



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

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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 comparison 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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APPROVAL

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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

	Page
ACKNOWLEDGMENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	ix
INTRODUCTION.....	1
LITERATURE REVIEW.....	3
Polymerase Chain Reaction (PCR).....	4
DNA-based Diagnostic Assays.....	5
Structure and Function of Ribosomal RNA.....	7
MATERIALS AND METHODS.....	10
Reagents.....	10
Partial Cloning of <i>T. foetus</i> 16S rRNA Gene.....	11
Preparation and Isolation of DNA.....	11
PCR Reaction and Thermal Profile.....	12
M13 Cloning and Sequencing of the PCR Products.....	13
Assay Development.....	14
PCR Diagnostic Assay Protocol.....	14
Slot-blotting of DNA.....	15
Hybridization of Probes.....	15
RNA Assay Protocol.....	16
Slot-blotting of RNA.....	16
Nucleic Acid Labelling.....	16
RESULTS.....	18
Partial cloning of <i>Trichomonas foetus</i> 16S rRNA Gene.....	18
Cross-Reactivity.....	27
Nucleic Acid Assay Development.....	31
PCR-based Assay.....	31
RNA Diagnostic Assay.....	37
Comparision of Total RNA Extraction Protocols.....	37

TABLE OF CONTENTS--Continued

	Page
Comparision of Different Membranes/UV versus Baking.....	38
Detection Limit Determination.....	40
RNA Stability in GUISCN and Reproducibility.....	43
Mock Testing.....	43
Field Experimentation.....	51
Case Study 1.....	51
Case Study 2.....	52
DISCUSSION.....	53
LITERATURE CITED.....	62

LIST OF TABLES

Table	Page
1. Homology analysis of <u>T. foetus</u> rRNA sequences against various rRNA sequences using the GENEPRO computer program.....	28
2. Mock <u>T. foetus</u> testing: Microscopic/culture versus RNA diagnostic Assays.....	50
3. A comparison of the microscopic/culture test with the RNA diagnostic Assay.....	59

LIST OF FIGURES

Figure	Page
1. Conservation of 16S-18S rRNA sequences from <u>T. brucei</u> , <u>E. gracilis</u> , <u>D. discoideum</u> , <u>Tetrahymena</u> , <u>P. falciparum</u> , human, <u>S. pustulata</u> , <u>O. nova</u> , <u>S. cerevisiae</u> and <u>E. coli</u>	19
2. Secondary structure map of <u>E. coli</u> 16S rRNA.....	20
3. Agarose gel electrophoresis analysis of PCR-amplified products from various starting concentrations of <u>T. foetus</u> (strain 85-330) genomic DNA.....	22
4. Agarose gel electrophoretic analysis of PCR-amplified products from various genomic DNA's.....	24
5. Sequence of <u>T.foetus</u> small-subunit 16S-18S rRNA aligned with bovine, <u>P. falciparum</u> and human 18S rRNA.....	25
6. Hybridization of synthetic oligonucleotide probes to various rRNA's.....	30
7. Detection limit of <u>T. foetus</u> parasites using a PCR assay protocol.....	33
8. Reproducibility of the PCR assay.....	34
9. Polyacrylamide gel electrophoretic analysis of PCR-amplified products from various DNA.....	36
10. Comparision of total RNA extraction protocols.....	39
11. A comparision of UV crosslinking versus baking on various membranes....	41
12. Detection limit of <u>T. foetus</u> parasites using the RNA assay.....	42
13. Stability of RNA in GUISCN and reproducibility of the RNA assay.....	44
14. Flow chart of the microscopic/culture test for the detection of <u>T.foetus</u> infection.....	46
15. Flow chart of the RNA assay for the detection of <u>T. foetus</u> infection.....	47
16. Detection of <u>T. foetus</u> parasites using the RNA assay.....	48

ABSTRACT

Oligonucleotide probes complementary to a unique region of *Trichomonas* 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 comparison 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.

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 artificial 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 available. 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 venereally 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 (T_m) 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 originally 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 kDa 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 myeloid (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 sensitive 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 μ g 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 structurally 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 lyophilized 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°C. Approximately 10⁸ bovine M617 cells and 10⁹ *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 μ g/ 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, 1 μ M 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 DevelopmentPCR 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 10 μ g/ml proteinase K. The sample was incubated at 37°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 μ g/ μ l, respectively. The DNA was precipitated by adding two volumes of cold (-20°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 μ l 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 MgCl₂ and 0.1% [wt/vol] gelatin) 200 μ M each of the dNTP's, 1 μ M of each of the primers, 25 μ l of the sample DNA, 2.5 units of AmpliTaq DNA polymerase and sterile water. Total reaction volume was 50 μ l. Template DNA's were initially denatured at 94°C for 5 min. Then a total of 25 PCR cycles was run under the following conditions: denaturation at 94°C for 1 min, primer annealing at 68°C for 1 min, and DNA extension at 72°C for 3 min.

Slot-blotting of DNA

To a 50 μ l PCR reaction, 5 μ l of 3M NaOH containing 0.1M EDTA were added, incubated at 60°C for 40 minutes, after which 55 μ l of 2M NH₄OAc were added. The sample was diluted with 250 μ l of 1M NH₄OAc. 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/cm² 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 10⁶ cpm/ml of end-labelled [γ -P³²]-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 ml 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°C) absolute ethanol (5).

Slot-blotting of RNA

RNA was resuspended in 160 μ l of RNase-free water and 40 μ l of 10x denaturation buffer (30% formaldehyde and 0.1M NaH_2PO_4 , pH 7.0), heated at 60°C for 20 minutes and 40 μ l 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 MgCl₂, 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 -³²P] dCTP (Dupont/NEN, Boston, MA) and terminal deoxynucleotidyl transferase (TdT). Fifty ng of oligonucleotide was labelled in a reaction containing 1.4 M potassium cacodylate, pH 7.2, 300mM Tris base, 40mM MgCl₂, 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 strategy 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 comparison 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 HOMOLGY	
Human 18S	UGAAACUUAAGGAAUUGACGGAA	100 %	24MER
<i>T.brucei</i>	 <u>A</u>	95.8%	
<i>D.discoideum</i>		100 %	
<i>E.gracilis</i>		100 %	
<i>Tetrahymena</i>		100 %	
<i>P.falciparum</i>	<u>A</u> <u>G</u> <u>A</u>	87.5%	
<i>S.pustulata</i>		100 %	
<i>O.nova</i>		100 %	
<i>S.cerevisiae</i>		100 %	
<i>E.coli</i>	.. <u>A</u> <u>C</u> .. <u>U</u> <u>GG</u>	79.1%	

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

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

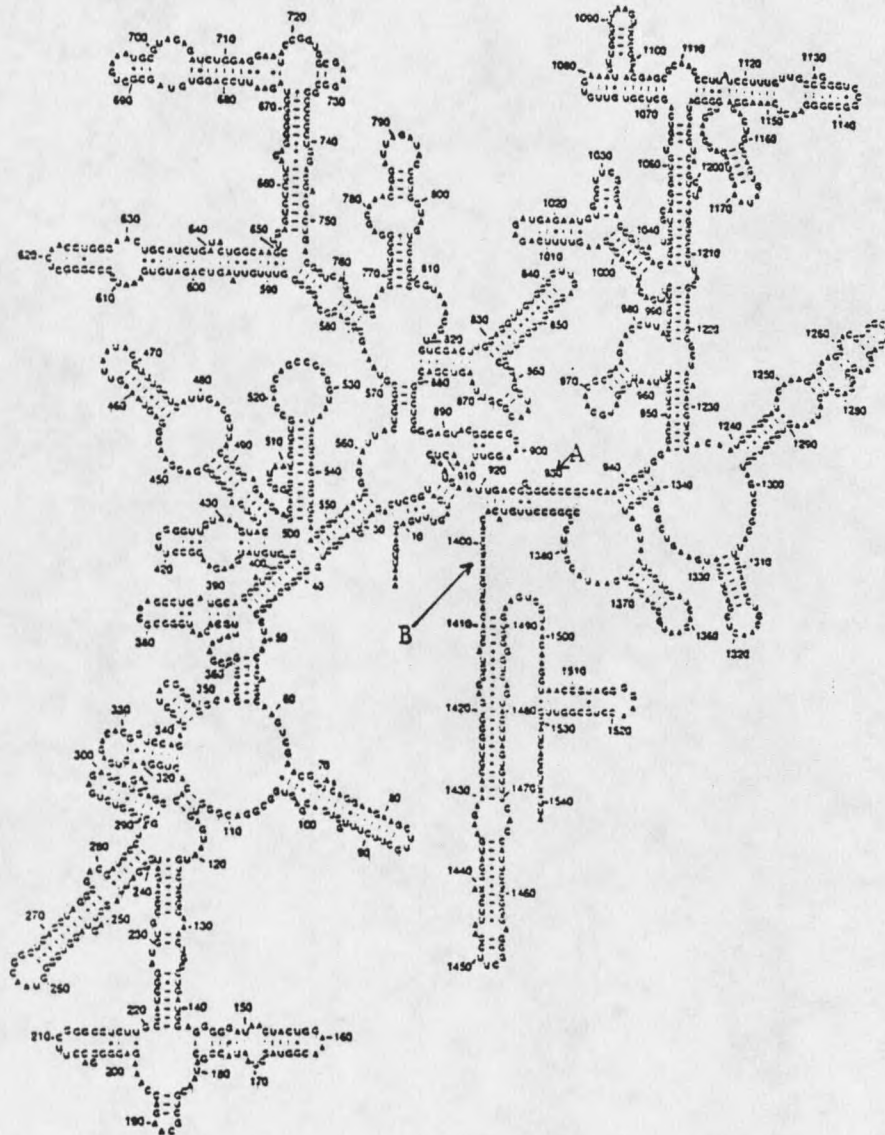


Fig. 2. Secondary structure map of *E. coli* 16S rRNA (Dalberg, A.E. 1989). Conserved region T.f. 1 (A). Conserved region T.f. 2 (B).

