (4°C) and were resuspended in 5 ml of NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris [pH 8]). Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.2% (wt/vol), and the solution was mixed gently before the DNA was extracted once each with an equal volume of phenol, a mixture of phenol-chloroform (1:1; vol/vol), and then chloroform. Two volumes of ice-cold 95% (vol/vol) ethanol and 0.1 volume of 3 M sodium acetate were added to precipitate the DNA. The DNA pellet obtained after centrifugation (12,000 × *g* for 20 min at 4°C) was rinsed with 70% (vol/vol) ethanol, air dried, and dissolved in 200 μl of TE (pH 8) buffer. DNase-free RNase (GIBCO BRL, Gaithersburg, Md.) was added to a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 30 min. Proteinase K (GIBCO BRL) was then added to a final concentration of 100 μg/ml, and the mixture was incubated at 37°C for 45 min. DNA was extracted with phenol, phenol-chloroform (1:1; vol/vol), and chloroform as described above, and the DNA was precipitated with 2 volumes of 95% (vol/vol) ethanol and 0.1 volume of 3 M sodium acetate. The DNA pellet was rinsed with 70% (vol/vol) ethanol, air dried, and dissolved in 200 μl of TE buffer. An average yield of 100 μg of DNA was obtained by this procedure. DNA was prepared from bovine thymocytes (kindly provided by Jeffery Stott, University of California, Davis) as described previously (8).

Acinetobacter calcoaceticus, *Campylobacter fetus* var. *veneralis*, *Corynebacterium pyogenes*, *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus somnus*, *Moraxella bovis*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus lactis*, and *Streptococcus viridans* (kindly provided by Veterinary Medicine Teaching Hospital Diagnostic Laboratory, University of California, Davis) were cultivated by standard methods (9, 49). Cells were harvested in the logarithmic growth phase, and genomic DNA was prepared as described previously (13).

Restriction enzyme digestion and cloning. The genomic DNAs of *T. foetus* isolates from various geographic locations were digested with either *Hin*dIII, *Eco*RI, *Hae*III, or *Sau*3A restriction endonuclease according to the manufacturer's instructions (GIBCO BRL). Digested DNA fragments were separated on a 1.4% (wt/vol) agarose gel (FMC Bioproducts, Rockland, Maine) in TBE (45 mM Tris-borate, 1 mM EDTA) at 40 V for 17 h. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination. A prominent band of 0.85 kb present in *Hin*dIII digests of the genomic DNAs of all *T. foetus* isolates was excised from agarose gels and purified by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.). The purified sequence was ligated into the *Hin*dIII site of the plasmid vector pUC19 by established methods (37). The ligated recombinant plasmids were introduced into competent *E. coli* JM83 cells, and individual transformants were selected after growth of the cells on Luria broth (LB) agar plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (37).

Southern blot hybridization. Restriction fragments separated on 1.4% agarose gels were denatured and transferred onto nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, Ill.) by Southern blotting as described previously (43). DNA was cross-linked to a nylon membrane by using a Stratalinker UV crosslinker (Stratagene, La Jolla, Calif.). Small-scale preparations of plasmid DNA were obtained by the alkaline miniprep method (37). The recombinant plasmid pUC19 was digested with *Hin*dIII, and the 0.85-kb fragment was recovered by using an NA45 DEAE membrane following the manufacturer's recommendation (Scheicher & Schuell, Keene, N.H.). The 0.85-kb fragment was labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by using a Random Primers DNA Labeling System (GIBCO BRL) and was hybridized to nylon membranes (Amersham Corp.) containing restriction endonuclease-digested *T. foetus* DNAs (37). Southern hybridization was carried out overnight in a 5× Denhardt's solution containing 5× SSC (20× SSC is 0.3 M sodium citrate [pH 7.0] and 3 M sodium chloride), 50% formamide, salmon testis DNA (100 μg/ml), 0.5% (wt/vol) nonfat dry milk, and 0.1% (wt/vol) SDS at 42°C. Membranes were washed twice in 4× SSC–0.1% SDS for 15 min at room temperature and were then washed twice in 1× SSC with 0.1% SDS for 30 min at 50°C. The autoradiographs were exposed to X-Omat film (Kodak, Rochester, N.Y.) with intensifying screens (DuPont Lighting Plus) for 24 to 72 h at −70°C.

