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PCR-Based Study of Conserved and Variable DNA Sequences of *Tritrichomonas foetus* Isolates from Saskatchewan, Canada

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The protozoan parasite *Tritrichomonas foetus* causes infertility and spontaneous abortion in cattle. In Saskatchewan, Canada, the culture prevalence of trichomonads was 65 of 1,048 (6%) among 1,048 bulls tested within a 1-year period ending in April 1994. Saskatchewan was previously thought to be free of the parasite. To confirm the culture results, possible *T. foetus* DNA presence was determined by the PCR. All of the 16 culture-positive isolates tested were PCR positive by a single-band test, but one PCR product was weak. DNA fingerprinting by both T17 PCR and randomly amplified polymorphic DNA PCR revealed genetic variation or polymorphism among the *T. foetus* isolates. T17 PCR also revealed conserved loci that distinguished these *T. foetus* isolates from *Trichomonas vaginalis*, from a variety of other protozoa, and from prokaryotes. TCO-1 PCR, a PCR test designed to sample DNA sequences homologous to the 5′ flank of a highly conserved cell division control gene, detected genetic polymorphism at low stringency and a conserved, single locus at higher stringency. These findings suggested that *T. foetus* isolates exhibit both conserved genetic loci and polymorphic loci detectable by independent PCR methods. Both conserved and polymorphic genetic loci may prove useful for improved clinical diagnosis of *T. foetus*. The polymorphic loci detected by PCR suggested either a long history of infection or multiple lines of *T. foetus* infection in Saskatchewan. Polymorphic loci detected by PCR may provide data for epidemiologic studies of *T. foetus*.

Tritrichomonas foetus is a venereally transmitted protozoan parasite found in infected reproductive tracts of male and female cattle. The parasite is considered to be a major cause of infertility and abortion, and thus economic loss, in many cattle producing areas (15, 16, 18, 19, 24, 33). Infection is generally nonpathogenic in bulls, in which the organism is usually restricted to the preputial cavity. Once they are infected, it is thought that bulls remain infected for life (24, 33). In cows, the primary sites of infection are the cervix and uterus (33), although colonization may also occur at other sites in the female reproductive system (24). It is believed that most infections in cows resolve spontaneously within 3 months (33).

Diagnosis may be difficult, as investigations are often initiated under harsh field conditions. Diagnosis currently relies on direct microscopic examination of cultures. After characteristically prolonged transit times and exposure to unfavorable sampling conditions, failure of *T. foetus* isolates to survive may result in false-negative culture results. For example, Saskatchewan was thought to be free of the parasite until recently, when *T. foetus* was diagnosed as the cause of low reproductive rates among naturally bred cattle residing on community pastures (5). For these reasons, DNA-based diagnostic methods, independent of organism survival, hold considerable appeal for confirmation of culture results or as an independent diagnostic method.

One approach to the development of DNA-based diagnosis is to use genetic loci conserved among *T. foetus* isolates. A common, major surface antigen was found among *T. foetus* isolates (4), while other antigens appear to vary in type or amount expressed (10, 27). Such antigenic variations need not reflect genetic variation, whose extent is still undetermined. There is limited information about possible genetic subtypes or variants within the species.

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Understanding of the genetic variability among *T. foetus* isolates may provide valuable tools for diagnostic and epidemiologic studies. Genetic variants may be directly studied at the DNA level. Methods applied with other parasite systems include random amplified polymorphic DNA (RAPD) analysis, a PCR method that detected genomic variants in a number of protozoa, fungi, and bacteria (3, 28, 30); M13 DNA fingerprinting, which detected genomic heterogeneity in *Giardia lamblia* isolates (6); and T17 PCR, which detected genomic variability in *Trichomonas vaginalis*, *G. lamblia*, and a variety of other protozoa (22).

Here we performed a culture-based study of *T. foetus* infection in Saskatchewan. We also evaluated single-band PCR tests as a complement to culture diagnosis. Finally, we used multiple PCR techniques, including T17 PCR, RAPD analysis, and PCR amplification of a target near conserved, cell division control-like sequences, to investigate genetic polymorphism among *T. foetus* isolates.

MATERIALS AND METHODS

Parasites. (i) *T. foetus* field isolates from Saskatchewan. In May 1993, five bulls from a community pasture in Saskatchewan were found to harbor *T. foetus* (5). This prompted testing of bulls from many community pastures in the province over the following year. The pastures were summer grazing and breeding areas for beef cows, calves, and yearlings from several owners. Bulls were supplied by the pasture managers.

The original diagnoses of *T. foetus* infections were made by inoculating preputial washings from bulls into CPLM medium (1) in screw-top glass tubes. The tubes were maintained at 37°C for 48 h prior to examination. *T. foetus* organisms were identified by their characteristic morphology and motility as viewed by dark-field microscopy.

