The development of a diagnostic assay for Tritrichomonas foetus
by William Edward Severson

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Science
Montana State University

Abstract:
Oligonucleotide probes complementary to a unique region of Tritrichomonad 16S-18S small-subunit ribosomal RNA were used to develop a nucleic acid-based assay for detection of T. foetus infection within the bovine. These probes bound specifically to whole-cell ribosomal RNA of T. foetus and not to rRNA from bovine cells or RNA from Trichomonas vaginalis. Initially, a diagnostic assay which utilized a polymerase chain reaction (PCR) format was examined and rejected due to recovery, reproducibility, and contamination problems. A RNA assay was developed that is able to detect as few as 50 organisms without amplification of the target nucleic acid. The sensitivity and accuracy of the RNA assay was examined in comparision to the conventional microscopic/culture assay. Mock testing results revealed a detection rate of 98.6% of T. foetus positive samples using the RNA assay compared to 27% by the conventional microscopic/culture assay. Storage of samples at room temperature or 4°C for four days had no effect on the RNA assay whereas detection by the microscopic/culture assay dropped to 7% indicating the sample used in the microscopic/culture assay must be kept at 37°C in a low oxygen environment to prevent analytical problems due to parasite death. Field studies included samplings of two herds (104 cows) from eastern Montana (case study 1) and one herd (86 cows) from western Texas (case study 2). The RNA assay detected a 17% infection rate in herd two of case study 1 and a 52% infection rate in case study 2. The levels of infection in these herds was generally below the estimated detection limit of the microscopic/culture assay indicating that a large number of false negatives would likely result using the conventional diagnostic protocol.

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A thesis submitted in partial fulfillment
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APPROVAL

of a thesis submitted by

William E. Severson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style and consistency and is ready for submission to the College of Graduate Studies.

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TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Oligonucleotide probes complementary to a unique region of Tritrichomonad 16S-18S small-subunit ribosomal RNA were used to develop a nucleic acid-based assay for detection of T. foetus infection within the bovine. These probes bound specifically to whole-cell ribosomal RNA of T. foetus and not to rRNA from bovine cells or RNA from Trichomonas vaginalis. Initially, a diagnostic assay which utilized a polymerase chain reaction (PCR) format was examined and rejected due to recovery, reproducibility, and contamination problems. A RNA assay was developed that is able to detect as few as 50 organisms without amplification of the target nucleic acid. The sensitivity and accuracy of the RNA assay was examined in comparision to the conventional microscopic/culture assay. Mock testing results revealed a detection rate of 98.6% of T. foetus positive samples using the RNA assay compared to 27% by the conventional microscopic/culture assay. Storage of samples at room temperature or 4^0C for four days had no effect on the RNA assay whereas detection by the microscopic/culture assay dropped to 7% indicating the sample used in the microscopic/culture assay must be kept at 37^0C in a low oxygen environment to prevent analytical problems due to parasite death. Field studies included samplings of two herds (104 cows) from eastern Montana (case study 1) and one herd (86 cows) from western Texas (case study 2). The RNA assay detected a 17% infection rate in herd two of case study 1 and a 52% infection rate in case study 2. The levels of infection in these herds was generally below the estimated detection limit of the microscopic/culture assay indicating that a large number of false negatives would likely result using the conventional diagnostic protocol.

INTRODUCTION

Bovine trichomoniasis, caused by the obligate protozoan parasite, Tritrichomonas foetus, is a significant disease problem of cattle throughout the United States (1,3,13,17,19,36). Clinically, the disease results in early embryonic death, transient infertility, pyometra and abortion. The disease is not manifested clinically in bulls and thus, they may perpetuate the infection undetected (36). The economic impact of this disease on the United States beef industry is estimated to be $650 million annually (37).

In a recent example, an epizootic of trichomoniasis was reported in a large dairy herd in California, which resulted in an estimated economic loss of $66,538. The greatest losses were attributed to infertility. The disease continued in the herd despite culling older bulls and the institution of an artifical insemination breeding program for two high-production strings (13).

In another example, approximately 5,000 cows and 300 bulls in the San Joaquin Valley of California were diagnosed as having trichomoniasis based on the isolation of the causative organism from bull preputial smegma samples. Two hundred eighty cows from this herd were chosen for investigation. The calving rate for the preceeding breeding season was 84%. During the season in question, the pregnancy rate for the same group was 78%. The 6% lower calving rate represents a $5,600 economic loss in this 280 cow herd (36).