Slot blot analyses. The specificity of the probe was tested by hybridization to slot blots of DNAs from various bacteria (5 μg), bovine thymocytes (5 μg), *T. vaginalis* (5 μg), and *T. foetus* (1 μg). DNAs were permanently bounded onto BA-S 85 nitrocellulose membranes by heating at 80°C for 30 min under vacuum according to the manufacturer's specifications (Scheicher & Schuell) and were hybridized with the [α-^{32}P]dCTP-labeled probe as described above.

Probe sensitivity was evaluated by hybridization of the [α-^{32}P]dCTP-labeled probe to DNA prepared from known numbers of in vitro-cultivated *T. foetus* mixed with smegma samples from healthy, uninfected bulls. Spiked smegma samples (200 μl) containing various numbers of *T. foetus* (10^3 to 10^7) were mixed with 2× NET buffer (200 μl), proteinase K (200 μg/ml), and 0.2% (wt/vol) SDS; incubated at 65°C for 15 min; and then extracted once with phenol, phenol-chloroform (1:1; vol/vol), and chloroform. DNAs were precipitated with 95% (vol/vol) ethanol and dissolved in 10 μl of TE buffer. Clinical specimens, negative smegma controls (no parasite DNA), and negative buffer controls (no DNA) were processed by the same procedure (36).

Selection and synthesis of primers. Plasmid DNA containing the probe was purified on a CsCl gradient as described previously (37) and was sequenced by the double-stranded

DNA dideoxynucleotide chain-termination sequencing method (38) with a Sequenase kit (Version 2.0; United States Biochemicals, Cleveland, Ohio). Aliquots of 3 to 5 μg of plasmid DNA were used for each reaction. The autoradiographs were developed at −70°C for 2 to 4 days. Oligonucleotide primers TF1 (5′-CATTATCCCAAATGGTATAAC-3′) and TF2 (5′-GTCATTAAGTACATAAATTC-3′) and an internal probe sequence (5′-CATCATTAATGCCTTTTG ATGGATCAGGCAACCATTTATA-3′) were synthesized by using a Cyclone Plus DNA Synthesizer (MilliGen/Millipore, Bedford, Mass.). Oligonucleotide products were purified with Oligo-Pak synthetic oligonucleotide purification columns as recommended by the manufacturer (MilliGen/Millipore).

Preparation of DNA samples for PCR. (i) Spiked smegma and culture samples. To test the sensitivity of the PCR detection system, various numbers of in vitro-cultivated *T. foetus* (1, 5, and 10 parasites) were mixed with 100-μl smegma samples from uninfected bulls. DNA was isolated by the rapid DNA extraction protocol by using an IsoQuick Nucleic Acid Extraction kit (MicroProbe Corp., Garden Grove, Calif.). Nucleic acid pellets were dissolved in RNase-free water for PCR. Negative smegma controls (no *T. foetus*) were included in each experiment. To investigate the effect of smegma samples on the sensitivity of the system, cultivated *T. foetus* cells were mixed with 100 μl of fresh culture medium, and DNA was isolated as described above.

(ii) Fresh clinical smegma samples. Smegma samples from infected and uninfected bulls were collected as described above. About 500 μl of each smegma sample was inoculated into Diamond's medium and observed daily in a separate laboratory (that of R.H.B.) for parasite growth. Another 500 μl of the sample was centrifuged at 4,000 × *g* for 5 min at room temperature. The supernatant was removed, leaving 100 μl of sample for DNA extraction by using the IsoQuick Nucleic Acid Extraction kit (MicroProbe Corp.). Briefly, the cell pellet was resuspended and mixed with 100 μl of lysis solution–700 μl of extraction matrix–400 μl of extraction buffer. Each sample was vortexed for 10 s and was centrifuged at 12,000 × *g* for 5 min. The aqueous phase was removed and mixed with equal volume of ice-cold isopropanol and 0.1 volume of sodium acetate to precipitate DNA at −20°C for 2 h or overnight for convenience. The DNA pellet obtained by centrifugation (12,000 × *g* for 10 min at 4°C) was rinsed with 70% (vol/vol) ethanol, air dried, and dissolved in RNase-free water for PCR (MicroProbe Corp.).

(iii) Bacteria samples. *Borrelia burgdorferi* DNA samples (isolates from Texas and Denmark) were obtained from R.B.L. *Escherichia coli* (isolates DH5α and T9100889), *Pasteurella multocida* (isolate P1059), and *Salmonella typhimurium* (isolates 845 and 1033) DNA samples were a gift kindly provided by Dwight Hirsh (University of California, Davis).