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

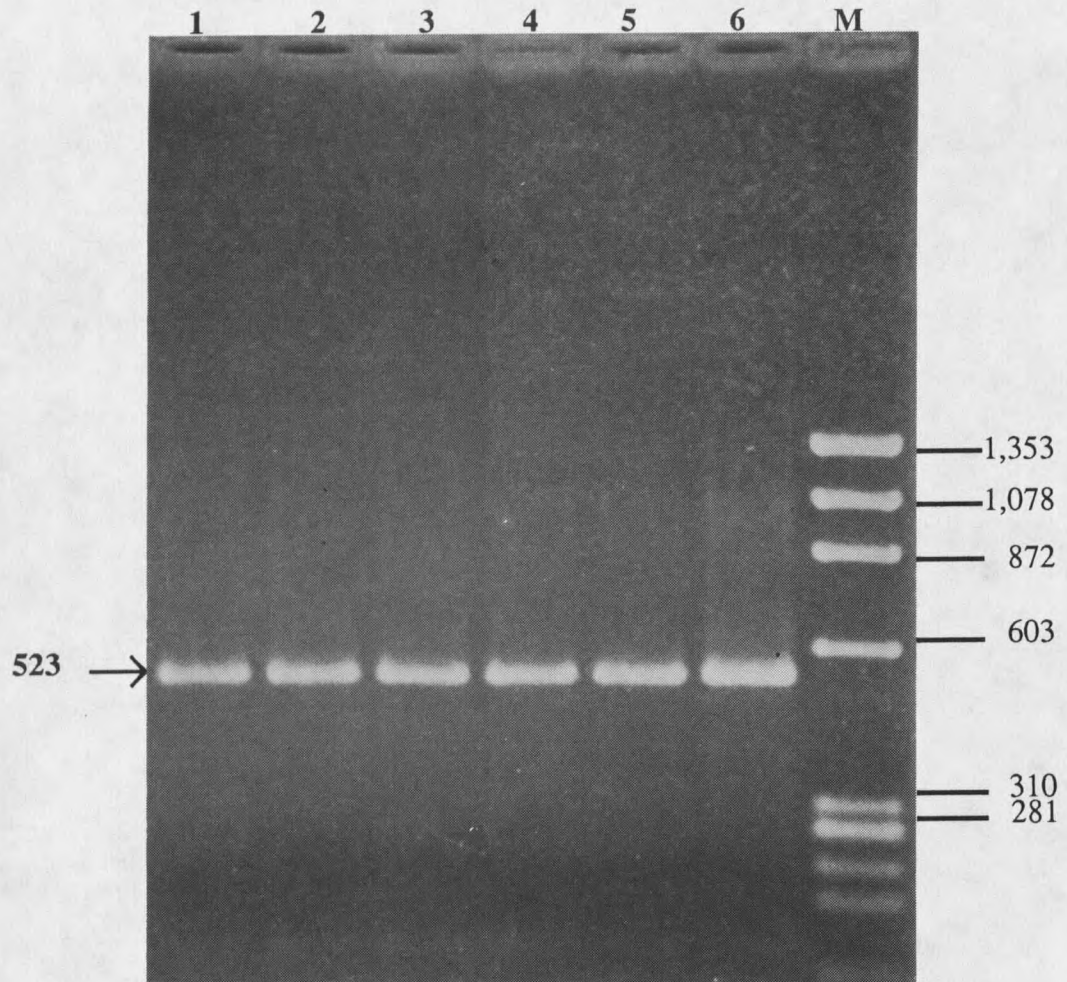


Fig. 3. Agarose gel (1.2%) electrophoretic analysis of PCR amplified products from various starting concentrations of *T. foetus* parasites (strain 85-330) genomic DNA. Lanes: 1 and 4, 10 ng *T. foetus* DNA; 2 and 5, 50 ng *T. foetus* DNA; 3 and 6, 100 ng *T. foetus* DNA; and M, Phi X174 RF/DNA Hae III standards.

sequences the various clones were screened for active genes by probing duplicate plaque lifts. Clones which hybridized to the internal probe were plaque purified and sequenced using published protocols (21). The T.f.1/T.f.2 primers have also been used to amplify a fragment of the 16S-18S rRNA genes from a diverse variety of cells. Figure 4 shows an ethidium bromide stained 1.2% agarose gel of PCR products using DNA from Fasciola hepatica, Trichomonas vaginalis, Tritrichomonas mobilensis, Tritrichomonas foetus, barley, rye and bovine cells (monocyte cell line M617). The PCR products from the different DNAs showed various fragment lengths; T. foetus (lane 6), T. mobilensis (lane 5), bovine M617 (lane 4), barley (lane 1) and rye (lane 2) fragments were approximately 500 base pairs in length; the fragment from F. hepatica (lane 3) was especially large, approximately 650 base pairs compared to 450 base pairs for T. vaginalis (lane 7). The minor upper band in the T. vaginalis lane has not been characterized and may be an artifact since the PCR annealing temperature (25°C) was relatively low.

The T. foetus fragment was completely sequenced and revealed a fragment of 523 nucleotides. The T. foetus sequence was aligned against bovine (also cloned and partially sequenced in this experiment), P. falciparum and human small-subunit rRNA sequences (Figure 5). It was necessary to introduce some "alignment gaps" in order to juxtapose regions of high similarity in the various sequences. Interestingly, in the P. falciparum sequence there was an addition of 47 nucleotides at T. foetus positions 361-371. Regions where the T. foetus rRNA sequence diverged from other rRNA sequences were chosen to design complementary oligonucleotides. The oligonucleotide designated MW24, was

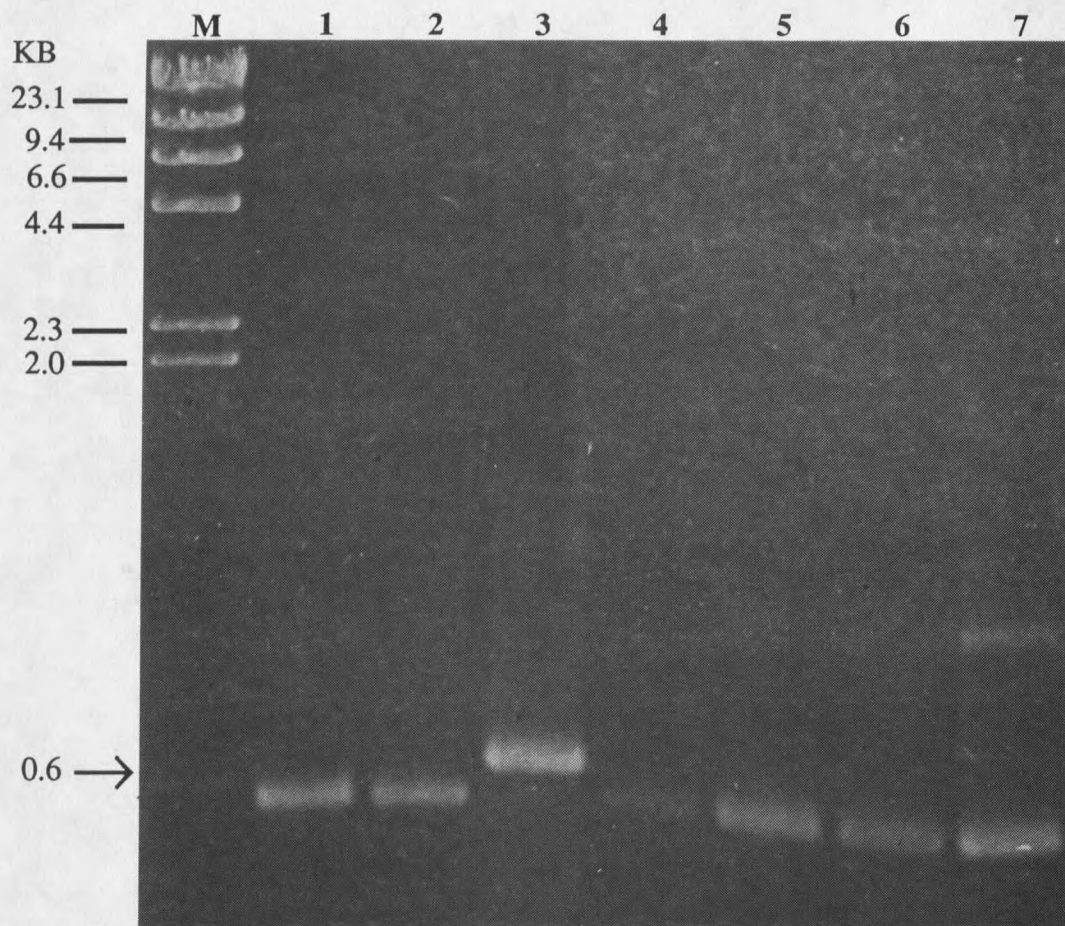


Fig. 4. Agarose gel (1.2%) electrophoretic analysis of PCR amplified products from various genomic DNAs using primers T.f. 1. and T.f. 2. Lanes: M, lambda Hind-III standards; 1, barley; 2, rye; 3, *E. hepatica*; 4, bovine monocyte cell line (M617); 5, *T. mobilensis*; 6, *T. foetus* (strain 85-330); and 7, *T. vaginalis*.

1	AACTTAAAGG AATTGACGGA AGGGCACCAC CAGGAGTGGA GCCTGCGGCT	Human
	-.....AN.. .N.....	Bovine
	..G.....A.C..... ..T.....	P.fal.
	-.....A-..G..... ..T..T....	T.foetus
51	TAATTTGACT CAACACGGGA AACCTCACCC GGCCCGGACA CGGACAGGAT	Human
	T.....	Bovine
	G ..A.....TA .TTTAA.... A.AGT.....	P.fal.
	A.G ..A..T...A ..A..A..TG TTTT..ATGAC	T.foetus
101	TGACAGATTG ATAGCTCTTT CTCGATTCCG TGGGTGGTGG TGCATGGCCG	Human
	A....C	Bovine
	AT....T.T ...A.....A	P.fal.
	GC.T CG-.G..... TCA.GA.GAC -TTT.....	T.foetus
151	TTCTTAGTTG GTGGAGCGAT TTGTCTGGTT AATTCCGATA ACGAACGAGA	Human
	(T)	
	..T.....C ...A.AT...	P.fal.
	..GG.G..GCGTT..C C....C.... G....A....G.....	T.foetus
	(AA)	
201	CTCTGGCATG CTAAGTAGTT ACGCGACCCC CGAGCGGTCTG GCGTCCCCCA	Human
	TCT.AACC..T...CG G..A.TA.A. TATACTTG.T TGA.TGAAA.	P.fal.
	T.A.C..CAA T...A..C.C GTNNNNNNNN NNNNNNNNNN.NNNNNNNNNN	T.foetus
251	ACTTCTTAGA GGGACA-AGT GCGGTTTCAGC CACCCGAGCT T-GAGCAATA	Human
	G..... ..A...TT.. .TGTC.A.CA ..AGGA..T. .AAG....C.	P. fal.
	NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN GGT.....	T.foetus
301	ACAGGTCTGT GATGCCCTT- AGATGTCCGG GGCTGCACGC GCGCTACACT	Human
	T..T....- ...T.AA.TA	P. fal.
	C.....T....-CT.T.N.....A.	T.foetus

Fig. 5. Sequence of *T. foetus* small-subunit 16S-18S rRNA aligned with *P. falciparum*, bovine, and human 18S rRNA. All sequences were aligned against the human 18S gene. Dots indicate sequence homology and N designates proprietary sequences.