At the present time the only accepted means of detecting the presence of T. foetus within the bovine is through microscopic examination. Microscopic detection often depends on successful culturing of T. foetus from venereal samples; a difficult and often

unreliable procedure. Although relevant antigens of T. foetus are being examined for diagnostic applications, no antibody-based test is currently commercially avaliable. An efficient diagnostic assay, however, is critically needed because the disease is increasing in prevalence across the western states. In addition, the evaluation of the efficacy of potential vaccines will be hampered without a reliable diagnostic method. A DNA or RNA-based assay could potentially fulfill this need. This type of assay will detect low numbers of T. foetus without the need to preculture and with the lack of background cross-reactivity which can affect immunological-based assays. Further, only actively infected cattle will show a positive response unlike a serotest which detects both currently infected cattle as well as cattle which have had previous contact with the pathogen.

LITERATURE REVIEW

Tritrichomonas foetus is a flagellate protozoan (Protozoa: Zoomastogophorea) which can be identified by its morphological characteristics, including a recurrent flagellum attached to the body by an undulating membrane and three anterior flagella (17). T. foetus multiplies exclusively by simple binary cell fission. The size of the organism is small, generally 10 to 25 by 3 to 15 μm, exists only in the form of a trophozoite. T. foetus is transmitted between cows to bulls by sexual intercourse (coitus), therefore the probability of their being transmitted through contaminated bedding or other species of animals is remote. Upon introduction into the reproductive tract of a cow, the Tritrichomonads reproduce in the vagina where they may cause vaginitis. If the animal is pregnant, the organisms may invade the uterus and infect the developing fetus. Usually when this happens, the cow will abort during the first 16 weeks of pregnancy (gestation in cows takes about 37 weeks). If abortion takes place, the cow will usually be free of infection after two estrus cycles (about 42 days). Infected bulls present a particularly difficult problem. Usually the bull is removed from the herd unless it is a particularly valuable breeding animal. In a clean herd it is essential that any bull being introduced into it be examined for Tritrichomonad infection. As a general rule, bulls to be purchased are examined for fertility and for venerally infectious agents such as T. foetus (22).

T. foetus has been the subject of biochemical investigations along with other flagellates of the Order Trichomonadida. The genome size of T. foetus, about 2.5×10^7 base pairs, is similar to Trypanosoma brucei, and about six times larger than E. coli.

T. foetus DNA shows a melting temperature (Tm) of 82°C corresponding to a 31% GC content. DNA isolated from T. foetus demonstrates 35-42% hyperchromicity when fully melted and Cot (concentration versus time) analysis indicates the presence of repetitive sequences accounting for approximately 46.7% of the DNA (44).

T. foetus is distinctive in that it possesses no mitochondrial structure but instead has many microbody-like cytoplasmic organelles termed hydrogenosomes. The hydrogenosomes contain, among many other enzymes, pyruvate dehydrogenase, ferredoxin and hydrogenase and constitutes an integral part of energy metabolism in this parasite. There is great interest in the biological origin of these hydrogenosomes and their relationship to mitochondria. This has led to a search for DNA in these organelles (44) and has resulted in controversy. Cerkasovava' et al. (4) have published electron observations on circular DNA from T. foetus hydrogenosomes, whereas Turner and Muller (44) were unable to detect extranuclear DNA in bulk DNA preparations.

Polymerase Chain Reaction (PCR)

The PCR was invented by Kary Mullis (25,26) and orginally applied by the Human Genetics Department at Cetus to amplify human B-globin DNA for diagnosis of sickle-cell anemia. The PCR technique is an in vitro method in which DNA sequences are enzymatically amplified (up to 10^{15} fold) directed by a pair of oligonucleotide primers. Primers are typically 15 to 30 nucleotides in length and are complementary to sequences defining the 5'-ends of the target DNA (49). Early experiments utilized the Klenow

fragment of E. coli DNA Polymerase I to extend the annealed primers. This enzyme was inactivated by the high temperature required to melt the two DNA strands at the onset of each PCR cycle consequently, fresh enzyme had to be added during every cycle. The discovery of a 94 kdal DNA polymerase (Taq polymerase) from a thermophilic bacterium, Thermus aquaticus has eliminated this problem (10). Taq polymerase has a specific activity of ca. 200,000 units/mg, good fidelity, a broad temperature optimum of 70°C-80°C (depending on template), and is currently the enzyme of choice for PCR (49). In a typical reaction, the components (template, primers, Taq polymerase, dNTP's and buffer) can be assembled and the amplification reaction carried out by simply cycling the temperature within the reaction tube. For each pair of oligonucleotide primers the optimal set of reaction parameters (eg. enzyme, primer and Mg^{++} concentration as well as the temperature cycling profile) must be determined to maximize the product yields.