Amplification by PCR and product detection. DNA amplification was carried out in a total volume of 50 μl by using either the DNA sample obtained from clinical or spiked smegma samples, 250 to 300 ng of bacterial DNA, or 100 ng of bovine cell DNA. The reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 0.1% Triton X-100, 1.25 mM magnesium chloride, 200 μM (each) deoxynucleoside triphosphates, 0.384 μM primer TF1, and 0.365 μM primer TF2. After precycle denaturation at 94°C for 4 min to reduce nonspecific amplification (29), 1.25 U of *Taq* DNA polymerase (Promega Corp., Madison, Wis.) was added and the mixture was overlaid with 50 μl of mineral oil. Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.) for 41 cycles, as follows: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. The last cycle was given a prolonged extension period of 7 min. Positive (100 ng of the 0.85-kb probe) and negative reagent controls were included with each batch of test samples. To avoid carryover of contaminating nucleic acids which may result in false-positive reactions (25, 32), the pre-PCR sample preparation, mixing of PCR reagents with the samples, and the final PCR thermal cycle reactions were performed in separate laboratories (24, 30).

After amplification, 5 μl of each sample or a BioMarker Low (BioVentures, Inc., Murfreeboro, Tenn.) DNA size standard was mixed with 1 μl of 6× loading dye and the mixture was electrophoresed on a 3% Nusieve 3:1 agarose gel (FMC Bioproducts). The gel was stained in a 0.5-μg/ml ethidium bromide solution for 30 min and was observed for the presence of amplification products under UV illumination. Amplification products were denatured in the gel and were transferred to nylon membranes (Amersham Corp.) by Southern blotting as described previously (37). DNA was cross-linked to a nylon membrane by using a Stratalinker UV crosslinker (Stratagene, La Jolla, Calif.). Prehybridization, preparation of the labeled internal probe, and hybridization were performed as recommended by the manufacturer of the Enhanced Chemiluminescence 3′-Oligolabeling and Detection Systems (Amersham). Labeled internal probe was added to a final concentration of 10 ng/ml of hybridization solution, and the mixture was incubated overnight at 50°C with gentle agitation. After hybridization, the membranes were washed twice for 5 min each time at room temperature in 5× SSC–0.1% (wt/vol) SDS and were then washed twice for 15 min each time at 60°C in 0.1× SSC–0.1% (wt/vol) SDS. Membrane blocking, antibody incubations, and signal generation and detection were performed as described by the manufacturer. Membranes were exposed to Kodak X-Omat film for 3 to 10 min.

RESULTS

Restriction enzyme digestion and Southern hybridization. Restriction endonuclease-digested DNAs from different *T. foetus* isolates were analyzed, and bands that were prominent and conserved among all of the isolates were identified as candidate *T. foetus* probes. Restriction fragment length polymorphisms were discernible when DNAs of *T. foetus* isolates from California (D1, RE2, and NA4), Idaho (RE20, RE23, and RE24), Nevada (RE19), and Costa Rica (CR1, CR2, and CR3) were digested with *Hin*dIII, *Eco*RI, *Hae*III, or *Sau*3A and the fragments were separated on 1.4% agarose gels (data not shown). A prominent band of 0.85 kb from *Hin*dIII-digested genomic DNAs which was present in all *T. foetus* isolates was purified and cloned into plasmid vector pUC 19. Figure 1 illustrates the typical Southern blot hybridization patterns observed when *Eco*RI-digested (lane 1) and *Hin*dIII-digested (lane 2) *T. foetus* DNA was hybridized with the radiolabeled 0.85-kb *T. foetus* sequence. The autoradiograph showed that the probe hybridized to a 0.85-kb fragment in *Hin*dIII-digested DNA (lane 2) as well as to multiple fragments at higher molecular masses in both digests. These hybridization patterns indicated that this may be a repetitive sequence in the *T. foetus* genome.