351	GACTGGCTCA	GCGTG-----	-----	-----	-----	Human
	..TATATAT.	A..A.TTTTT	AAAAATATGC	TTATATTGTA	TCTTTGATGC	P. fal.
	.TTA..ATC.	.A...-----	-----	-----	-----	T.foetus
367	-----	--TGCCTACC	CTACGCCGGC	AGGCGCGGGT	AACCC-GTTG	Human
	TTATATTTTG	CA.A.T.TT.	..C.....AA	TA...	..T.TTA.C	P. fal.
	-----	-----NNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	T.foetus
401	AACCCCATTC	GTGATGGGGA	TCGGGGATTG	CAATTATTCC	CCATGAACGA	Human
	..TATATA..		.A.ATT....	AA	T.T.....	P. fal.
	NNNNNNNNNN	..AG.T....	.T.A.....	T...C...-	T.....C.	T.foetus
451	GGAATTCCCA	GTAAGTGC GG	GTCATAAGCT	TGCGTTGATT	AANTCCCTGC	Human
	G..T.	CAT.A	T....C..A.	..T.C...C.		P. fal.
	C..TT	A....T	AC...G	C.....A.	.CG.....	T.foetus
501	CCTTTGTACA	CACCGCCCGT	CGCTA	525		Human
						P. fal.
			...			T.foetus

Fig. 5. Continued.

complementary to a 22 nucleotide region (22 mer) beginning at map position 108 and ending at position 130; MW25 was a 20 mer corresponding to position 153-173. MW40 was an 18 mer, map position 275-292; MW41 an 18 mer, map position 253-271; MW42 a 20 mer, map position 372-391. MW25 was an oligonucleotide designed from a variable or semiconserved sequences (approximately 50% homology). MW40, MW41 and MW42 were oligonucleotides designed from hypervariable regions which showed little sequence conservation.

Cross-Reactivity

Previous reports have demonstrated that oligonucleotides complementary to "variable" regions of rRNAs can be used as probes for detecting different organisms (12 and 46). In order to obtain a reliable estimation of the cross reactivity of the oligonucleotide probes from the T. foetus 16S-18S rRNA sequences, a computer analysis was performed to determine the homology of the four oligonucleotides (MW25, MW40, MW41 and MW42) to known rRNA sequences (Table 1). MW25 shows the best homology to T. thermophilus matching 14 out of 20 nucleotides for a 70% correlation. Other organisms match 13 out of 20 or show a 67% homology. Oligonucleotides (MW40, MW41 and MW42) designed from a hypervariable region of the T. foetus 16S rRNA sequence were then compared. Surprisingly, rRNA sequence from N. gonorrhoeae matches 15 out of 18 nucleotides to MW40 (80% homology). Also the non-vertebrates, D. discoideum and S. costatum and the prokaryotes, P. aeruginosa and T. thermophilus matched 14 out of 18 bases (78% homology). MW41 (18 mer) proved to be

Table 1. Homology analysis of *T. fetus* sequences against various rRNA sequences using the Genepro^R computer program. MW40 is an 18 mer, MW25 a 20 mer, MW41 an 18 mer and MW42 an 18 mer.

<u>Organisms</u>	<u>MW40</u>	<u>MW25</u>	<u>MW41</u>	<u>MW42</u>
Eukaryotes:				
Vertebrates:				
<i>Human</i>	11	13	10	11
Non-Vertebrates:				
<i>Chlorella ellipsoidea</i> (green alga)	13	12	11	11
<i>Skeletonema costatum</i> (alga)	14	13	12	10
<i>Dictostelium discoideum</i> (mold)	14	13	13	12
<i>Saccaromyces cerevisia</i> (yeast)	12	13	10	11
Protozoans:				
<i>Oxytricha nova</i> (ciliate)	11	13	11	11
<i>Stylonychia pustulata</i> (ciliate)	11	13	11	11
<i>Tetrahymena pigmentosa</i> (ciliate)	10	13	11	11
<i>Trypanosome brucei</i> (flagellate)	10	12	11	10
<i>Tritrichomonas fetus</i> (flagellate)	18	20	18	18
<i>Plasmodium falciparum</i> (sporozoan)	11	10	10	11
Prokaryotes:				
<i>Streptomyces coelicolor</i>	13	12	11	12
<i>Pseudomonas aeruginosa</i>	14	12	9	11
<i>Escherica coli</i>	13	13	9	11
<i>Clathrochloris sylfurica</i>	11	13	10	11
<i>Thermus thermophilus</i>	14	14	13	10
<i>Neisseria gonorrhoeae</i>	15	12	9	11
<i>Brucella abortus</i>	11	12	11	9
Primitive Prokaryotes:				
<i>M. soehngenii</i> (archaebacterium)	10	12	10	10

Conversion to Melting Temperature [1% Difference=1°C Change].

more specific for T. foetus since the best match against the various sequences was 13 out of 18 for a 72% homology with D. discoideum and T. thermophilus. The lowest percent homology compared against the various organisms rRNA 16S sequences was with oligonucleotide MW42. D. discoideum and S. coelicolor sequences match 12 out of 18 nucleotides for a 67% homology. Oligonucleotide, MW42, was designed from the region corresponding to the P. falciparum 47 nucleotide insertion site indicating the lack of sequence conservation of this region. This analysis indicated that oligonucleotide MW42 maybe the best choice to be used as a probe for the detection of T. foetus infection.

Hybridization experiments were performed using total RNA from T. foetus strains Montana 85-330 [85-330], California 1984 [CA84], California VMC 2031 [VMC] and Nevada [NV], T. mobilensis, T. vaginalis and bovine M617 cells (See Materials and Methods). Total RNA from each organism was slot-blotted onto nitrocellulose using the Biorad slot-blot apparatus (5 μ g RNA per slot) and the nitrocellulose was cut into five identical strips, which were then probed with oligonucleotides T.f.2, MW25, MW40, MW41 and MW42 that had been end-labelled with [γ -P³²] ATP and T4 polynucleotide kinase. The slot-blots were hybridized at 53°C using standard protocols (See Materials and Methods) and exposed to X-ray film (See Materials and Methods). The autoradiograph from this experiment (Figure 6) showed that oligonucleotide T.f.2 gave positive hybridization signals in all lanes confirming the conservation of this sequence. Oligonucleotides MW25 and MW41 hybridized to Trichomonas and Tritrichomonas species, but not to bovine RNA (Figure 6B and 6D). MW40 hybridized to Tritrichomonas species and faintly with T. vaginalis, whereas MW42 hybridized to

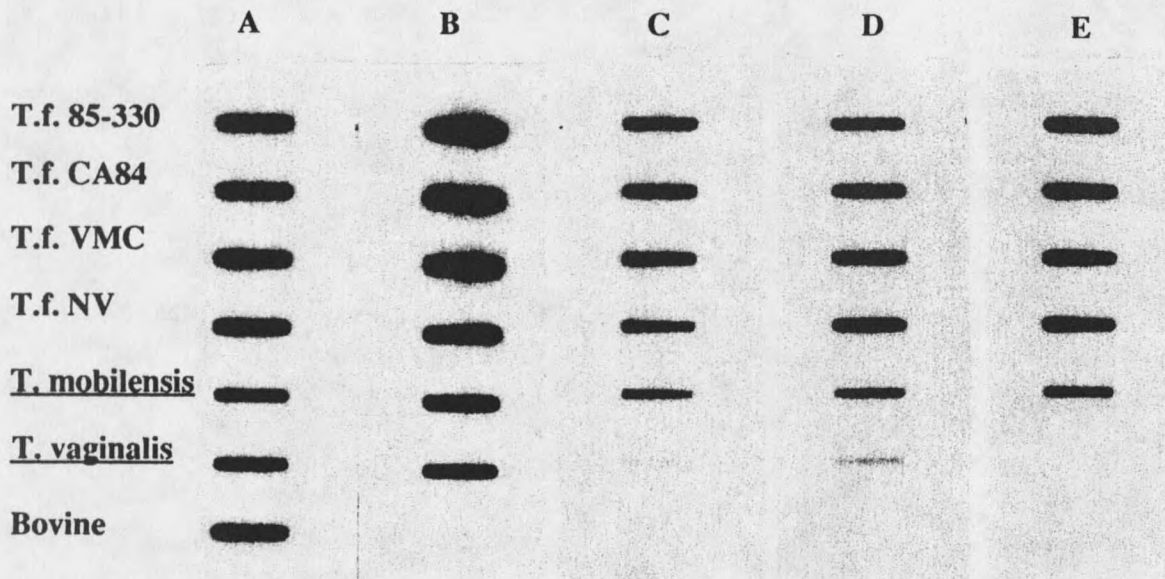


Fig. 6. Hybridization of synthetic oligonucleotide probes to various RNAs. Total RNA was extracted, bound to nitrocellulose filters by slot-blotting and hybridized to 5'-end labelled oligonucleotides T.f. 2. (column A), MW25 (column B), MW40 (column C), MW41 (column D) and MW42 (column E). Lanes: 1, T. foetus, Montana strain 85-330 (T.f. 85-330); 2, T. foetus, California strain 1984 (T.f. CA84); 3, T. foetus California strain VMC (T.f. VMC); 4, T. foetus, Nevada strain (T.f. NV); 5, T. mobilensis; 6, T. vaginalis; and 7, bovine cells.