Since early 1994, preputial scrapings have been used instead of preputial washings, and a commercial system (2) (In-Pouch; Biomedical Diagnostics, San Jose, Calif.) has replaced CPLM medium for primary cultures. Isolates from 16 bulls from various locations throughout the province collected during 1993 and 1994 were maintained at 37°C in CPLM medium (1). The isolates examined in these studies included the following: *T. foetus* MC11, *T. foetus* SC, *T. foetus* excel, *T. foetus* BoR1, *T. foetus* Colo, *T. foetus* R6, *T. foetus* MC14, *T. foetus* R2, *T.*

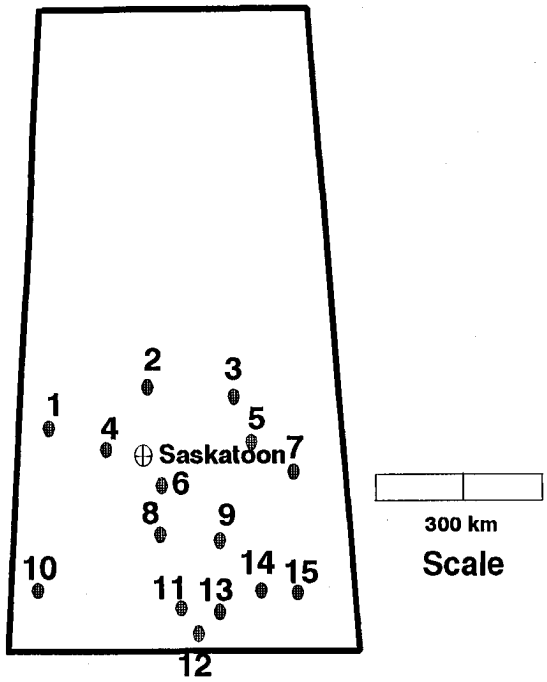

FIG. 1. Distribution of *T. foetus* in bulls in Saskatchewan sampled between May 1993 and April 1994. Hatched ovals indicate positive *T. foetus* cultures. *T. foetus* was found throughout southern Saskatchewan, where cattle are often maintained in community pastures. Northern Saskatchewan is heavily forested and has few commercial cattle herds. Locations: 1, Unity; 2, Shellbrooke; 3, Tisdale; 4, Borden; 5, Kelvington; 6, Dundurn; 7, Preeceville; 8, Central Butte; 9, Regina; 10, Maple Creek; 11, Assiniboia; 12, Rockglen; 13, Ogema; 14, Weyburn; 15, Stoughton.

foetus MC30, *T. foetus* R1, *T. foetus* R3, *T. foetus* R7, *T. foetus* B2, *T. foetus* R4, *T. foetus* R5, and *T. foetus* MC52 (Fig. 1).

(ii) Other trichomonads. An Austrian isolate, *T. foetus* 50151, was obtained from the American Type Culture Collection (Rockville, Md.). *T. vaginalis* isolates used for comparison in these studies included clinical *T. vaginalis* S933, S1047, S1114, S1126, and S1163 from patients attending the Harborview Medical Center Sexually Transmitted Diseases Clinic and the University of Washington Medical Center Prostatitis Clinic (14).

(iii) Other microorganisms. We evaluated a number of other protozoa, including amoebas, yeasts, cilliates, *Giardia* spp., *Leishmania* spp., and *Trypanosoma* spp. as well as a variety of prokaryotes.

Acanthamoeba castellani, *Acanthamoeba rhisodes*, *Acanthamoeba hatchetti*, and *Acanthamoeba culbertsoni* cultures were generous gifts from Thomas Fritche (University of Washington). *Acanthamoeba* cells were washed twice in sterile phosphate-buffered saline (PBS) and subsequently treated in the same way as *T. foetus* cells.

G. lamblia cysts H7 (from a human in West Virginia), K1, P1 (from a human in Portland, Oregon), PB1 (from a human in Portland, Oregon), WB1B (from a human in Afghanistan), BE1 (from a beaver in Canada), BE4 (from a beaver in Canada), and BE5 (from a beaver in Canada) and *Giardia muris* CuH1 DNAs were obtained from Jerry E. Ongerth and Mansour Samadpour (University of Washington). Trophozoites were cultivated in TYI-S33 medium (29) with 10% calf serum supplemented with ampicillin (50 μg/ml) and streptomycin (40 μg/ml).