DNA-based Diagnostic Assays

PCR has enabled scientists to realize the potential of clinical DNA-based diagnosis by producing enough of the target DNA sequences so that simple and rapid methods for identifying these sequences could be employed. The specific amplification of DNA sequences provides not only dramatic increases in the number of copies but concomitantly provides a nearly equivalent reduction in the complexity of the nucleic acid to be probed. Either DNA or RNA (following the production of complementary DNA using reverse transcriptase) can be used as a template for amplification. The sensitivity of detection can

be as low as a single pathogenic organism or virus particle per sample. Since the first clinical application of PCR for the prenatal diagnosis of sickle-cell anemia (34), there has been a proliferation of PCR applications. PCR has been applied to the detection of serum hepatitis B virus DNA in patients with chronic hepatitis(16). In the case of chronic myeloid leukemia (CML) and some forms of acute lymphocytic (ALL) and acute myliod (AML) leukemia, a chimeric mRNA (BCR-ABL) indicating a particular chromosomal translocation is found only in the leukemic cells of these patients. Since the specific amplification of a "fusion" sequence (eg., BCR-ABL) is accomplished by using one primer complementary to the BCR sequence and the other to the ABL sequence, the PCR amplification of this unique translocation encoded fragment is a powerful and sensitve way to monitor minimal residual disease. This assay is capable of detecting one cell in a million containing the fusion transcript (6).

Another area where PCR is being applied is to distinguish closely related organisms. An example is the phylogenetic evidence for the acquisition of ribosomal RNA introns subsequent to the divergence of some of the major Tetrahymena groups (40). The PCR method can also be used to detect the presence of parasitic organisms in the nucleic acid background of the host organism. With the PCR method and a multicopy conserved gene such as the ribosomal RNA genes, the number of cells required is remarkably low. The PCR amplification of a 16S-like rRNA coding region from a typical eucaryotic microorganism with a genome complexity of 1×10^8 bp may require fewer than 100 cells to produce 0.5 μg of product (24). A relatively small number of nucleotides (approximately 80-100 nucleotides) representing a unique region of the rRNA genes of

an organism are the only requirements needed to detect an infecting organism and consequently, differentiate between the parasite and the host (20).

Structure and Function of Ribosomal RNA

Ribosomes are large ribonucleoprotein structures that are involved in the translation of the genetic code (50). Ribosomes are divided, both structually and functionally, into a large and a small subunit. The best understood ribosomal particle is the E. coli 30S small subunit which is involved in the early steps of translational initiation and is the site of codon-anticodon interaction (41). The E. coli 30S subunit is composed of a 16S ribosomal RNA (rRNA) and 21 different ribosomal proteins which are assembled into a complex three-dimensional structure held together by noncovalent interactions. In recent years, the focus of research on ribosomal mechanisms of action has shifted from an emphasis on ribosomal proteins to ribosomal RNA as a result of a growing body of evidence suggesting that rRNAs are the function-determining molecules in ribosomes (43).

Conserved nucleotide sequences in the small-subunit rRNA's reflect conserved function. An example of this is the interaction between the 16S rRNA of the E. coli 30S subunit and mRNA. Direct evidence for the role of 16S rRNA in mRNA translation was provided by mutations within the pyrimidine-rich antiShine-Dalgarno (anti SD) region at the 3'-end of the molecule. A single base mutation of C to U at position 1538 dramatically reduced the synthesis of the cellular proteins examined. This was not due

to a reduction in the level of mRNA's encoding these proteins but rather a reduction in initiation of mRNA translation (7). Efficient translation of a mRNA with an altered SD sequence (UGUGU) complementary to the mutated anti-SD sequence of the plasmid-borne 16S rRNA gene, demonstrated the functional capacity of the mutant ribosomes and the importance of the SD sequence for proper initiation (7).