Tritrichomonas species only with no cross hybridization with either T. vaginalis or bovine RNA (Figure 6E).

Nucleic Acid Assay Development

With the identification of tritrichomonas specific oligonucleotide probes, two assay formats were examined for the detection of T. foetus infection. The first format involved the amplification of appropriate nucleic acid target sequences using the PCR. The second format involved the direct hybridization of RNA. The PCR format has been used in the clinical detection of pathogens such as Toxoplasma gondii in HIV infected patients and for the detection of serum hepatitis B virus DNA in patients with chronic hepatitis (16). The PCR focuses on amplifying a single fragment of a specific gene, thus, only one gene copy per cell is theoretically required. The direct assay of rRNA depends on the content of cellular rRNA; there are approximately 1×10^6 copies of rRNA molecules per cell in human cells.

PCR-based Assay

We first examined the PCR format for T. foetus detection. Samples containing various amounts of T. foetus (strain 85-330) parasites were extracted and the partially purified DNA amplified using primers T.f.1 and MW25 in a standard PCR protocol (See Materials and Methods). The samples were ethanol precipitated, the DNA recovered by centrifugation and slot-blotted. The slot-blot was probed with end-labelled oligonucleotide

MW25. The blot was exposed to X-ray film with an intensifying screen and densitometric absorbance values were determined by scanning the autoradiograph with an Ultrosan XL Enhanced Laser Densitometer (Pharmacia, Bromma, Sweden). The results showed that 10 parasites could be detected at a relative absorbance of 2.5-fold over background (Figure 7).

Although the previous experiment examined the sensitivity of the PCR format, the reliability of this format is dependent on the efficiency of DNA extraction, cross contamination of samples and reaction to reaction variability. We examined the reliability of the PCR and the DNA extraction protocol in the following experiment: 1.) 10 samples in Diamonds medium (8) (100 *T. foetus* parasites/sample) were processed according to the PCR diagnostic assay protocol (See Materials and Methods) and the reactions slot-blotted onto nitrocellulose. 2.) one sample containing 1000 parasites in Diamonds medium (8) was processed according to the PCR diagnostic assay protocol and the DNA split into ten identical PCR reactions and the reactions slot-blotted. Oligonucleotide MW25 was end-labelled and the blot hybridized at 60°C (Figure 8). The results showed problems in DNA recover and in the PCR reaction. The variable signal intensity in column A and the lack of signal in column A2 indicated a problem in DNA recovery. The DNA recovery was improved in the sample with 1000 parasites split into ten reactions (column B), however, these reactions showed additional reproducibility problems since there was reduced or absent signal in columns B5 and B9. The lack of PCR amplification in columns B5 and B9 could be a problem with the PCR reaction components or a problem with the thermal cycler since a recent report (31) suggested that

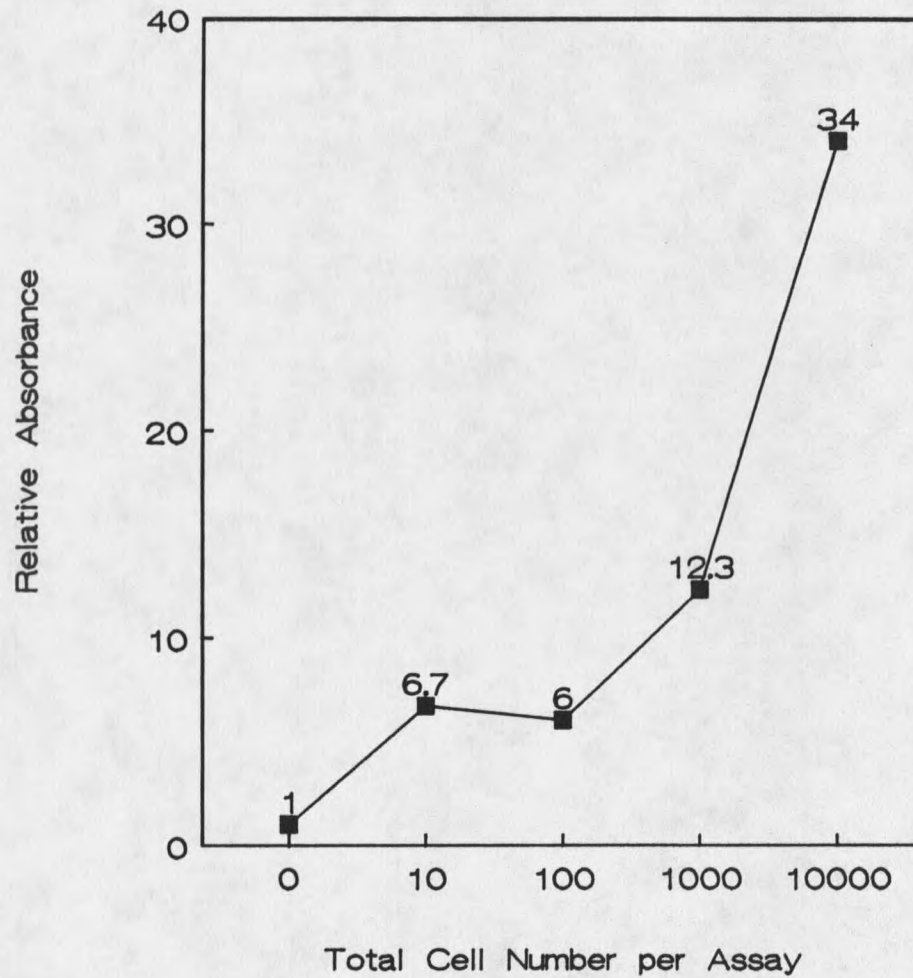


Fig. 7. Detection limit of *T. foetus* parasites using a PCR assay protocol.

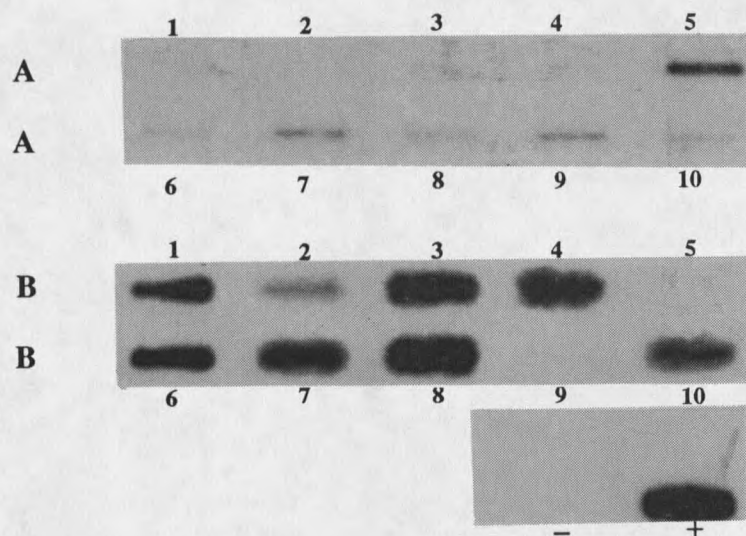


Fig. 8. Reproducibility of the DNA-based PCR assay. Column A: 10 samples (100 *T. foetus* parasites strain 85-330/sample) processed according to the DNA-based PCR Assay (See Materials and Methods), slot-blotted onto nitrocellulose and probed with 5'-end labelled oligonucleotide MW25. Column B: One sample containing 1000 *T. foetus* parasites, processed according to the DNA-based PCR Assay protocol, split into ten identical PCR reactions, slot-blotted and probed with 5'-end labelled oligonucleotide MW25.

thermal cycler blocks may not amplify uniformly.

Although extraneous nucleic acid from multiple sources may serve as a template for amplification thereby leading to false positives, most of false positives appear to result from pipetting contamination from adjacent samples (10). DNA from bovine M617 cells, bacteriophage λ DNA, T. vaginalis, T. mobilensis and T. foetus were used in three different PCR reactions using three sets of primers, T.f.1 and T.f.2, T. foetus variable region primer MW24 and T.f.1, and T. foetus variable region MW25 and T.f.1. We expected to amplify a fragment of approximately 500 base pairs using primers T.f.1 and T.f.2, a 130 base pair fragment using MW24 and T.f.1 and a 170 base pair fragment using MW25 and T.f.1. PCR products were separated on a 6% polyacrylamide gel and detected by staining with ethidium bromide (Figure 9). There should not be bands in lanes 2, 3, 4 or 5 since DNA from bacteriophage λ or bovine M617 does not contain complementary sequences to oligonucleotide primers MW24 and MW25. Thus, the presence of an approximately 130 base pair band in lanes 2 and 4 and 170 base pair band in lanes 3 and 5 indicates that DNA contamination occurred in this experiment. Also, the disappearance of a 130 base pair band in lane 9 could be from the inability of the thermal block to amplify uniformly. Because of the reliability and cross contamination problems the PCR format did not appear to be a suitable protocol. We then explored a protocol to detect rRNA directly without amplification.