Tetrahymena thermophilia CU427 DNA was obtained from Meng Chao Yao (Fred Hutchinson Cancer Research Center, Seattle, Wash.). *Paramecium tetraurelia* DNA was a gift from Robert Hinrichsen (Fred Hutchinson Cancer Research Center). *Leishmania donovani* 2D and DDB DNAs were gifts from Cindy Tripp and Kenneth Stuart (Seattle Biomedical Research Institute, Seattle, Wash.). *L. donovani* DDB is a human isolate (India). *Trypanosoma equiparitum* and *Trypanosoma cruzi* DNAs were gifts from Harvey Eisen (Fred Hutchinson Cancer Research Institute). *Saccharomyces cerevisiae* DNA was a gift from Stephan Zweifel and Walton Fangman (University of Washington).

In addition, we evaluated prokaryotes that inhabit the genitourinary and gastrointestinal tracts. Prokaryotic DNAs were obtained from Marilyn Roberts and George Kenny (University of Washington, Seattle). Prokaryotic samples included DNAs from the prokaryotes *Escherichia coli* DH5α, *Gardnerella vaginalis* GV9016B, *Bacteroides intermedius* 25261, *Peptostreptococcus anaerobius* Clt 17.5, *Bacteroides disiens* Clt 75.5, *Peptostreptococcus tetradius* Clt 92.2, *Fusobacterium nucleatum* 6601 TE10, *Veillonella parvula* 10028, *Enterococcus faecalis* DS160, *Mobiluncus curtisii* 73, *Streptococcus agalactiae* AES 14, *Mycoplasma fermentans*, *Bacteroides fragilis*, *Bacteroides intermedius* 25361, and *Ureaplasma urealyticum* UU5 (22).

Molecular methods. (i) DNA isolation. *T. foetus* DNA and other protozoan DNAs were purified by a previously described method (21) unless otherwise specified. Briefly, mid-log-phase cultures (75 to 150 ml) were harvested by centrifugation at 900 × *g* for 5 min at 4°C. The cellular pellet (0.5 ml) was washed twice by centrifugation in 40 volumes of cold (4°C), sterile PBS (pH 7.4) and suspended in 1 ml of PBS. A 20-μl portion of diethylpyrocarbonate (Sigma, St. Louis, Mo.) was added to the cell suspension. The cells were subjected to two cycles of centrifugation (1,300 × *g* for *T. foetus*; 900 × *g* for *T. vaginalis*) for 3 min at 4°C in 1% (vol/vol) Triton X-100 (Sigma) in reticulocyte standard buffer (10 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$) that had been treated with 100 μl of diethylpyrocarbonate.

The resulting pellet was suspended in a solution containing 0.5 ml of 10 mM EDTA and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and then incubated at 65°C for 15 min to inactivate the diethylpyrocarbonate. The suspension was then treated with 0.2 mg of proteinase K (Bethesda Research Laboratories, Gaithersburg, Md.) per ml at 37°C for 1 h and subjected to three cycles of phenol (Bethesda Research Laboratories)-chloroform (Sigma)-isoamyl alcohol (Sigma) (1:1:0.02 [vol/vol]) extraction, two cycles of chloroform-isoamyl alcohol (1:0.04 [vol/vol]) extraction, and 80% (vol/vol) ethanol precipitation. Cultures were harvested in logarithmic phase when the total cell number was approximately 10^7 *T. foetus* or *T. vaginalis* cells as determined by hemocytometer counts. Each culture yielded 100 to 200 μg of trichomonad DNA (as determined by the A_{260}). Both single- and multiple-band PCR tests employed 10 ng of template DNA per reaction.

(ii) Agarose gel electrophoresis. Agarose gels consisted of 1.4% (wt/vol) agarose (Fisher Scientific, Fair Lawn, N.J.) in a solution containing 90 mM Tris buffer (Fisher Scientific), 90 mM boric acid (Sigma), and 25 mM EDTA (pH 8.3). A 20-μl portion of each PCR mixture was combined with 5 μl of a solution containing 0.5% SDS, 50 mM EDTA (pH 7.4, adjusted with NaOH) 45% (vol/vol) glycerol (Sigma), and 0.1% (wt/vol) bromophenol blue (Sigma). Molten gel solutions and electrophoretic running buffers contained 0.5 μg of ethidium bromide (Sigma) per ml for visualization of nucleic acids under UV illumination. Electrophoresis was conducted at 100 V for 3.5 h.

(iii) Southern blot hybridization. Single-band PCR products were verified by Southern blotting and hybridization with labeled oligonucleotide probes. PCR products were transferred from agarose gels to Nytran (Schleicher and Schuell) by using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 mM sodium citrate) as the blotting medium. The internal probe for the TF1-TF2 PCR product was TF3, with the sequence GGATCAGGCAACCATTTATA (9). The probe for S-TCO-1 PCR products was CDO7 (TGGGCGATTCTCAAGTTG). CDO7 is complementary to the coding region of a known trichomonad cell division control (CDC2) homolog (20). Prior to hybridization, membranes were rinsed with 4× SSC, air dried, and DNA cross-linked to the membrane by 2 min of irradiation on a UV transilluminator (Fisher). Membranes were prehybridized in a solution containing 6× SSC, 5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll), 0.1% SDS, and 100 μg of salmon testis DNA per ml for 4 h at 45°C.