Specificity test of the probe by slot blot hybridization. To evaluate the specificity of the probe, the radiolabeled 0.85-kb DNA probe was hybridized to slot blots of DNAs prepared

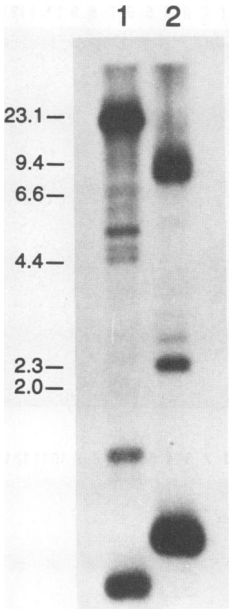

FIG. 1. Southern blot of *Eco*RI or *Hin*dIII-digested *T. foetus* DNA hybridized with a radiolabeled 0.85-kb *T. foetus* probe. Lanes: 1, *T. foetus* D1 DNA digested with *Eco*RI; 2, *T. foetus* D1 DNA digested with *Hin*dIII. Molecular sizes are indicated on left (in kilobases).

from *T. foetus* (isolates D1, RE19, CR1, CR2, CR3, CR4, CR5, CR6), *T. vaginalis* (isolates T015 and T048), bacteria (*Acinetobacter calcoaceticus*, *Campylobacter fetus* var. *veneralis*, *Corynebacterium pyogenes*, *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus somnus*, *Moraxella bovis*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus lactis*, *Streptococcus viridans*), bovine thymocytes, negative buffer controls (no DNA), and a positive control (0.85-kb probe). Figure 2 shows that the probe hybridized to DNAs of *T. foetus* isolates from various geographic areas (A1 to A8) but not to DNAs of *T. vaginalis* (A9 and A10), bovine thymocytes (A11), or different bacteria (B1 to B11).

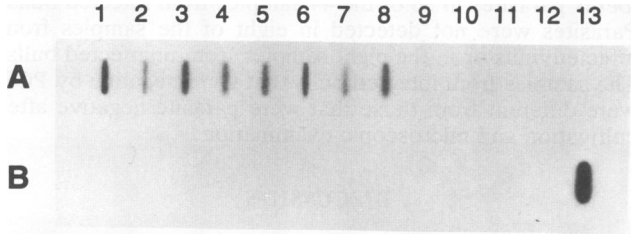

FIG. 2. Specificity test of *T. foetus* probe hybridized to slot-blotted DNAs of trichomonads, bacteria, and bovine cells. A1, *T. foetus* D1; A2, *T. foetus* RE19; A3, *T. foetus* CR1; A4, *T. foetus* CR2; A5, *T. foetus* CR3; A6, *T. foetus* CR4; A7, *T. foetus* CR5; A8, *T. foetus* CR6; A9, *T. vaginalis* T048; A10, *T. vaginalis* T015; A11, bovine thymocytes; B1, *Acinetobacter calcoaceticus*; B2, *Campylobacter fetus* var. *veneralis*; B3, *Corynebacterium pyogenes*; B4, *Enterobacter aerogenes*; B5, *Escherichia coli*; B6, *Haemophilus somnus*; B7, *Moraxella bovis*; B8, *Staphylococcus epidermidis*; B9, *Staphylococcus saprophyticus*; B10, *Streptococcus lactis*; B11, *Streptococcus viridans*; A12, A13, and B12, negative buffer controls (no DNA); B13, positive control (0.85-kb probe).

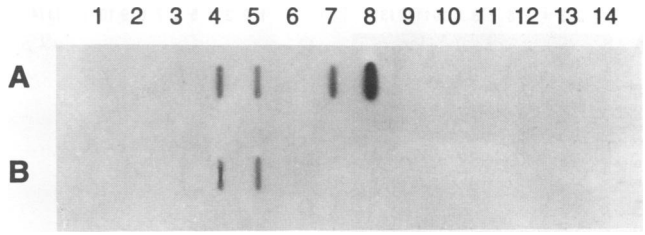

FIG. 3. Sensitivity test of *T. foetus* probe by hybridization to slot-blotted DNAs of spiked and clinical smegma samples. A1 and B1, 10^3 *T. foetus*; A2 and B2, 10^4 *T. foetus*; A3 and B3, 10^5 *T. foetus*; A4 and B4, 10^6 *T. foetus*; A5 and B5, 10^7 *T. foetus*; A6, negative smegma control; A7, 1 μg of *T. foetus* DNA; A8, 0.85-kb probe; A9 to A14 and B9 to B12, smegma samples from *T. foetus*-infected bulls; B13 and B14, smegma samples from healthy, uninfected bulls; B6 and B8, negative buffer control (no DNA); B7, bovine thymocytes.