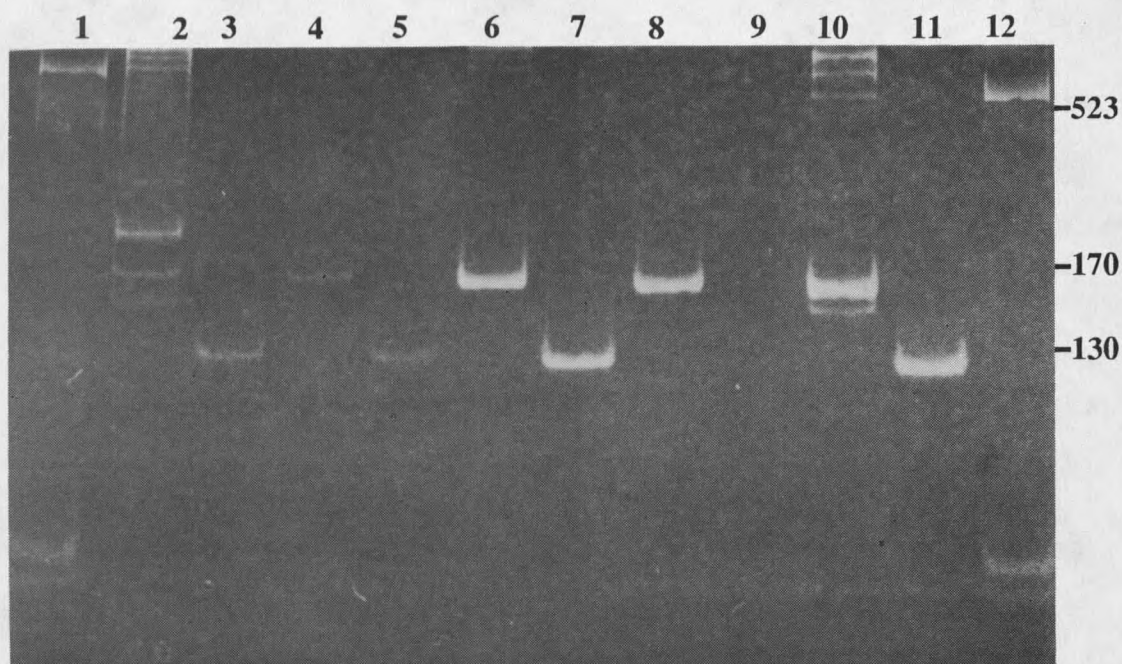


Fig. 9. Polyacrylamide gel (6%) electrophoretic analysis of PCR amplified products from various DNA. Lanes 1, bovine M617 DNA and 12, *T. foetus* DNA (strain 85-330) were amplified using primers T. f. 1. and T.f. 2. Lanes 2, M617 DNA, 4, bacteriophage lambda DNA, 6, *T. vaginalis* DNA, 8, *T. mobilensis* DNA and 10, *T. foetus* DNA (strain 85-330) were amplified using primers T.f. 1. and MW25. Lanes 3, M617 DNA, 5, bacteriophage lambda DNA, 7, *T. vaginalis* DNA, 9, *T. mobilensis* DNA and 11, *T. foetus* DNA were amplified using primers T.f. 1. and MW24.

RNA Diagnostic Assay

Comparision of Total RNA Extraction Protocols

In the development of the RNA assay, the extraction of RNA from a sample was the first procedure studied. We utilized three types of samples. The first one consisted of T. foetus parasites suspended in Diamonds medium (8). This type of sample contained low levels of protein, thus, a single GUISCN extraction was sufficient to recover the RNA. However, the most common types of field samples received for diagnosis of T. foetus infection were cervical mucus from cows and smegma from bulls. Both of these sample types are high in protein which presents problems in the extraction of total RNA. Therefore, an additional step was needed to recover the RNA efficiently. Experiments were performed to explore ways of extracting RNA from different sample types. All extraction protocols began with the removal of mucous proteins by solubilization in the reducing agent β -mercaptoethanol (final concentration 5%). Upon microscopic examination of samples treated in this manner, we found that trophozoites of T. foetus were not affected (there is no membrane lysis or parasite death) by brief treatment with 5% β -mercaptoethanol. The sample was then centrifuged for 5 min to concentrate the parasites, the supernatant removed and the cell pellet lysed in 0.3 ml of 4M GUISCN solution (See Materials and Methods). Two methods were employed to remove proteins. One protocol involved repeated extraction and ethanol precipitation with a GUISCN solution. In the second protocol, proteins were removed by phenol/chloroform extraction (See Materials and Methods). Samples of T. foetus (strain 85-330, 1000 parasites/sample)

were processed according to the two different protocols and slot-blotted onto nitrocellulose. An autoradiograph of slot-blots hybridized to the end-labelled oligonucleotide MW40 is shown in Figure 10. Slots 1 and 4 were parasites suspended in Diamonds medium and processed by a single 4M GUISCN extraction; slots 2 and 5 were suspended in bovine cervical mucus and proteins removed by the repeated GUISCN extraction protocol; slots 3 and 6 parasites were suspended in bovine cervical mucus and proteins removed by phenol extraction. No hybridization signal occurred in slots 2 and 5 indicating that the repeated GUISCN extraction was not successful in removing excess proteins, however, the phenol extraction protocol produced excellent results as the signal in slots 3 and 6 were as strong as in slots 1 and 4.

Comparision of Different Membranes/UV vs. Baking

One of the critical steps in solid-phase hybridization assays of nucleic assays is the immobilization of DNA or RNA fragments on a solid support. With the advent of nylon membranes and the UV cross-linking alternative to vacuum baking there are a number of choices which we attempted to resolve (27). We chose three different membranes which were either UV cross-linked or baked at 80°C for 1 hr to compare the efficiency of immobilization of RNA. Nitrocellulose, BA85 0.45 μ m (Schleicher & Schuell Inc., Keene, NH), Gene Screen Plus (Dupont, Boston, MA) and Duralose 0.45 μ m nylon supported nitrocellulose (Stratagene, La Jolla, CA) were used to slot-blot total RNA from 12,000 *T. foetus* trophozoites, in Diamonds medium or in bovine cervical mucus. These samples were split into three sets and slot-blotted onto the three membranes which were

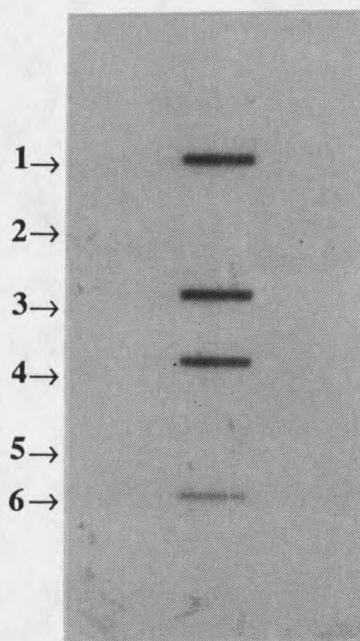


Fig. 10. Comparison of total RNA extraction protocols. Samples of *T. foetus* parasites (strain 85-330, 1000 parasites/sample) processed according to two different protocols, slot-blotted onto nitrocellulose and probed with 5'-end labelled MW40. Slots: 1 and 4, parasites suspended in diamonds media and processed by a single guanidine isothiocyanate (GUISCN) extraction protocol; 2 and 5, parasites suspended in bovine cervical mucus and proteins removed by the GUISCN extraction protocol; 3 and 6, parasites suspended in bovine cervical mucus and proteins removed by the phenol extraction protocol (See Materials and Methods).

then split into two strips per membrane type. One strip of each membrane was baked at 80°C for 1 hr while the other strip was UV cross-linked at 120mJ/cm² for 30 sec. The membranes were hybridized to 5'-end labelled oligonucleotide MW40 (Figure 11). The highest signals were obtained when RNA was immobilized on Duralose which had been UV cross-linked (lane 5) or UV cross-linked nitrocellulose (lane 1). Since nylon supported nitrocellulose (Duralose) is significantly stronger than regular nitrocellulose this would be the membrane of choice. The signals in lane 4, C and D, were weak, this could be due to a component in the mucous which prevented the binding of the nucleic acids to the nylon membrane.

Detection Limit Determination

We examined the RNA assay format for the detection of T. foetus. Total RNA was extracted from samples containing various amounts of T. foetus (strain 85-330) and M617 bovine cells according to the RNA assay protocol and slot-blotted (See Materials and Methods). The slot-blot was probed with end-labelled oligonucleotide MW25 and exposed to X-ray film with an intensifying screen. Quantitative values were determined by densitometric scanning of the autoradiograph from this experiment. A graph of relative absorbance against cell number showed that 10 parasites gave a 4.2 fold signal over background (Figure 12).

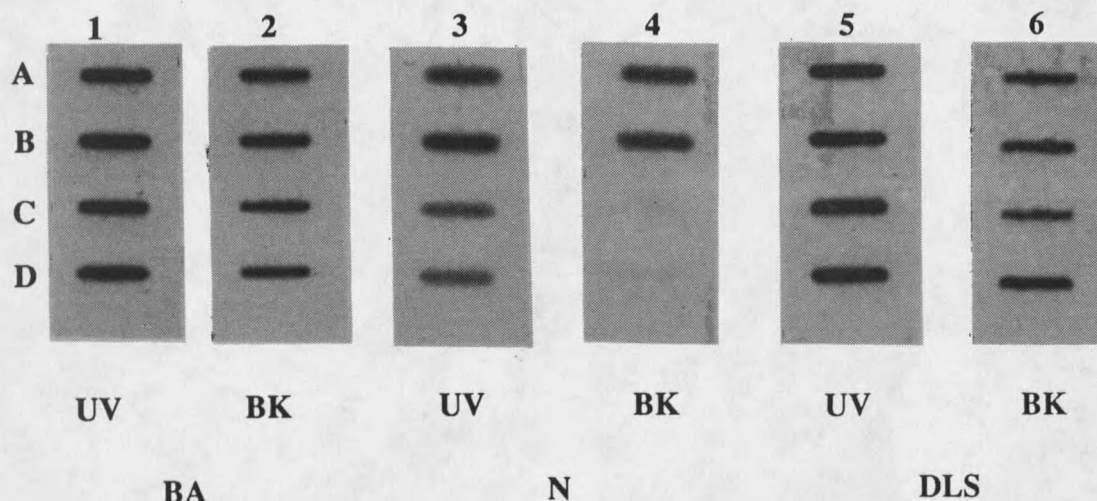


Fig. 11. A comparison of UV crosslinking versus baking on various membranes. Three membranes were UV-crosslinked at $120\text{mJ}/\text{cm}^2$ for 30 sec (UV) or baked at 80°C for 1 hr (BK); BA85 nitrocellulose (BA), Gene Screen Plus nylon membrane (N) and Duralose nylon supported nitrocellulose (DLS). Total RNA was extracted from 12,000 *T. foetus* parasites (strain 85-330) in media (Columns A and B) or in bovine cervical mucus (Columns C and D). The samples were split into three sets and slot-blotted onto the three types of membranes. The membranes were hybridized to a 5'-end labelled oligonucleotide MW40.