Footprinting (tRNA protection) experiments with structure-specific chemical probes to study the molecular structure of the decoding region identified three classes of sites in 16S rRNA that are shielded as a consequence of the nonenzymatic (factor independent) interaction of tRNA with the subunit: A site, P site and overlapping tRNA and 50s subunit regions. The experiments reveal nearly all of the sequences around 1400 (1392-1400) are conserved in E. coli and mutations in this region affect subunit association. Substitution of a G or A for C at position 1400 was found to inhibit ribosomal activity by 80% and 50%, respectively (7). Insertions and deletions between 1397 and 1404 completely blocked initiation-dependent peptide synthesis (fMet-Ser) but markedly stimulated the initiation-independent reaction (Phe-Val). A modification that blocked all ribosomal function was the deletion of G at 1401 (7).

These conserved regions in rRNA have allowed the alignment of sequences from different organisms revealing variable regions which have been used to evaluate evolutionary relationships (38). The rRNA genes are universally distributed and functionally equivalent in all known organisms and do not appear to undergo lateral transfer between species. The 16S-18S small-subunit rRNA's in contrast to the 5S and 5.8S species, are particularly useful because they are relatively large (approximately 1500

nucleotides) and provide a statistically significant number of variable nucleotide positions (39).

While there is tremendous conservation of sequence within the rRNAs these considerable species-specific regions form the targets and the basis for evolutionary distance measurements and potential use in diagnostic applications. These regions are used to design complementary DNA oligonucleotides which are in turn radiolabelled and used to probe extracted RNA from a sample. To date researchers have developed this approach successfully for the detection of the human malaria parasites (20), Neisseria gonorrhoeae infection (33) and Chlamydia (30).

There are several advantages of rRNA-based diagnostic assays including the ability to detect small numbers of cells. It has been estimated that there are about 10^6 copies of the rRNA molecules per Plasmodium parasite which equates to roughly 0.2pg. This allows the detection of fewer than 20 parasites, which represents minimally a 100-fold increase in sensitivity over published procedures based on the hybridization to repetitive DNA molecules (2), thus, low levels of parasitemia may be detected (48).

MATERIALS AND METHODS

Reagents

AmpliTaq thermal-stable DNA polymerase was purchased from Perkin-Elmer Cetus Corporation (Norwalk, Conn). The deoxynucleotide triphosphates (HPLC grade) were purchased from United States Biochemical (USB) (Cleveland, Ohio). Yeast transfer ribonucleic acid (tRNA) was purchased from Sigma (St. Louis, Missouri). Guanidine isothiocyanate was obtained from Fluka (Ronkonkoma, New York). Diethyl pyrocarbonate (DEP) was purchased from Aldrich Chemical Company (Milwaukee, WI). T4 polynucleotide kinase and terminal deoxynucleotidyl transferase were purchased from Pharmacia (Uppsala, Sweden). Oligonucleotides were synthesized with an ABI Model 381A DNA synthesizer (Applied Biosystems Inc., Foster City, CA) by the Veterinary Molecular Biology Laboratory (Montana State University, Bozeman, MT). Oligonucleotide purification was performed by two methods: 1.) Desalting(21); The lyophylized crude oligonucleotide mix from the synthesizer is resuspended in TE buffer(50mM Tris pH 7.5, 2mM EDTA) and NaCl is added to final concentration of 0.25M NaCl. The oligonucleotide is precipitated with 2.5 vols of ethanol, collected by centrifugation, washed with 70% ethanol (v/v), and vacuum dried. The oligonucleotide is then dissolved in TE buffer and the concentration determined by UV spectrophotometry. 2.) Detritylation; During oligonucleotide synthesis the final 5'-trityl group is not removed. Shorter oligonucleotides do not contain 5'-trityls and, therefore,

may be removed by reverse phase column chromatography using the Nensorb™ prep column as described by the Nensorb™ prep instruction manual (DuPont/NEN, Boston, MA). All solutions for DNA work were autoclaved prior to use. Diethylpyrocarbonate was used in the preparation of RNase free water according to published procedures (21).