Sensitivity test of the probe by slot blot hybridization. Repeated experiments were conducted to determine probe sensitivity by hybridization of the radiolabeled 0.85-kb probe to smegma samples spiked with various numbers of *T. foetus* parasites. Figure 3 shows that the probe reliably detected DNA from 10^7 (A5 and B5) and 10^6 (A4 and B4) *T. foetus* organisms. The minimum number of parasites detectable by this method was 10^5 (A3 and B3) organisms. The DNA probe was further used to analyze clinical smegma specimens from 10 infected (Fig. 3, A9 to A14 and B9 to B12) and 2 uninfected (Fig. 3, B13 and B14) bulls. The probe showed no reactivity with the clinical specimens under these experimental conditions.

PCR amplification of *T. foetus* DNA. To improve the sensitivity of the detection system, the probe sequence was used to identify two oligonucleotide primers (TF1 and TF2) and a 162-bp target sequence for a PCR-based amplification system. By using these primers, PCR products of approximately 162 bp were amplified from the DNAs of all nine *T. foetus* isolates from California, Nevada, Idaho, and Costa Rica (Fig. 4A, lanes 2 to 10). No PCR products were produced with DNAs from the different bacteria tested (Fig. 4B, lanes 2 to 8). Bovine cell DNA had an amplification product of approximately 400 bp (Fig. 4B, lane 9). Multiple amplification products were obtained with *T. vaginalis* DNA (Fig. 4B, lane 10), and these were easily distinguished from the PCR products produced with *T. foetus* DNA (162 bp) or the 0.85-kb probe controls (Fig. 4A and B, lanes 11). In repeated experiments, amplification products were not produced with any of the negative reagent controls (Fig. 4A and B, lanes 12 to 14). Only the amplification products produced with *T. foetus* DNAs (Fig. 4C, lanes 2 to 10) and the 0.85-kb probe controls (Fig. 4C and D, lanes 11) showed hybridization signals with the chemiluminescent internal probe.

To test the sensitivity of the PCR detection system, 1:10 serial dilutions of cultivated *T. foetus* parasites were added to smegma samples from uninfected bulls. All samples were tested in triplicate in repeated experiments. PCR amplification products were observed in an ethidium bromide-stained gel from all three samples containing 10 *T. foetus*, one of three samples containing 5 *T. foetus*, and one of three samples containing 1 *T. foetus* (Fig. 5A, lanes 2 to 10). No amplification product was produced from smegma sample controls (Fig. 5A, lanes 11 to 13). To determine the effect of smegma on the sensitivity of the PCR detection system, 1:10 serial dilutions of *T. foetus* parasites were added to culture

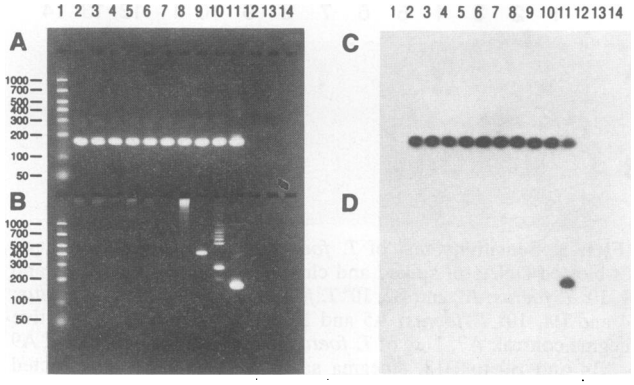

FIG. 4. Specificity of *T. foetus* detection by PCR amplification of *T. foetus*, *T. vaginalis*, bacteria, and bovine cell DNAs. Amplification products were electrophoresed on a 3% Nusieve 3:1 agarose gel stained with ethidium bromide (A and B) and hybridized to a chemiluminescent internal probe (C and D). (A) Lanes: 2 to 10, *T. foetus* D1, NA4, RE19, RE20, RE23, RE24, CR1, CR2, and CR3, respectively; 11, 0.85-kb probe control; 12 to 14, negative reagent controls. Molecular sizes are indicated on the left (in base pairs). (B) Lanes: 2 and 3, *Borrelia burgdorferi* isolates from Texas and Denmark, respectively; 4 and 5, *Escherichia coli* DH5α and T9100889, respectively; 6, *Pasteurella multocida* P1059; 7 and 8, *Salmonella typhimurium* 845 and 1033, respectively; 9, bovine cells; 10, *T. vaginalis*; 11, 0.85-kb probe control; 12 to 14, negative reagent controls. Molecular sizes are indicated on the left (in base pairs).

medium. The result showed that in the absence of smegma, amplification products could be produced from one *T. foetus* organism (Fig. 5B, lanes 8 to 10). Thus, substances in the smegma may be responsible for the inhibition of *Taq* DNA polymerase or the thick, mucus-like smegma may interfere with DNA isolation and thereby reduces the sensitivity of the test.