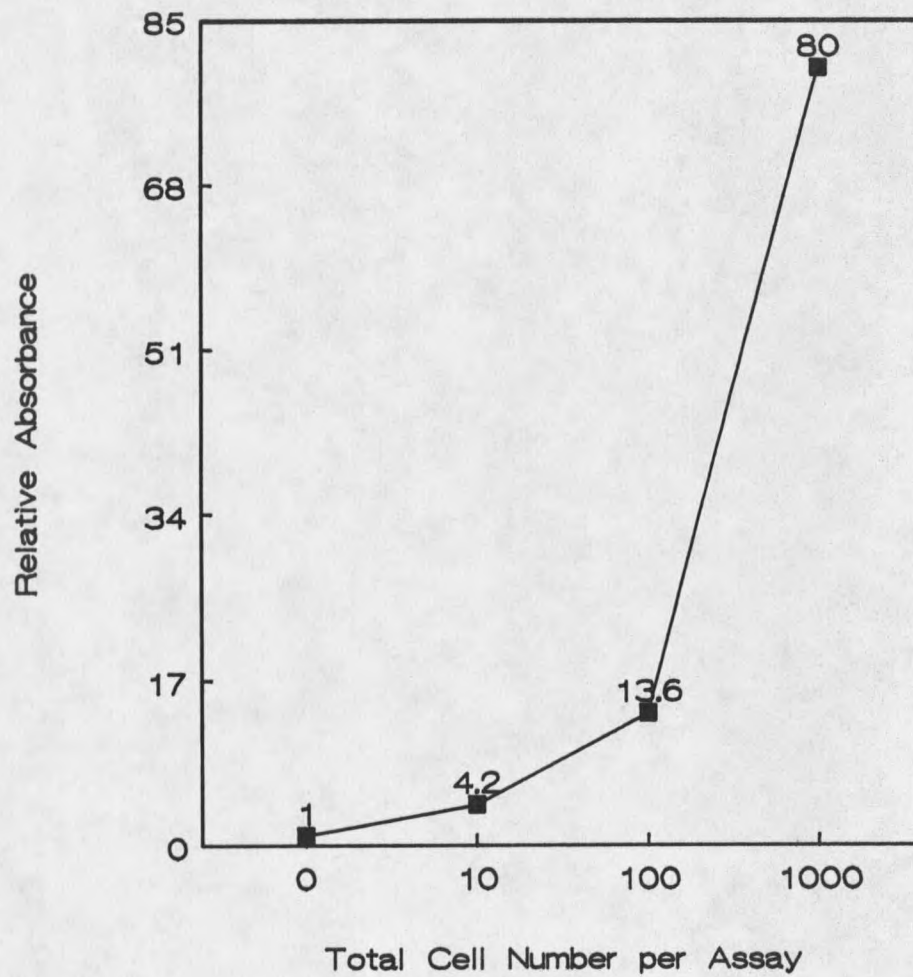


Fig. 12. Detection limit of *T. foetus* parasites using the RNA assay.

RNA Stability in GUISCN and Reproducibility

The RNA assay was tested for reproducibility under a variety of conditions. Briefly, two tubes, one containing 500 *T. foetus* parasites (Set A) and 5000 parasites (Set B) were lysed in 1.0 ml of 4M GUISCN solution. A 0.2 ml sample was removed every day for 4 days and total RNA processed. Set A was stored at room temperature while set B was stored at 4°C throughout the experiment. The samples were slot-blotted onto nitrocellulose and probed with end-labelled oligonucleotide MW25. The slot-blots were hybridized at 60°C using standard protocols (See Materials and Methods) and exposed to X-ray film with an intensifying screen. The autoradiograph from this experiment showed that the samples stored in GUISCN solution had no RNA degradation when stored at room temperature or 4°C suggesting that samples can be lysed in the field and shipped to the testing laboratory without loss of signal. No reproducibility problems were detected since there was an equal signal seen in all lanes of sets A and B (Figure 13).

Mock Testing

Currently, the only accepted diagnostic test for the detection of *T. foetus* infection within the bovine is a microscopic/culture test. A field sample is typically collected using saline with an infusion pipette and contains cervical mucus (cow) or smegma (bull). The sample is shipped to a diagnostic laboratory and 20-30 fields from a drop of the sample examined by dark-field phase-contrast microscopy. A 0.5 ml sample is placed in Diamonds medium, cultured for 1-7 days at 37°C, and examined again by dark-field

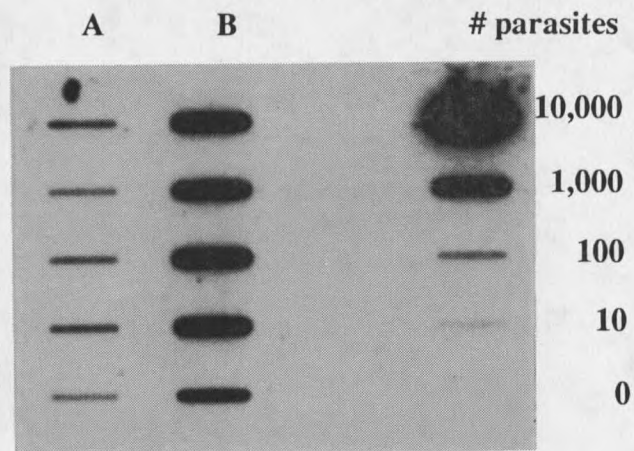


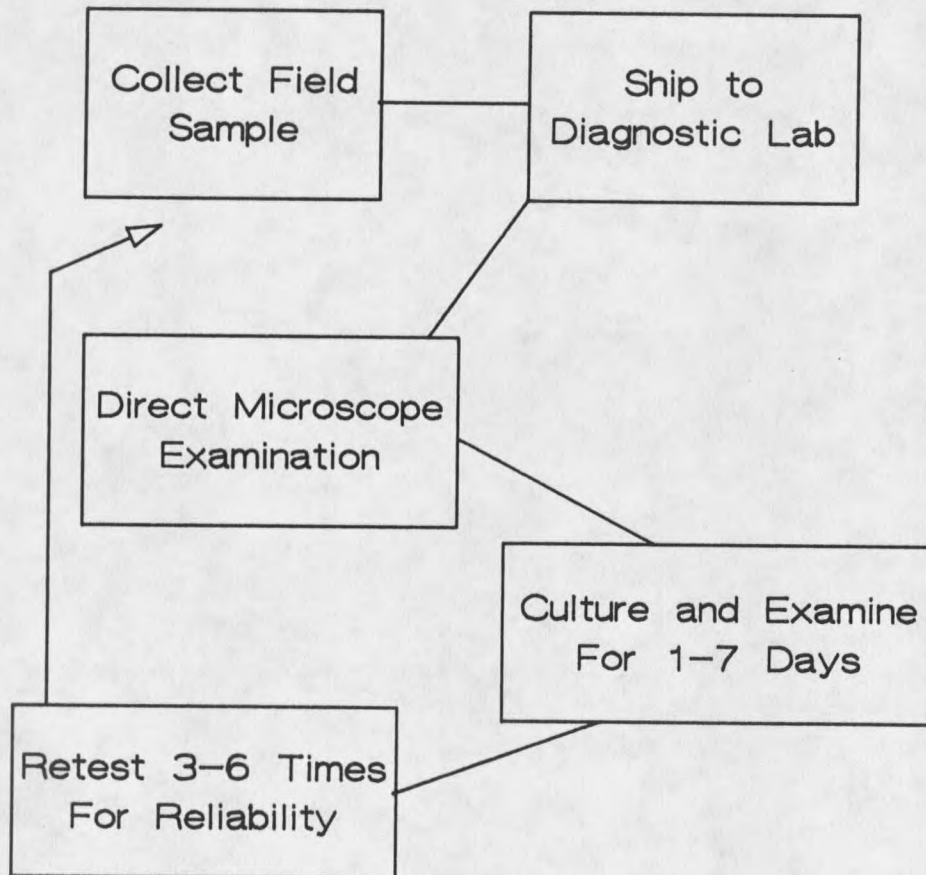
Fig. 13. Stability of RNA in GUISCN and reproducibility of *T. foetus* RNA assay. Two samples, one containing 500 *T. foetus* parasites (strain 85-330) (Set A) and the other containing 5000 parasites (Set B) were lysed in 1.0 ml of 4M GUISCN solution. Set A was stored at room temperature while set B was stored at 4°C. A 0.2 ml sample was removed every day for 4 days and total RNA processed (See Materials and Methods). Samples were slot-blotted onto nitrocellulose and probed with 5'-end labelled oligonucleotide MW25. A standard curve of known parasites was included in the experiment.

microscopy for the presence of T. foetus. A sample is considered positive if a motile organism containing three flagella and an undulating membrane is detected (Figure 14). For comparison, Figure 15 shows a flow chart for the RNA assay.

To further test the RNA assay, mock samples were set up that contained T. foetus parasites plus bovine cells or cervical mucus. Each mock test contained thirty samples, fifteen of which contained 50, 100, 200 or 1000 parasites and fifteen contained no parasites. All samples contained 1000 bovine cells also. Figure 16 shows the autoradiograph from one mock test using 5'-end labelled oligonucleotide MW25. A standard curve of known parasites is included in the experiment. The data indicate a 100% detection with no false positives. Figure 16 also indicates that the DNA probe can easily detect samples corresponding to 100 parasites/sample.

A total of five mock tests were performed comparing the microscopic/culture protocol to the RNA assay (Table 2). Mock test sets 3, 4 and 5 were processed by the Montana State Veterinary Diagnostic Laboratory (Livestock Health Division) in Bozeman, MT according to the standard microscopic/culture assay along with the RNA assay. Test set 1 was microscopically examined by Dr. C.A. Speer (Head, Veterinary Molecular Biology, MSU). Briefly, twenty to thirty fields of a drop of each sample were viewed by microscopy for the presence of a motile organism containing three flagella and an undulating membrane. A 0.5 ml sample was removed and cultured at 37°C in 5 ml of Diamonds medium (8) for seven days. At the end of this time, the cultured sample was again examined by microscopy for the presence of T. foetus. The remaining sample from the initial microscopic examination was processed according to the RNA assay and

Microscopic/Culture Test



Positive = Motile organism containing
three flagella and undulating membrane

Fig. 14. Flow chart of the microscopic/culture test for the detection of *T. foetus* infection.