Partial Cloning of T. foetus 16S rRNA Gene

Preparation and Isolation of DNA

T. foetus, strains 85-330, CA84, VMC and NV (American Type Culture Collection, Rockville, MD) were used in the following studies. The parasites were cultivated at 37°C in Diamonds medium (8), pH 7.4, containing 5% fetal bovine serum (HyClone Laboratories, Logan, Utah). Bovine monocyte cell line M617 (American Type Culture Collection, Rockville, MD) was cultivated in RPMI-1640 Medium (Gibco, BRL. Grand Island, N.Y.), 15% fetal calf serum at 38.5^{0}C. Approximately 10^8 bovine M617 cells and 10^9 T. foetus parasites were lysed and DNA was isolated by the method of Wang et al (47). Briefly, the cells were lysed in 1.0 ml of a 4.0M guanidinium thiocyanate solution (GUISCN); (4M guanidine thiocyanate containing 0.5% sarcosyl (w/v), 8% β-mercaptoethanol (v/v) and 24mM Na citrate) at room temperature and vortexed for 10 sec. To the lysate CsCl was added to a final concentration of 0.4 g/ ml and dissolved at room temperature. A cushion of 2.5 mls of 5.7M CsCl containing 0.1M EDTA pH 7.5 was placed at the bottom of a Beckman SW41 ultracentrifuge tube followed by 8 mls of the CsCl/ cell-lysate mixture. The tube was centrifuged in a Beckman SW-41 rotor at

34,000 r.p.m. for 16 hr at 20°C. The viscous DNA band was collected carefully with a pasteur pipette and the DNA solution extracted with an equal volume of water-saturated phenol/chloroform (1:1). The layers were separated by centrifugation in the Sorvall HB-4 rotor at 10,000 r.p.m. for 10 min. and the phenol/chloroform extraction repeated. The final aqueous layer was then removed and immediately overlaid with two volumes of cold (-20°C) absolute ethanol and the DNA recovered by spooling onto a glass rod. The DNA was dissolved in sterile TE buffer (50mM Tris-HCl pH 7.2, 2mM EDTA) containing 0.5% SDS, digested with 0.1 mg/ml proteinase K for 1 hr at 37°C, extracted with an equal volume phenol/chloroform (1:1) and precipitated with 0.6 volumes of isopropanol. The DNA precipitate was then collected by centrifugation in a Microfuge E vertical rotor (Beckman Instruments, Palo Alto, CA) at 12,000 r.p.m. for 10 min., washed twice with 1.0 ml of 70% ethanol, air dried for 2 min and dissolved in 0.5 ml of TE buffer. The DNA was checked by electrophoresis through a 0.8% agarose gel, using a 89mM Tris-borate/2.5mM EDTA (TBE buffer) pH 8.3 running buffer and compared to λ Hind-III DNA fragments size standards (9). The gel was run at 50 volts for 2.5 hours, stained with 0.5 μg/ ml of ethidium bromide in water for 30 min and viewed with a UV transilluminator.

PCR Reaction and Thermal Profile

Target sequences were amplified in a 0.1 ml reaction volume containing 10 to 100 ng of either T. foetus or bovine M617 DNA, 2.5 units of AmpliTaq polymerase, 0.2 mM of each dNTP, 1μM of each primer, 50 mM KCl, 10mM Tris-HCl, 1.5mM MgCl and

0.1% w/v gelatin. The PCR thermal profile was as follows; after initially heating the DNA sample to 95°C for 7.5 min, the reaction was cooled to 25°C for 4.25 min to allow hybridization of the primer/template. The temperature was then raised to 68°C for 7.5 min to allow for polymerization and finally raised to 95°C for 2.5 min to begin the cycle again. The cycle was repeated 40 times in a programmable DNA thermal cycler (Perkin-Elmer, Norwalk, CT). PCR products were analyzed on a 1.5% agarose gel and compared to DNA standards.

M13 Cloning and Sequencing of the PCR Products

The PCR products were extracted with TE buffer saturated phenol and concentrated by precipitation with ethanol (21). The samples were then resuspended in TE buffer and digested with Hind-III. The digested samples were extracted with phenol/chloroform (1:1), concentrated by ethanol precipitation and ligated into the replicative form (RF) of M13mp19 (28) using T4 DNA ligase (New England Biolabs, Beverly, MA) (21). Single-stranded templates for directing DNA synthesis in sequencing protocols were prepared using established protocols (51). Sequencing of the PCR products was performed using the Sequenase enzyme and reaction protocols (51). Sequencing products were electrophoresised on a 8% polyacrylamide/8M urea sequencing gel containing a continuous gradient of TBE buffer (0.5x-2.5x) (20). After electrophoresis the gel was soaked in 12% methanol/10% acetic acid and then vacuum dried onto 3mm filter paper and the radioactive bands visualized by autoradiography.