Oligonucleotide probes were end labeled by using T4 polynucleotide kinase (Bethesda Research Laboratories) and gamma-labeled [^{32}P]ATP (New England Nuclear). Hybridization was performed overnight at 45°C in buffer identical to the prehybridization buffer with the addition of 10^6 cpm of boiled oligonucleotide probe. Membranes were rinsed once in 2× SSC–0.01% SDS at 22°C and then twice in the same buffer at 45°C.

(iv) PCR. The amount of template DNA was constant at 10 ng for both single-target and multiple-target PCRs. Oligonucleotide primers were synthesized with a Cyclone DNA synthesizer (Millipore, Bedford, Mass.). The TF1-TF2 single-band PCR test used published primer sequences (9). TCO-1 amplifications used the 57-mer, GGATCCATCGGAATTAAAAAACCGAAAAA AAAATTTAGGGTAAAAAAAATCATTACA, complementary to the control region of a trichomonad cell division control-like sequence (20). The T17 PCR primer set sequences were previously reported (22). RAPD PCR primers TAP5 and TAP6 had the sequences ATGTTCTATCTTTTCA and ACCACCTTAG TTTACA, respectively, while S-TCO-1 amplifications used the TCO-1 57-mer described above but at higher stringency as specified below.

PCR buffer for TF1-TF2 PCR consisted of 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 1.25 mM $MgCl_2$. PCR buffer for T17, RAPD, TCO-1, and S-TCO-1 PCR consisted of 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.; 1 to 5 U per 100 μl of reaction mixture) was used as the thermostable enzyme.

Thermal cycles for TF1-TF2 PCR (9) consisted of 94°C for 5 min followed by 41 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min followed by a 7-min extension at 72°C. Thermal cycles for T17 PCR consisted of 7 min at 94°C, followed by 40 cycles of sequential variation between 94, 50, and 72°C (1 min each), followed by a 7-min extension cycle at 72°C. Thermal cycles for RAPD PCR consisted of 3 min at 94°C, followed by 46 cycles of 94°C for 20 s, 47°C for 20 s, and 67°C for 20 s, followed by a 5-min extension cycle at 72°C.

Thermal cycles for TCO-1 PCR were 3 min at 94°C, followed by 46 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a 5-min extension cycle

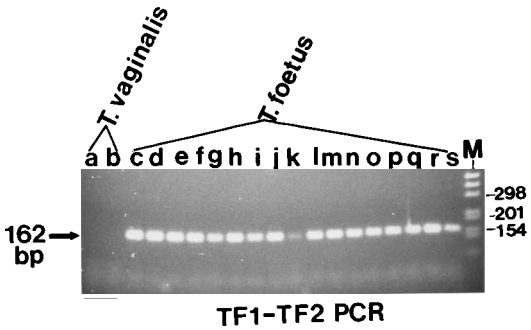

FIG. 2. Single-site PCR test for identification of *T. foetus* isolated from bulls. DNA was purified from two *T. vaginalis* isolates and one *T. foetus* American Type Culture Collection isolate and from cultures of preputial washes of 16 bulls from the province of Saskatchewan, Canada. Ten nanograms of each DNA was subjected to PCR using primers TF1 and TF2 (9). PCR products were electrophoresed in 1.4% agarose for 3.5 h at 90 V and then subjected to ethidium bromide staining, UV illumination, and photography. The expected PCR product size was 162 bp (arrow). Lanes: a, *T. vaginalis* isolate S933; b, *T. vaginalis* S1047; c, *T. foetus* 50151 (American Type Culture Collection); d, *T. foetus* MC11; e, *T. foetus* SC; f, *T. foetus* excel; g, *T. foetus* BoR1; h, *T. foetus* Colo; i, *T. foetus* R6; j, *T. foetus* MC14; k, *T. foetus* R2; l, *T. foetus* MC30; m, *T. foetus* R1; n, *T. foetus* R3; o, *T. foetus* R7; p, *T. foetus* B2; q, *T. foetus* R4; r, *T. foetus* R5; s, *T. foetus* MC52. M, 1-kb molecular size marker from Bethesda Research Laboratories. Molecular standard sizes in base pairs are indicated on the right.

at 72°C. While these conditions would be considered normal stringency for 20-base PCR primers, they constitute low to moderate stringency for the TCO-1 57-mer. In separate experiments, termed S-TCO-1 PCR, the same primer was used with the annealing temperature raised to 52°C, a more stringent condition that limited the number of targets amplified.