RNA ASSAY

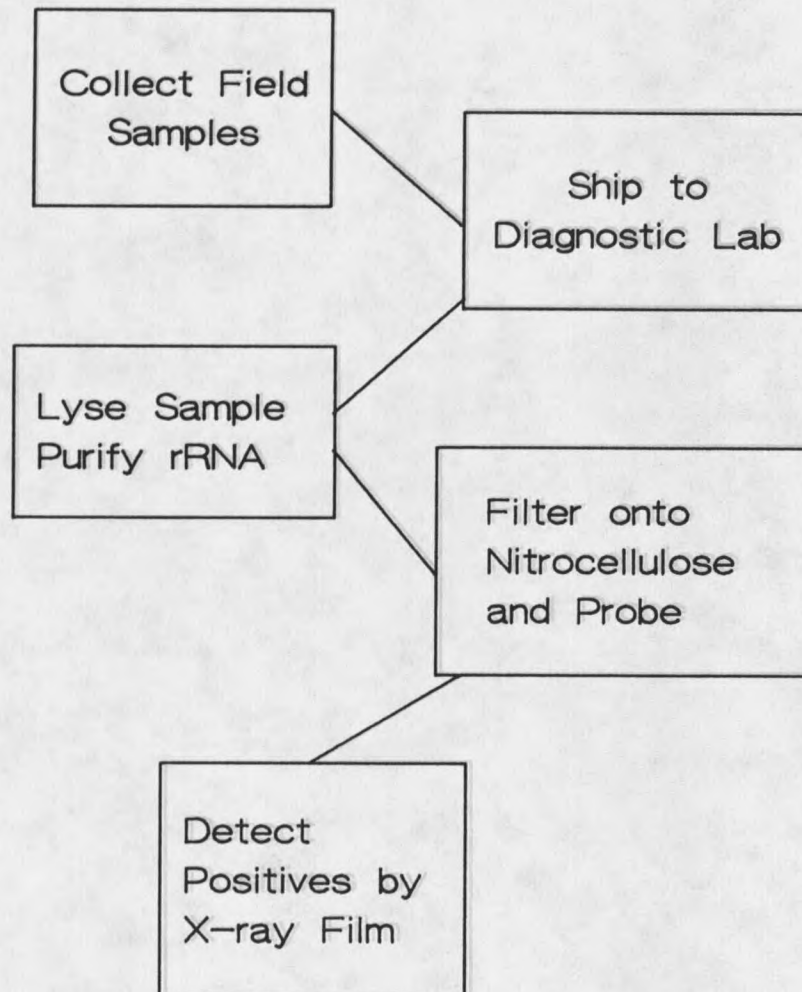


Fig. 15. Flow chart for the RNA Assay for the detection of *T. foetus* infection.

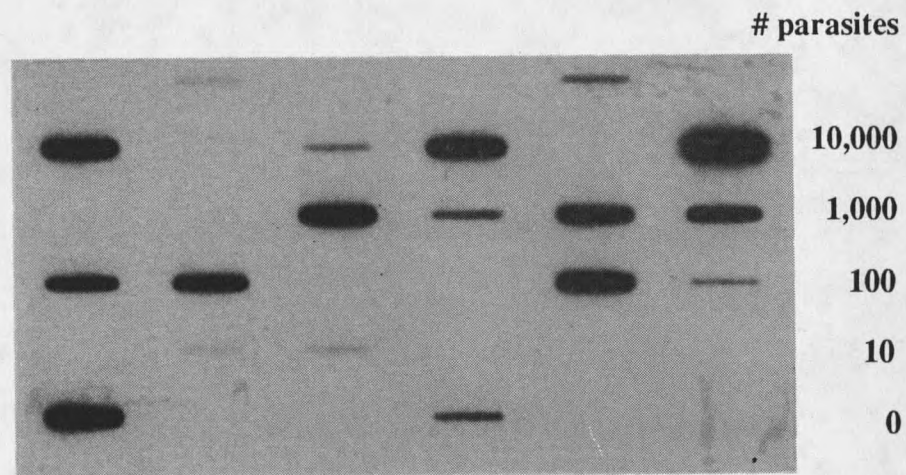


Fig. 16. Detection of *T. foetus* parasites using the RNA assay. The experiment consisted of 30 test samples, 15 containing 50, 100, or 1000 parasites per sample and 15 samples containing no parasites. All samples contained 1000 bovine cells. Total RNA was extracted, slot-blotted onto nitrocellulose and probed with 5'-end labelled oligonucleotide MW25. A standard curve of known parasites was included in the experiment.

slot-blotted. The slot-blots were probed with 5'-end labelled oligonucleotide MW25. A standard curve was included in each of the test sets. Tests 2 and 3 were 60 total samples, 10 samples containing 50, 100 or 1000 parasites and 30 samples containing no parasites. The samples in test sets 2 and 3 were first stored at 4°C for 72 hours prior to testing by the microscopic/culture and/or RNA assay protocols. Test sets 1, 4 and 5 were processed immediately after aliquoting parasites at room temperature using a log phase T. foetus culture. All samples of the mock tests were performed without the operators knowledge of which samples were positive and after completion of the assays the results were decoded. The RNA assay utilized 5'-end labelled oligonucleotide MW25. The overall success of the microscopic culture assay (120 samples, 60 positives) was 27% of the total positives with no false positives, while the RNA assay found 98.6% of the positives with no false positives (150 total samples, 75 positives). When the samples were first stored at 4°C for 72 hrs (test sets 2 and 3), the detection rate for the microscopic/culture assay dropped to 7% confirming a major limitation in the current assay such that the parasites must be alive to be culturable. The detection rate for the RNA assay was unaffected by storage at 4°C. No pattern of detection with regards to parasite concentration was observed in the test sets assayed by the Montana State Veterinary Diagnostic Laboratory (Sets 3, 4 and 5). A similar number of 1,000 parasites/ml and 100 parasites/ml were detected indicating that the microscopic/culture assay is essentially random at these parasite levels.

Table 2. Mock testing: Microscopic/culture vesus the RNA assay.

<u>TEST</u>	<u>Number Positives</u>	<u>Percent Positive</u>	
		<u>Micro</u>	<u>rRNA Assay</u>
Test 1	15	53	100
Test 2 72h cold	15	N/A	100
Test 3 72h cold	15	7	100
Test 4	15	13	100
Test 5	<u>15</u>	<u>27</u>	<u>93</u>
Total = 75		AVE = 27	98.6
Total samples = 150			

Field ExperimentationCase Study 1

The samples for the first case study were from two herds physically separated and located in eastern Montana. Herd 1 consisted of 38 open heifers from a group of 180 head having a 79% pregnancy rate. The heifers were given Preg guard 9, wormed with Safe guard and poured for lice on April 1, 1990. They were moved to pasture May 10. Nine, 3 year old bulls were placed with the heifers on May 20, 1990 and pulled on July 9. The heifers were pregnancy tested August 21 and trichomoniasis tested on September 6, 1990 using the RNA assay. Samples of the open heifers were collected with a human pap smear scraper and flushed with saline. The RNA assay results showed there was no evidence of T. foetus infection in these samples.

Herd 2 consisted of 66 open heifers from a group of 217 head having a 70% pregnancy rate. These heifers were managed like the heifers in herd 1. Eleven bulls were placed with the heifers on May 22 and 10 were pulled on July 8. The last bull was found in a separate herd and pulled on July 31. The heifers were pregnancy tested August 23 and trichomoniasis September 7, 1990. The first 14 samples were collected by a Kalajian 3000 culture swab. The remaining 42 samples were collected by repeatedly injecting and aspirating the vaginal area with 10 ml PBS until the sample appeared cloudy; 5 of the 14 swabs were positive by the RNA assay and 7 of the 42 saline flushes were positive for a 17% infection rate. The results indicate that open cows from herd 1 have no T. foetus infection while herd 2 showed a 17% infection rate which correlated with the relative

pregnancy rates. The highest level of infection in herd 2 was approximately 250 parasites/ml suggesting that the microscopic test would miss many of these samples. The microscopic/culture assay was not performed on the above samples.

Case study 2

The samples for the second case study were from a single herd located in western Texas. This herd consisted of 11 open heifers from a group of 86 head having a 87% pregnancy rate. The heifers were managed like the heifers in case study one. The heifers were pregnancy tested August 1, and trichomoniasis tested August 16, 1990 using the RNA assay protocol. Samples 1-86 were collected with saline flushes through an infusion pipette. Nine duplicate samples were collected with cotton tipped swabs of the cervix of randomly chosen heifers from samples 1-86. Of the 86 mucus samples, 47 were positive for a 52% infection rate. Seven of the nine swabs correlated with the results of the saline-flush samples. One swab sample was received without identification and the final swab was negative while the duplicate saline-flush was weakly positive. The intensity of the positive signal from the swabs correlate well with the level of intensity of the paired-saline flush. The level of parasites per sample was low (approximately 100 parasites/sample), which would be difficult for the microscopic/culture assay to detect. However this high infection rate (52%) did not correlate with the pregnancy rate (87%) in these animals. An independent microscopic/culture test was not performed on the above samples and during the preparation of the manuscript the calving rate for this herd was unavailable.

DISCUSSION

For the past fifty years, the only means of diagnosing Tritrichomonas foetus infection within the bovine has been by microscopy. Our approach of DNA hybridization using specific probes to target nucleic acid molecules of T. foetus offers significant advantages over conventional microscopy. DNA hybridization using specific probes is extremely sensitive (50 parasites/sample) and can be performed relatively quickly on a large number of samples. While the microscopic/culture assay relies on culturing a small aliquot of a sample rendering the system vulnerable to contamination, the high specificity of DNA hybridization precludes the need for pure cultures. Recently, an rRNA-directed DNA probe assay for the nonculturable laboratory diagnosis of N. gonorrhoeae was evaluated; the assay was shown to be 99% specific and 93% sensitive (33).