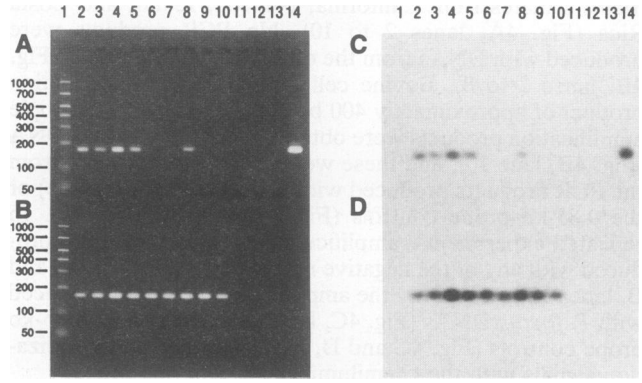

FIG. 5. Sensitivity of *T. foetus* detection by PCR of spiked smegma and cultured samples. Amplification products were electrophoresed on a 3% Nusieve 3:1 agarose gel stained with ethidium bromide (A and B) and hybridized to a chemiluminescent internal probe (C and D). (A) Lanes: 2 to 4, 10 *T. foetus* added to smegma samples; 5 to 7, 5 *T. foetus* added to smegma samples; 8 to 10, 1 *T. foetus* added to smegma samples; 11 to 13, smegma sample-negative controls; 14, 0.85-kb probe control. Molecular sizes are indicated on the left (in base pairs). (B) Lanes: 2 to 4, 10 *T. foetus* in culture medium; 5 to 7, 5 *T. foetus* in culture medium; 8 to 10, 1 *T. foetus* in culture medium; 11 to 13, culture medium-negative controls; 14, negative reagent controls. Molecular sizes are indicated on the left (in base pairs).

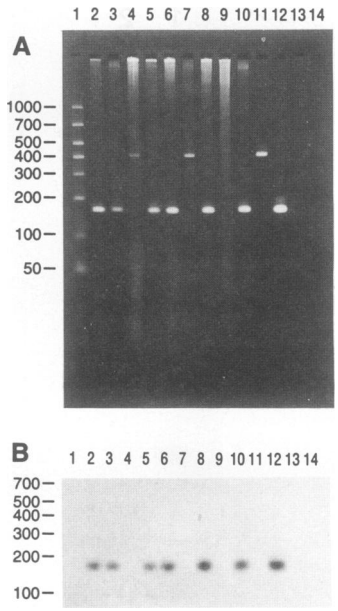

FIG. 6. Detection of *T. foetus* in clinical smegma samples by PCR amplification. Amplification products were electrophoresed on a 3% Nusieve 3:1 agarose gel stained with ethidium bromide (A) and hybridized to a chemiluminescent internal probe (B). (A) Lanes: 2, 3, 5, 6, 8, and 10, smegma samples from infected bulls showing amplification of *T. foetus* DNA by PCR; 4 and 7, smegma samples from uninfected bulls; 9, smegma sample from infected bull with no detectable PCR amplification product; 11, bovine cell DNA; 12, 0.85-kb probe control; 13 and 14, negative reagent controls. Molecular sizes are indicated on the left (in base pairs).

Detection of *T. foetus* in clinical smegma samples by PCR amplification. Smegma samples from 10 infected bulls and 2 healthy uninfected bulls were collected weekly and evaluated by PCR and by in vitro cultivation for the presence of parasites. The PCR detection system identified 39 of the 44 samples from infected bulls as positive, whereas no parasite DNA was amplified from the other 5 samples from the infected bulls or any of the 8 samples from uninfected bulls (Fig. 6). No false-positive samples were reported by the PCR detection system. In vitro cultivation and microscopic examination of aliquots from the same samples revealed *T. foetus* parasites in 36 of the 44 samples from infected bulls. Parasites were not detected in eight of the samples from infected bulls or in the eight samples from uninfected bulls. The samples from infected bulls that were negative by PCR were different from those that were parasite negative after cultivation and microscopic examination.