T17 PCR product patterns including minor bands were insensitive to the amount of template DNA used within the range 2 to 100 ng. In contrast, some minor bands within RAPD PCR product patterns increased in intensity with larger amounts of DNA template within the range of 40 to 100 ng. For this reason, the RAPD PCR template amount was maintained at 10 ng of DNA for isolate comparisons.

RESULTS

Identification of *T. foetus* by culture. *T. foetus* isolates were recovered from bulls throughout the agricultural region of Saskatchewan (Fig. 1). Between May 1993 and April 1994, 65 (6%) of 1,048 bulls tested by the Provincial Veterinary Laboratory and the Western College of Veterinary Medicine Laboratory were culture positive for *T. foetus*.

Since some trichomonads are enteric, it was desirable to confirm these isolates as *T. foetus* by an independent method. We were able to maintain 16 positive cultures by serial passage. These cultures were submitted to PCR analysis.

Single-band *T. foetus* PCR tests. DNA was purified from the 16 Saskatchewan isolates and the American Type Culture Collection Austrian isolate, 50151. These DNAs were subjected to the *T. foetus*-specific single-band PCR test of Ho et al. (9). The expected 162-bp PCR product was strongly amplified from 16 isolate DNAs (Fig. 2). The remaining isolate, R2, produced a weak, 162-bp product. Hybridizations using the probe TF3 confirmed the identity of the 162-bp PCR product for all isolates except isolate R2, which hybridized only weakly compared with other isolate PCR products after normalization for the diminished amount of R2 product (data not shown). Sample pouch media from 11 culture-negative bulls were negative by PCR. These results strongly suggested that at least 16 of the 17 isolates examined were *T. foetus*. The weak signal produced by isolate R2 suggested either genetic variation at the locus amplified by primers TF1 and TF2 or that isolate R2 was not *T. foetus* (see Discussion).

Upstream of the *T. vaginalis* cyclin-dependent kinase-like sequence (20) are potential controlling sequences with homology to the upstream sequences near other trichomonad genes (12, 17). We reasoned that such sequences might be conserved and thus may be useful targets for PCR detection of trichomonads.

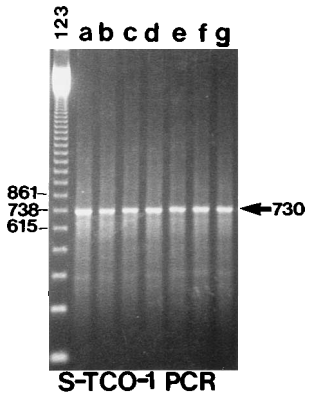

FIG. 3. S-TCO-1 PCR analysis of DNA from *T. foetus* isolates. The TCO-1 primer was used at high stringency, yielding a single PCR product for each of the 17 isolates (7 shown) studied. Ten nanograms of template DNA was used for each isolate. Lanes: 123, molecular size standards consisting of the 123-bp ladder (Bethesda Research Laboratories); a, *T. foetus* 50151; b, *T. foetus* MC11; c, *T. foetus* SC; d, *T. foetus* BoR1; e, *T. foetus* Colo; f, *T. foetus* R6; g, *T. foetus* MC14. Electrophoresis conditions were as described in the legend to Fig. 2.

The 57-mer TCO-1 (see Materials and Methods) is complementary to the 5′ flank of the trichomonad CDC2 homolog. PCRs were performed at high stringency (termed S-TCO-1) with TCO-1 as the sole primer to amplify sequences between adjacent TCO-1 complementary sites on opposite DNA strands. All 17 *T. foetus* isolates (7 are shown in Fig. 3) exhibited a single, predominant PCR product at 730 bp confirmed by hybridization with CDO-7. These results suggest that a 730-bp sequence flanked by sites complementary to TCO-1 was conserved and potentially useful for PCR-based diagnosis of *T. foetus* infection. However, to extend the versatility of *T. foetus* PCR, we sought multiple-band PCR tests with potential for both *T. foetus* identification and isolate-specific DNA fingerprinting.

***T. foetus* T17 PCR.** T17 PCR produces multiple product bands whose sizes result in both species- and isolate-specific patterns (22). The *T. foetus* isolate DNAs were subjected to T17 PCR, as were three *T. vaginalis* isolate DNAs for comparison. All 17 *T. foetus* isolates tested exhibited a 220-bp PCR product (Fig. 4). Additionally, 13 (76%) of the 17 isolates exhibited a product at 330 bp, and 5 (29%) of the 17 exhibited a 105-bp product that was prominent in intensity for a small DNA fragment expected to bind less ethidium bromide per molecule than larger fragments.