Assay Development

PCR Diagnostic Assay Protocol

A 10x extraction buffer (0.1 M Tris-HCl [pH8.0], 0.1 M EDTA, 0.1M NaCl, 10% SDS and 300 mM DTT) and proteinase K was added to each sample at a final concentration of 1x buffer and 10μg/ml proteinase K. The sample was incubated at 37^{0}C for 24 hours and then extracted with an equal volume of TE saturated phenol/chloroform (1:1). The aqueous layer was removed and NaCl and yeast tRNA were added to final concentrations of 0.1M and 2.0 μg/μl, respectively. The DNA was precipitated by adding two volumes of cold (-20^{0}C) absolute ethanol. The sample was centrifuged for 1 hour at 10,000 x g, washed once with 1.0 ml of 70% ethanol, air dried for 2 min and dissolved in 50 μl of TE buffer. PCR amplification was performed using a DNA thermal cycler and AmpliTaq polymerase. The PCR solution contained 1x PCR amplification buffer (10x buffer contains 50mM KCl, 100mM Tris hydrochloride [pH 8.13], 15mM $MgCl_2$ and 0.1% [wt/vol] gelatin) 200 μM each of the dNTP's, 1 μM of each of the primers, 25 μl of the sample DNA, 2.5 units of AmpliTaq DNA polymerase and sterile water. Total reaction volume was 50 μl. Template DNA's were initially denatured at 94^{0}C for 5 min. Then a total of 25 PCR cycles was run under the following conditions: denaturation at 94^{0}C for 1 min, primer annealing at 68^{0}C for 1 min, and DNA extension at 72^{0}C for 3 min.

Slot-blotting of DNA

To a 50 μl PCR reaction, 5 μl of 3M NaOH containing 0.1M EDTA were added, incubated at 60°C for 40 minutes, after which 55 μl of 2M NH_4OAc were added. The sample was diluted with 250 μl of 1M NH_4OAc. The sample was slot-blotted on nitrocellulose filters (Schleicher & Schuell Inc., Keene, NH) as described by the Bio-Dot SF microfiltration instruction manual (Biorad Laboratories, Richmond, CA), air dried for 20 minutes and baked at 80°C for one hour or UV crosslinked at 120mJ/cm^2 for 30 sec. Immobilization of DNA fragments by UV crosslinking on the various membranes was performed with the Stratalinker 1800 (Stratagene, La Jolla, CA).

Hybridization of Probes

Nitrocellulose or Duralose filters were prehybridized in the following solution: 50mM PIPES (piperazine-N-N'-bis [2-ethane sulfonic acid]), 100mM NaCl, 50mM sodium phosphate, 1mM EDTA and 5.0% SDS, for 60 minutes at the hybridization temperature. The prehybridization buffer was then discarded and replaced with fresh hybridization buffer (same as above) containing 10^6 cpm/ml of end-labelled [γ-P^{32}]-oligonucleotide. Hybridization was carried out in a water bath for two hours at the appropriate temperature. Following hybridization, the filters were washed once for 10 minutes in 50 ml of 5% SDS, 1x SSC (0.15M NaCl and 0.015M Na Citrate) at room temperature, followed by two washes of 20 minutes each in 200 ml of 5% SDS, 1x SSC at the hybridization temperature (45). The filter was then exposed to X-ray film at -70°C

using a Dupont Lighting-Plus intensifying screen.

RNA Diagnostic Assay Protocol

A cervical mucus (cow) or smegma (bull) sample was treated with β-mercaptoethanol (BME) to a final concentration 8% (v/v) and centrifuged in a Sorvall H1000B rotor at 2,500 r.p.m. for three minutes at room temperature to pellet parasites and bovine cells. The pellet was lysed with 1.0 ml of 4M GUISCN solution, followed by the addition of 0.1 mls of 2M NaOAc (pH 4.0), vortexed for 5 seconds and then extracted with 1.0 ml of water-saturated phenol/chloroform (10:1). The aqueous layer was removed and RNA precipitated with 2 volumes of cold (-20^{0}C) absolute ethanol (5).

Slot-blotting of RNA

RNA was resuspended in 160 μl of RNase-free water and 40 μl of 10x denaturation buffer (30% formaldehyde and 0.1M NaH_2PO_4, pH 7.0), heated at 60^{0}C for 20 minutes and 40 μl of 5M NH_4OAc was added to a final concentration of 1M NH_4OAc. The RNA was slot-blotted onto duralose (nylon supported nitrocellulose from Stratagene, La Jolle, CA) using the Bio-Dot SF microfiltration apparatus (Biorad Laboratories, Richmond, CA). Hybridization of RNA blots was identical to DNA blots (see above).