DISCUSSION

Accurate diagnosis is essential for the prevention and control of bovine trichomoniasis, particularly in the absence of an approved treatment method (4) or a completely efficacious vaccine (6, 19, 23, 39). Currently, a dependable immunological test for bovine trichomoniasis is not available (50). Cultivation and microscopic examination are the standard methods used for the diagnosis of *T. foetus* infection in cattle. The technique is specific; however, it is time-consuming and lacks sensitivity (15, 41, 42). Development of highly sensitive and specific DNA probes was undertaken as a means of improving the diagnosis of *T. foetus*. The 0.85-kb

sequence was selected as the best candidate probe on the basis of its specificity for the parasite and no cross-reactivity with bovine cell DNA or DNAs from pathogenic and nonpathogenic bacteria which inhabit the bovine reproductive tract (9, 49).

Although the 0.85-kb probe was highly specific for *T. foetus*, the sensitivity of the system was limited to the detection of 10^5 *T. foetus* organisms. To overcome this problem, we used PCR technology, similar to that which has been used for the improved diagnosis of other pathogenic protozoa (3, 18, 33, 35). The application of this technology allowed for the reliable detection of a single *T. foetus* organism in culture medium or 10 *T. foetus* organisms in spiked smegma samples. The PCR system amplified the DNAs of *T. foetus* isolates from different geographic areas. In addition, the system amplified *T. vaginalis* DNA to produce a unique amplification product pattern which was easily distinguished from that produced with *T. foetus* DNA. Riley et al. (35) reported that notable genomic differences exist among *T. vaginalis* isolates, and not all PCR primer pairs in their study amplified products with the same intensity. Therefore, the TF1 and TF2 primers should be further evaluated to determine their usefulness in the detection of different *T. vaginalis* isolates.

In the initial development of the PCR-based detection system for *T. foetus*, we attempted to use an easy method of sample preparation by heating samples at 97°C for 15 min during the precycle denaturation step (20). Unfortunately, no PCR amplification products were obtained by this method with known infected bull samples or culture-derived parasites (data not shown). This may be because trichomonads, such as *T. foetus* and *T. vaginalis*, contain relatively large amounts of endogenous nucleases which digest their DNAs completely in a short period of time after cell lysis occurs (26, 28, 47, 48). Therefore, a fast, easy, and reliable method for sample preparation was needed to isolate intact genomic DNA from trichomonads. Previously described methods for the isolation of trichomonad DNA are effective (34, 36, 48), but time-consuming. With the rapid procedure (IsoQuick Nucleic Acid Extraction kit) used in our study, large numbers of samples can be handled simultaneously. DNA isolation is reliable and easy to perform by using guandinium thiocyanate in the lysis solution to inhibit nuclease activity and an organic extraction matrix to remove protein and cell debris without using phenol or chloroform.

This is the first report of a PCR-based amplification system for the detection of *T. foetus* infections in cattle. Speer and colleagues (44, 45) previously reported the development of a *T. foetus*-specific DNA probe. However, a recent comparison of different diagnostic methods showed that this DNA probe lacks the sensitivity needed for the effective diagnosis of bovine trichomoniasis (2).

In a comparison of the PCR-based system with in vitro cultivation, neither system showed 100% sensitivity in detecting *T. foetus* in clinical samples from infected bulls. Although a single *T. foetus* trophozoite was detected with the PCR-based amplification system, test sensitivity may be lower because of inhibition of *Taq* DNA polymerase by inhibitors (16, 17, 20) in the bovine smegma that are not removed in the process of DNA isolation. The sensitivity of the cultivation method may be restricted by a lack of sufficient numbers of viable parasites in some smegma samples that are able to adapt to culture and grow to detectable numbers. Overall, the PCR-based *T. foetus* detection system appears to be highly specific and at least as sensitive as the cultivation method, with the advantage that this molecular method can accommodate a large number of samples, is easy to perform, and can be performed relatively quickly. The total time required for sample collection, DNA isolation, and visualization of an amplification product on an ethidium bromide-stained gel was 1 working day. Confirmation of *T. foetus*-specific amplification products by hybridization with the chemiluminescent internal probe could be completed on the next day. Therefore, by standardizing the method of smegma collection and processing, the PCR detection system described here may provide a powerful, efficient, and reliable alternative method for detecting *T. foetus* infections in cattle.

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