Characteristic *T. foetus* T17 PCR products were distinct from characteristic *T. vaginalis* T17 PCR products (Fig. 4) and from T17 PCR products of DNA from *G. lamblia*, *G. muris*, *T. equiparitum*, *T. cruzi*, *Trypanosoma herpetomonas*, *L. donovani*, *L. brazeliensis*, *Leishmania tarentolae*, *A. castellani*, *A. rhisodes*, *A. hatchetti*, *A. culbertsoni*, *P. tetraurelia*, *T. thermophilia*, and *S. cerevisiae* (data not shown). For example, in contrast with the *T. foetus* 220- and 330-bp T17 PCR products, *T. vaginalis* T17 PCR products included prominent 740- and 440-bp products common to all *T. vaginalis* isolates tested to date (Fig. 4) (22). *T. foetus* isolate R2, which produced a weak signal in the single-band, TF1-TF2 PCR test, clearly exhibited both 220- and 330-bp T17 PCR products, supporting the identification of this isolate as *T. foetus* (see Discussion).

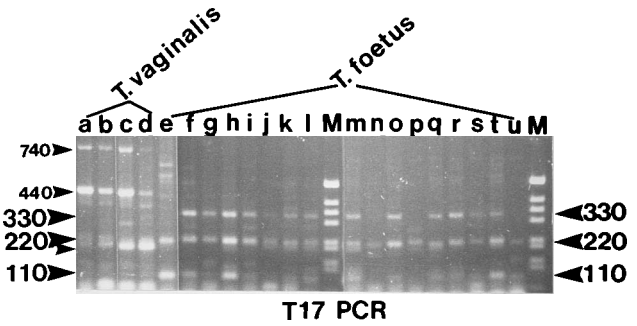

FIG. 4. T17 PCR analysis of DNA from *T. vaginalis* and *T. foetus* isolates. Purified DNA samples were subjected to T17 PCR using primers TR7 and TR8. Common *T. foetus* PCR products are indicated with large arrowheads and numbers on both the left and the right. The smaller arrowheads and numbers on the left indicate characteristic T17 PCR products observed among *T. vaginalis* isolates described previously (22) as well as among the *T. vaginalis* isolates shown here. The small arrowhead just below the large arrowhead at 220 bp indicates a variably present *T. vaginalis* product that is intense in the case of a limited number of isolates, such as those of lanes c and d. Ten nanograms of template DNA was used for each isolate. Lanes: a, *T. vaginalis* S933; b, *T. vaginalis* S1047; c, *T. vaginalis* S1126; d, *T. vaginalis* S1163; e, *T. foetus* 50151; f, *T. foetus* MC11; g, *T. foetus* excel; h, *T. foetus* Colo; i, *T. foetus* MC14; j, *T. foetus* MC30; k, *T. foetus* R3; l, *T. foetus* R2; M, 1-kb molecular size markers (Bethesda Research Laboratories); m, *T. foetus* SC; n, *T. foetus* BoR1; o, *T. foetus* R6, p, *T. foetus* R2; q, *T. foetus* R1; r, *T. foetus* R7; s, *T. foetus* R4; t, *T. foetus* R5; u, *T. foetus* MC52. Electrophoresis conditions were as described for Fig. 2.

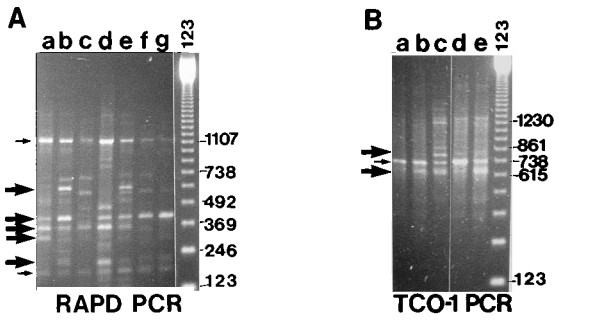

FIG. 5. RAPD and TCO-1 PCR analysis of DNA from *T. foetus* isolates. (A) RAPD PCR primers TAP5 and TAP6 were used to PCR amplify multiple genomic sites in DNA from *T. foetus* isolates. Small arrows indicate PCR products common to the isolates studied. Large arrows indicate variable PCR products observed for some but not other isolates. Ten nanograms of template DNA was used for each isolate. Lanes: a, *T. foetus* 50151; b, *T. foetus* MC11; c, *T. foetus* excel; d, *T. foetus* Colo; e, *T. foetus*, MC14; f, *T. foetus* MC30; g, *T. foetus* R3; 123, molecular size standards consisting of the 123-bp ladder (Bethesda Research Laboratories). Molecular sizes in base pairs are indicated on the right. (B) TCO-1 PCR analysis of *T. foetus* isolates. The primer TCO-1, complementary to potential 5′ controlling sequences, was used at low stringency in PCRs with *T. foetus* genomic DNAs. Ten nanograms of template DNA was used for each isolate. Lanes: a, *T. foetus* 50151; b, *T. foetus* MC11; c, *T. foetus* SC; d, *T. foetus* BoR1; e, *T. foetus* Colo; 123, molecular size standards consisting of the 123-bp ladder (Bethesda Research Laboratories). Electrophoresis conditions were as described in the legend to Fig. 2.