Nucleic Acid Labelling

Oligonucleotides were 5'-end labelled with [γ-^{32}P] ATP, 6,000 ci/mMole,

(Dupont/New England Nuclear, Boston. MA) and T4 polynucleotide kinase. Briefly, 25 ng of oligonucleotide was end-labelled in a reaction containing 50mM Tris-HCl [pH 7.6], 10mM $MgCl_2$, 5mM dithiothreitol, 0.1M spermidine HCl, 0.1mM EDTA [pH 8.0]) and 10 units T4 polynucleotide kinase according to previously published protocols (21). Oligonucleotides were 3'-end labelled with [α-^{32}P] dCTP (Dupont/NEN, Boston, MA) and terminal deoxynucleotidyl tranferase (TdT). Fifty ng of oligonucleotide was labelled in a reaction containing 1.4 M potassium cacodylate, pH 7.2, 300mM Tris base, 40mM $MgCl_2$, 1mM DTT and 30 units TdT according to previously published protocols (32). The labelled products were purified from unincorporated nucleotides on Elutip-D columns (Schleicher and Schuell, Inc., Keene, NH) according to manufacturer protocols.

RESULTS

Partial Cloning of Tritrichomonas foetus 16S rRNA Gene

The goal of this study was to develop a nucleic acid based diagnostic assay for the detection of Tritrichomonas foetus infection within the bovine. Since ribosomal RNAs (rRNA) are the most abundant constituent nucleic acid in most organisms, it constitutes a logical target for a diagnostic assay (48). Our stategy was to clone and sequence a segment of the small-subunit (16S-18S) rRNA gene from T. foetus, which contains "variable" and "hypervariable" nucleotide sequences with respect to evolutionary conserved regions within the rRNA gene. The published sequences of the 16S-18S small-subunit rRNA genes of T. brucei, D. discoideum, E. gracilis (38), Tetrahymena (39), P. falciparum, human (23), S. pustula, O. nova, S.cerevisiae and E. coli (11) were compared to determine conserved regions. The evolutionary divergence between these organisms is large, however, a comparision of these ten 16S-18S rRNA sequences reveals that there are highly conserved sequence elements. Two regions in the human, T. brucei and E. coli genes show a 80% conservation of nucleotide sequence (Figure 1). This is a very high degree of conservation considering the extreme evolutionary diversity of these cell types and Figure 2 shows that these two regions are involved in joining three of the four arms of the E. coli 16S secondary structure indicating the functional importance of the sequences (7). The nucleotide sequences between these two conserved regions show different degrees of variability.

REGION #1		PERCENT HOMOLOGY	
Human 18S	UGAAACUUAAAGGAAUUGACGGAA	100 %	24MER
T.brucei	.....A.....	95.8%	
D.discoideum	.....	100 %	
E.gracilis	.....	100 %	
Tetrahymena	.....	100 %	
P.falciparum	A....G.....A.....	87.5%	
S.pustulata	.....	100 %	
O.nova	.....	100 %	
S.cerevisiae	.....	100 %	
E.coli	.A.....C...U.....GG	79.1%	

REGION #2		PERCENT HOMOLGY	
Human 18S	GUCCCUGCCCUUUGUACACACCGCCCGUCG	100 %	30MER
T.brucei	.....A.....	96.6%	
D.discoideum	.....	100 %	
E.gracilis	.....A.....	96.6%	
Tetrahymena	.....G.....	96.6%	
P.falciparium	.....	100 %	
S.pustulata	.....	100 %	
O.nova	.....	100 %	
S.cerevisiae	.....	100 %	
E.coli	..U..C.GG.C.....A	80.0%	

Fig. 1. Conservation of 16S-18S rRNA sequences.