We chose the small-subunit major rRNA gene as a target since these genes show regions of nearly absolute conservation as well as regions of hypervariability. Thus, oligonucleotides T.f.1 and T.f.2 were designed from conserved regions and used in a standard PCR protocol to amplify a segment of the 16S-18S rRNA gene from T. foetus. Upon subcloning, sequencing and comparison of the T. foetus 16S sequences to a data bank of rRNA sequences, we identified conserved, variable and hypervariable regions (Table 1). These findings led us to design oligonucleotides that corresponded to the variable regions. Oligonucleotides T.f.2, MW25, MW40, MW41 and MW42 were examined in cross-reactivity experiments (Figure 6). Oligonucleotide T.f.2 recognized all RNA from the various protozoans and cell lines consistent with the conserved nature

of these sequences. Oligonucleotides MW25 and MW41 detected all strains of *Trichomonads* thereby representing variable regions of sequence and could be compared to the variable region at map positions 1410 to 1490 of the *E. coli* rRNA gene (7). Oligonucleotides MW40 and MW42 are *Tritrichomonas* specific and represent hypervariable sequences which are comparable to map positions 840 to 900 in the 16S rRNA sequences of *E. coli*. This region may contain 60 nucleotides as in *E. coli* (11) to as few as 25 nucleotides in the 16S rRNA of *D. discoideum* (38).

Our first attempt at designing a diagnostic assay for the detection of *T. foetus* infection within the bovine involved using the polymerase chain reaction (PCR). The PCR, since its introduction a few years ago has already become a widespread research tool. The popularity of the PCR is due primarily to its simplicity and high probability of success. Scientists have used PCR for research applications in direct sequencing of in vitro amplified DNA (42), mutational detection (25) and evolutionary analysis (14). They have also used the PCR in medical applications to diagnose monogenic disease (18), for DNA typing in relationship to disease susceptibility (35) and the detection of human infectious diseases (16). Utilizing these applications, we combined two areas, evolutionary analysis and detection of infectious disease, to develop a DNA-based PCR assay. The PCR assay protocol was evaluated with the *T. foetus* variable region oligonucleotide, MW24, and the conserved primer T.f 1 which amplified a region of the *T. foetus* small-subunit rRNA gene. These reactions were probed with an internal variable region oligonucleotide, MW25. Our results with the PCR assay indicate there are problems with recovery of the sample DNA and reproducibility of sample

amplification (Figure 8). Pipetting contamination from adjacent samples was also a major problem associated with the PCR (Figure 9). While problems of DNA recovery are approachable, sample contamination represents a serious problem in livestock disease diagnosis. The lack of control over sampling techniques (each veterinarian has there own method of sampling an animal) would make it difficult to set a rigid sampling protocol. Also, the sanitary conditions in the field are relatively poor. Since the PCR amplifies extremely low concentrations of DNA from a sample, the lack of good sanitary conditions for sampling animals could lead to many false positives. The high cost and impracticability of multiple samplings are also major limitations. The PCR is an expensive system and if retesting was routinely required the dollar cost could be prohibitive. In addition, resampling would substantially increase injuries to handlers since sampling is often dangerous and difficult due to the size and strength of the animal involved. Unlike the PCR test for the detection of HIV infection in AIDS diagnosis, multiple blood samples are easily obtained and the cost of the tests are typically covered by health insurance. Thus, we concluded that the PCR format was not suitable for the diagnosis of bovine trichomoniasis.

Our next attempt at designing a diagnostic assay for the detection of T. foetus infection within the bovine involved a nonamplification format. This was achieved by targeting rRNA molecules of T. foetus with specific oligonucleotide probes. Probes for rRNA were first described in the diagnosis of Plasmodium falciparum (20) and Chlamydia trachomatis (30). Unlike restriction fragment length polymorphisms and repetitive DNA nucleic acid targets which are used in criminology to identify an

individual, the rRNA probe, depending on the probe specificity, can be used to identify the presence of different groups of organisms. A limitation in this type of assay is that the detection rate is dependent upon the content of cellular RNA. Under conditions of low organism number or low RNA content or both, the sensitivity may not be adequate without an amplification step.

Development of the RNA assay for bovine trichomoniasis involved the identification of appropriate probes and the development of suitable protocols to recover RNA from venereal samples. The process of extracting RNA from bovine cervical mucus or smegma samples was one of the problems encountered. We found phenol extraction was required for extracting RNA in protein rich samples (Figure 10). Duralose (nylon supported nitrocellulose) membranes which were UV cross-linked proved to be the most efficient membrane for immobilization of extracted RNA compared to nitrocellulose and nylon membranes (Figure 11). The detection limit of *T. foetus* parasites using the DNA-based PCR assay (Figure 7) versus the RNA assay (Figure 12) showed that the RNA assay was comparable to the DNA-based PCR assay without the need for amplification. In both assay formats (PCR versus RNA) the results from standard curves showed a limited, linear dynamic range due to the properties of X-ray film. Less than five percent of the β particles emitted by a P^{32} labelled sample actually interact with the emulsion of a standard X-ray film to form a latent image center (15). For dynamic purposes, the range of film is limited to about 300 to 1 latent images, and is complicated by the characteristic sigmoidal density versus log exposure (15). This is due to the saturation of the film by labelled sample over long time periods of exposure. Since our goal was to develop a

qualitative assay, we did not perceive the limited linear range of X-ray film as a substantial problem. The sample reproducibility with the RNA assay was considerably better than the PCR protocol (Figures 8,13). RNA extracted in GUISCN solution was stable for at least four days indicating that a sample could be shipped at room temperature for evaluation of T. foetus infection without concern for RNA degradation.

Our data showed that the RNA assay was equally sensitive and more practical than a PCR assay and superior to the conventional microscopic/culture assay. The results of five mock tests showed that the RNA assay has a higher level of sensitivity (Table 2) as compared to the microscopic/culture assay. The RNA assay detected 98.6% of the positives and as few as 50 T. foetus parasites per sample consistently, while the microscopic/culture assay detected 27% of the positive samples that contained 50 to 1000 parasites per sample. Although the results showed a detection range of 10 to 20 T. foetus parasites per sample in the mock testing studies, background noise decreased the level of detection in field samples. Overall these results showed the RNA assay to be at least 3.5 times more sensitive than the microscopic-culture assay. The higher detection rate for the microscopic assay in test set 1 (Table 2) was attributed to a more complete analysis of the samples and the greater skill and knowledge of the operator. Dr. Speer analyzed the samples in test set 1 and has over 25 years experience in identifying parasites and in microscopy in general. While it is standard to examine 20-30 fields of a drop (0.1 ml) of sample fluid, Dr. Speer examined the entire coverslip taking 3.5 hours to examine 30 samples. Dr. Speer detected 4 out the 5 of the 1,000 parasite/ml samples and 3 out of 5 samples at 200 parasites/ml and 1 out of 5 samples at 100 parasites/ml

indicating that with care and time the microscopic assay can detect 200-1,000 parasites/ml fairly reliably. We confirmed that the examination of a complete coverslip would result in a higher detection rate by repeating a 30 sample mock test using another operator (Dr. Kent Thomas). Again the overall detection rate in the test was higher (58%) with a 80% detection at the 1,000 parasite/ml level. A detection rate of 80% positives is very close to the published detection limit of 78% (37) indicating that under optimal conditions, with sufficient parasite numbers and time for assay the microscopic/culture protocol can detect 8 out of 10 positives.

The RNA assay offers several other significant advantages over the microscopic assay (Table 3). Due to the low reliability, the microscopic assay requires approximately 3-6 samplings to achieve >80% accuracy. The RNA assay can achieve 98% accuracy in one sample at parasite numbers >50 parasites/ml. The time factor is another advantage for the RNA assay. Turnover time for the RNA assay is relatively short, approximately 3 days, while the assay time for the complete microscopic/culture assay is 5-7 days for one test or a total of 3-4 weeks (3 samples) to completely test one animal. The sample taken for microscopy does not represent the total sample since the operator examines and cultures a small portion (0.1-0.5 ml) of a sample. The RNA assay utilizes at least 50% of the entire sample for diagnosis provided half the sample is saved for potential reconfirmation, a feature only possible for the RNA assay. The sample received for the microscopic assay must be kept at room temperature or 37°C in a low oxygen environment to avoid the death of T. foetus organisms because the microscopic assay requires the presence of a motile flagellated organism in order to be considered positive.

Table 3. Comparision of the microscopic/culture assay with the RNA assay.

	<u>Micro</u>	<u>rRNA Assay</u>
1. Live Organisms	Yes	No
2. Number of Samples	3-6	1
3. Detection Limit	500-1000	50
4. Assay Time	1 test=7 days Total=3-4 weeks	3 days
5. Operator Error	high	low

Since the RNA assay detects a nucleic acid, the organism can be dead and still be detected. The sample for the RNA assay can be stored at either room temperature or 4°C for a minimum of four days without decrease in signal intensity (Figure 13).

The RNA assay was examined using field samples. These initial field samples (case study 1 and 2) were not confirmed by the microscopic/culture assay. In case study 1, herd 1, there was no T. foetus infection detected while in herd 2, 17% of the animals were positive by the RNA assay. The infection found in herd 2 correlated with the lower reproductive rate (70%) versus herd 1 (79%). Since these herds were sampled differently, it was not possible to determine how sampling might have affected the outcome of the assays. Of the eleven bulls placed with the heifers of herd 2 (case study 1) on May 22, ten were pulled on July 8. The last bull was pulled on July 31 after he had escaped and was found in a third herd near Columbus. The Columbus herd was part of a 1700 cow operation located in seven ranches in eastern Montana. In February, Agricultural Bioengineering Inc. (Bozeman, MT) tested 58 bulls for trichomoniasis from this 1700 cow operation using the RNA assay. The only bulls tested positive in this group were from the herd north of Columbus where the escaped bull was found. The results indicated that the escaped positive bull from herd 2 (case study 1) had an active infection which was spread to the Columbus herds. The pregnancy rate in the Columbus herd was 97% in September, however, data on the calving rate was unavailable. These initial field samples are minimal and unconfirmed by any independent assay, full epidemiology and testing of the RNA assay remains to be completed. In conclusion, the diagnosis of T. foetus infection, with the RNA probe test (RNA assay) has significant advantages over

a PCR-based DNA assay and conventional microscopy. These studies also indicate that DNA hybridization can provide a sensitive alternative diagnostic method which should be especially useful in the analysis of large numbers of samples. Although the current protocol relies on radioactive isotopes, adaptation to a highly sensitive nonradioactive detection system should be the next step in the development of this assay.

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