T17 PCR products were absent when prokaryotic DNAs from *E. coli* DH5α, *G. vaginalis* GV9016B, *B. intermedius* 25261, *P. anaerobius* Clt 17.5, *B. disiens* Clt 75.5, *P. tetradius* Clt 92.2, *F. nucleatum* 6601 TE10, *V. parvula* 10028, *E. faecalis* DS160, *M. curtisii* 73, *S. agalactiae* AES 14, *M. fermentans*, *B. fragilis*, *B. intermedius* 25361, and *U. urealyticum* UU5 were examined. We conclude that *T. foetus* T17 PCR resulted in a potentially characteristic pattern of *T. foetus* PCR products.

In addition, there were isolate-specific variations among *T. foetus* T17 PCR product patterns. Specifically, several T17 PCR products, including both faint and prominent product bands (e.g., the 105-bp product), varied in appearance and/or size among isolates (Fig. 4). These variations were reproducible in all of three experiments, indicating that the patterns were not due to variations in individual PCRs. These results suggested that T17 PCR detected common or frequently present loci (the 220- and 330-bp targets) in the *T. foetus* genome that distinguished *T. foetus* from other microorganisms tested and that it also detected loci that vary among *T. foetus* isolates.

RAPD PCR analysis. The general goal of RAPD PCR is to detect genomic polymorphism in order to distinguish and further identify isolates. Primers for RAPD PCR are chosen on the basis of their ability to detect polymorphic regions of the genome (3, 28, 30). We tested numerous potential RAPD PCR primer sets, searching for sets that yielded multiple PCR products that varied among *T. foetus* isolates. Another criterion was that the RAPD PCR product patterns be reproducible in sequential PCRs performed in separate experiments.

The RAPD primer set TAP5-TAP6 produced multiple product bands that were distinguishable and reproducible for individual *T. foetus* isolates. As shown in Fig. 5A, RAPD PCR using these primers led to hypervariable patterns, although all of the *T. foetus* isolates exhibited a common RAPD PCR 1,100-bp product. The other RAPD PCR products generated by TAP5-TAP6 varied dramatically among isolates. The isolate-specific RAPD PCR patterns were reproducible in independent experiments. No variation in these patterns was observed with template DNAs within the range of 2 to 40 ng. Above 40 ng of template DNA, some band intensity variations were observed. The results suggested that the TAP5-TAP6 primer set recognized exceedingly polymorphic, or hypervariable, regions of the *T. foetus* genome.

TCO-1 PCR analysis. Although knowledge of the function of genomic sites is unnecessary for their use in DNA fingerprinting, we were interested in evaluating potential genomic variability at loci with suggested functions. We explored sequences related to the TCO-1 sequence by using the TCO-1 primer in PCRs at decreased stringency.

At stringency decreased from that for Fig. 3, TCO-1 PCR resulted in multiple product bands, which reflected genomic variability among *T. foetus* isolates (Fig. 5B), and a background smear, suggesting many sequences in the *T. foetus* genome partially related to TCO-1. Several of these bands hybridized to the probe, CDO7 (see Materials and Methods), suggesting that these sequences may be related to the CDC2 family sequence previously reported (20). TCO-1 PCR of *T. vaginalis* DNA exhibited multiple, high-molecular-weight bands superimposed on a background smear (data not shown). The results suggested that sequences flanked by TCO-1 hybridizing sites in *T. foetus* are conserved (Fig. 3) while related sequences (Fig. 5B) observed at moderate stringency are polymorphic.

We conclude that, while isolates of *T. foetus* exhibit conserved genetic loci, such as the 220-bp target detected by T17 PCR, the 730-bp S-TCO-1 target, and to some extent the 162-bp TF1-TF2 target, *T. foetus* isolates also exhibit a high level of genetic polymorphism detected by PCR targeting a variety of unrelated genetic loci.

DISCUSSION

Bovine trichomoniasis is currently diagnosed on the basis of morphological identification of viable organisms isolated from infected animals and cultured. Sampling may occur under harsh climatic conditions, and it often involves transportation from remote areas. Wild-type parasites may prove difficult to culture even under optimal conditions. These problems may

have contributed to the earlier belief that the community pastures of Saskatchewan were free of *T. foetus*.