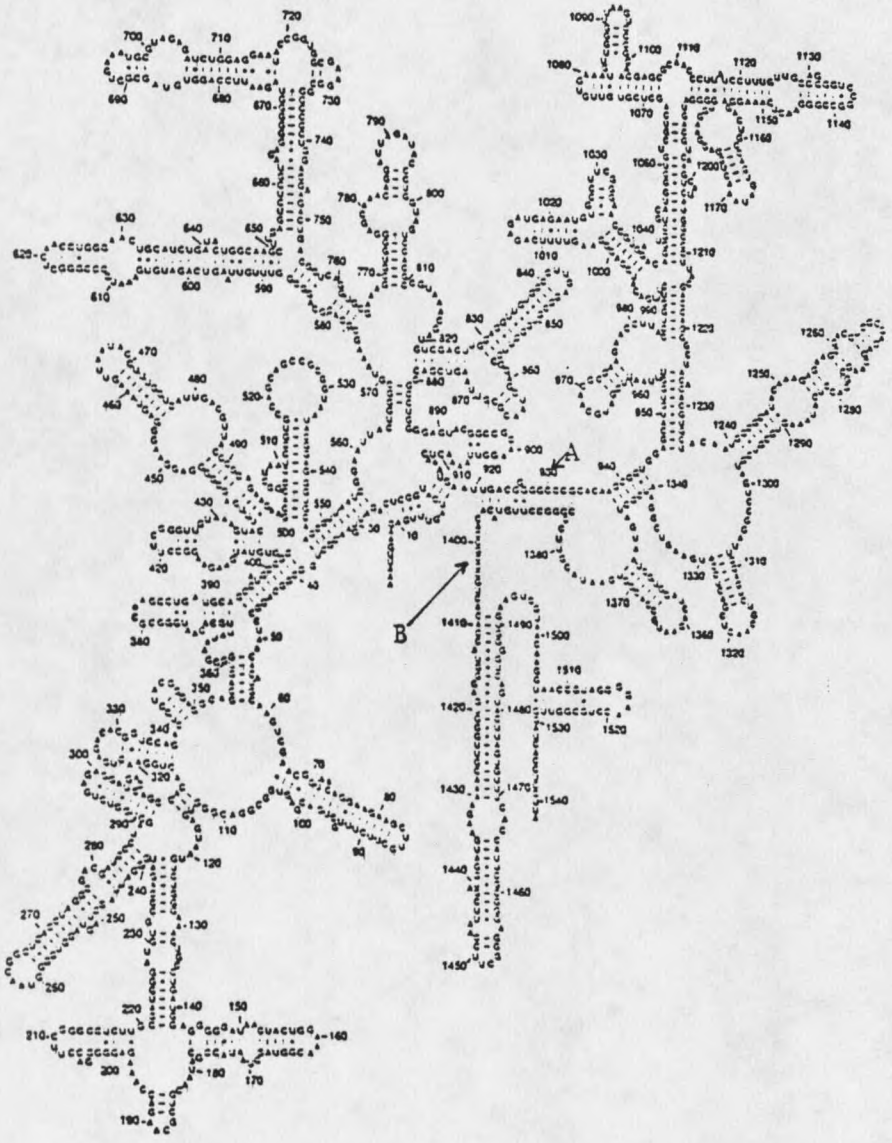

Fig. 2. Secondary structure map of E. coli 16S rRNA (Dalberg, A.E. 1989). Conserved region T.f. 1 (A). Conserved region T.f. 2 (B).

Two synthetic oligonucleotide primers were designed to amplify a fragment of the Tritrichomonas foetus 16S-18S rRNA gene. The first primer designed T. f. 1.(5'-GCGG GCAAGCTTACTTAAAGAAATTGACGGAA-3') is complementary to a 20 nucleotide region in the small subunit rRNA gene of T. brucei and contains a Hind-III restriction site for subcloning. T. f. 1. sequence corresponds to map position 1510 of the T. brucei 18S rRNA published sequence (38, also Figure 1) and correlates with map position 905 of the E. coli 16S gene. A second primer T. f. 2. (5'-GAAACCAAGCTT CGACGGGCGGTGTGTACAAA-3') is complementary to a 20 nucleotide region of the noncoding strand(38) near the 3' terminus of the T. brucei 18S rRNA gene (map position 2101), includes a Hind-III restriction site and correlates with position 1379 of the E. coli 16S rRNA gene.

Using the two universal primers T.f.1 and T.f.2, we amplified a segment of the T. foetus 16S-18S rRNA gene using the polymerase chain reaction (PCR) (See Materials and Methods). Figure 3 shows the results from six separate PCR reactions using various starting concentrations of T. foetus strain 85-330 DNA separated on a 1.2% agarose gel and stained with ethidium bromide. The size of the fragment amplified was approximately 500 base pairs in length. The starting concentration of T. foetus genomic DNA was not an important factor since 10 ng (lanes 1 and 4) gave as strong a signal as 100 ng of DNA (lanes 3 and 6). The amplified DNA fragments were then ethanol precipitated, recovered by centrifugation and subcloned into vector Mp13mp19 after Hind-III digestion. Pseudogenes presented a problem in the cloning of the T. foetus 16S-18S rRNA gene. By designing oligonucleotides from internal, evolutionarily conserved