More extensive sampling revealed the presence of trichomonads throughout the community pastures of southern Saskatchewan. The 6% prevalence among 1,058 bulls should be viewed as a minimal estimate because of harsh sampling conditions and long transport times. Thus, it was desirable to begin the evaluation of tests that can detect parasite artifacts, such as DNA, even when the parasite expires outside the host.

The single-band PCR (9) is potentially faster than culture and may detect trichomonads whose survival is uncertain after removal from the host. The T17 and RAPD PCR tests described here offer these advantages and the possibility of distinguishing genetic variants of *T. foetus*. TCO-1 PCR could be used to detect a conserved 730-bp locus at high stringency and multiple, polymorphic loci at lowered stringency. Because of their larger numbers of discrete and variable or hypervariable PCR products, T17 and RAPD PCRs may be superior to TCO-1 PCR for isolate fingerprinting. High-stringency TCO-1 PCR has potential as an alternative, single-locus *T. foetus* PCR test, but further study of heterologous species is needed.

In addition to supporting culture diagnoses, the results presented here strongly suggest genomic heterogeneity among *T. foetus* isolates. The T17 and RAPD PCR product patterns were similar in appearance to hypervariable minisatellite repeat patterns that have often been used to type or fingerprint other organisms (7, 8, 11, 13, 23, 25, 26, 32). Genomic variability is a well-known feature of the human genome and is widely used to identify or DNA fingerprint individuals (7, 8, 11, 29, 31). Generally, the more variable a genomic site is, the higher the value of that site for purposes of individual identification. With the more recent discovery of genomic variability in the protozoa comes the possibility of tracking individual isolates through lines of infection and of detecting the most virulent or, alternatively, the least harmful isolates. With the heterogeneity that we observed in T17, RAPD, and reduced-stringency TCO-1 PCR, it is doubtful that trichomonads in southern Saskatchewan arose from a single, recent line of infection.

Although individual isolates exhibited variations, a typical *T. foetus* T17 pattern was observed. Specifically, PCR-amplified products at 220 and 330 bp were present in the majority (76%) of the *T. foetus* isolates, including an isolate that produced only a weak single-band PCR product with the TF1-TF2 primer set. Reduced hybridization with the TF3 probe, specific for the TF1-TF2 PCR product, suggested that isolate R2 exhibited genetic variation at the locus defined by the TF1-TF2 primer set. The 220-bp T17 PCR product was produced by all *T. foetus* isolates studied. Occasionally (4 of 17 isolates [23%]) *T. foetus* isolates, including the Austrian isolate 50151, exhibited a PCR product of 105 bp. The T17 PCR clearly distinguished *T. foetus* isolates from *T. vaginalis* with its characteristic T17 PCR products of 440 and 740 bp (Fig. 4) (22).

On the basis of this and previous studies, T17 PCR distinguishes a wide variety of protozoa, each exhibiting a characteristic pattern. The patterns of intense T17 PCR products and, in addition, several of the less intense bands distinguished *T. foetus* isolates from *T. vaginalis*, *G. lamblia*, *G. muris*, *T. equiparitum*, *T. cruzi*, *T. herpetomonas*, *L. donovani*, *L. brazeliensis*, *L. tarentolae*, *A. castellani*, *A. rhisodes*, *A. hatchetti*, *A. culbertsoni*, *P. tetraurelia*, *T. thermophilia*, and *S. cerevisiae* (22). T17 PCR also distinguished *T. foetus* from the prokaryotes *G. vaginalis*, *B. intermedius*, *P. anaerobius*, *B. disiens*, *P. tetradius*, *F. nucleatum*, *V. parvula*, *E. faecalis*, *M. curtisii*, *S. agalactiae*, *M. fermentans*, *B. fragilis*, *B. intermedius*, and *U. urealyticum*.

In summary, the culture prevalence of *T. foetus* infection in southern Saskatchewan was 6%, a minimal estimate because culture results depend on the survival of parasites during sampling and transit and because only bulls, and not cows, were sampled. Each of the 1,058 bulls tested sexually contacts an average of 30 cows per season (unpublished observation). Genetic heterogeneity among cultured *T. foetus* isolates was observed by T17, RAPD, and low-stringency TCO-1 PCRs, suggesting a long history of *T. foetus* infection in Saskatchewan, multiple lines of infection, or both. These techniques may assist in typing and treatment studies or in the delineation of genetic types potentially associated with virulence. In addition, PCR methods may prove valuable as a complement to culture or in primary diagnosis of *T. foetus* infection.

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