Detection of *Tritrichomonas foetus* in Bovine Semen by PCR Amplification

by

Chance Lee Armstrong

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 10, 2016

Keywords: Trichomoniasis, *Tritrichomonas foetus*, Bull, PCR, Semen

Approved by

Dwight F. Wolfe, Chair, Professor of Clinical Sciences
Misty A. Edmondson, Associate Professor of Clinical Sciences
Thomas Passler, Associate Professor of Clinical Sciences
Kellye Joiner, Associate Professor of Pathobiology
Soren P. Rodning, Associate Professor of Animal Sciences
Robert L. Carson, Professor of Clinical Sciences

Abstract

Bovine trichomoniasis caused by *Tritrichomonas foetus*, is a true venereal disease of cattle that is spread only through coitus. The objective of the study was to determine if a *T. foetus* infection could be detected in pre-seminal fluid and a seminal sample using polymerase chain reaction (PCR) collected from known positive bulls. Diagnostics have recently improved with the emergence of a more sensitive and specific PCR techniques. This is an improvement from traditional culture methods, but better diagnostics and collection methods are still needed given the serious consequences of inaccurate diagnosis.

Mature beef bulls (n=20) of various breeds from several south Florida ranches that were previously diagnosed to be positive for *T. foetus* by routine culture and real-time PCR on preputial smegma at an external state diagnostic laboratory were used for this study. These bulls underwent routine electroejaculation, and a dry preputial scraping sample was collected from each bull using a 52.5 cm infusion pipette with a flex adaptor and a 20 ml syringe. The samples collected from urethral emissions were fractionated into a pre-seminal sample and seminal sample based on gross appearance. The bulls all achieved an erection and extended completely outside of the sheath. The preputial sample was immediately suspended in 2 ml of Trypticase- Yeast Extract- Maltose Medium (TYM) with agar (Diamond's media). The samples were transported to the laboratory in a

commercial incubator at 37^{o} C. Samples from each bull were centrifuged at 4000g for 9 minutes at room temperature and the resulting pellet was used for DNA isolation prior to processing for conventional PCR. Overall, 13 of 20 bulls were positive by traditional preputial scraping resulting in a test sensitivity of 0.65 (95% CI: 0.41-0.84). Four of these 13 positive bulls were also positive on the pre-seminal sample. One bull tested positive on the pre-seminal sample, but was negative on preputial scraping. The test sensitivity and specificity for detection of *T. foetus* by conventional PCR in pre-seminal fluid was 0.30 (95% CI: 0.1-0.61) and 0.86 (95% CI: 0.42-0.99), respectively. None of the semen samples were found to be positive for *T. foetus*. The combined sensitivity for the preputial scrape and pre-seminal fluid in this study was [1 - (1- 0.308) x (1- 0.65) = .758], suggesting that the diagnosis of *T. foetus* in infected bulls can be improved by additionally testing pre-seminal fluid during routine collection of a preputial scrape.

Acknowledgments

The author would like to thank the entire Food Animal Department at Auburn University's College of Veterinary Medicine for their mentorship and guidance during my clinical training and research. Special thanks goes to my graduate committee members Dwight Wolfe, Robert Carson, Misty Edmondson, Thomas Passler, Kellye Joiner, and Soren Rodning for their patience and continued support. Although not on my formal committee, thanks go to Herris Maxwell and Dan Givens who contributed significantly to my training with their expertise and time. I would like to express my sincere appreciation to all the staff at the college of veterinary medicine that have been a source of support for many years. A special thanks to my resident mate and dear friend, Jennifer Koziol, without whom I would have never accomplished my goals during the past three years. My sincere appreciation goes to my friend Andrew Lovelady that has been there through the peaks and valleys to help me thorough this season of my career. Thank you to my grandfather Pete Coates for teaching me how to work with livestock and that all things are possible with hard work, focus, and determination. Thank you to my colleagues Clare Scully and Matt Welborn for your unrelenting support. I could not have completed my project without the both of you.

A special appreciation goes to my family for teaching me that love and respect for your fellow man are the most important things in life. Your love and support have always been an inspiration to persevere through adversity and always strive for excellence.

Dedicated to Dr. Robert Lea Carson

The teacher and mentor who always inspired

me to be better than I was at that moment.

Table of Contents

List of Tables

List of Illustrations

List of Abbreviations

CSS	Certified Semen Services
USDA	United States Department of Agriculture
CVM	Cervical Vaginal Mucus
PCR	Polymerase Chain Reaction
FDA	Food and Drug Administration
TYM	Trypticase Yeast extract Maltose
SEM	Scanning Electron Microscopy
T. suis	*Tritrichomonas suis*
T. foetus	*Tritrichomonas foetus*
T. vaginalis	*Trichomonas vaginalis*
TEM	Transmission Electron Microscopy
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
DNA	Deoxyribonucleic Acid
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
TMA	Transcription Mediated Amplification
NAATS	Nucleic Acid Amplification Tests
STD	Sexually Transmitted Disease

Chapter 1: Introduction

Bovine Trichomoniasis

Bovine trichomoniasis caused by *Tritrichomonas foetus (T. foetus),* is a true venereal disease of cattle that is spread only through coitus. This disease results in significant economic loss due to embryonic death, abortions, and reduced weaning weights of calves born late in the calving season from cows that could not maintain pregnancy because of infection early in the breeding season. The bull is an asymptomatic carrier of the disease. *T. foetus* persists on the epithelial surface of the prepuce and penis of the bull and has been isolated on rare occasions from the distal urethra (1, 2).

Trichomonas vaginalis (T. vaginalis), the most common non-viral, venereally transmitted pathogen of humans, is similar in its pathogenesis to *T. foetus* and is known to cause urethritis and prostatitis in men. Recent studies of men infected with *T. vaginalis* demonstrated that PCR testing of centrifuged samples of semen or urine had increased sensitivity for detection of *T. vaginalis* as compared to culture of urine and urethral wet-mount scrapings (3).

Currently the gold standard for detection of *T. foetus* in the United States artificial insemination industry has been six consecutive cultures of a dry scraping of the prepuce in Diamond's media taken one week apart (4). Diagnostics have recently improved with the emergence of more sensitive and specific PCR techniques. Despite these improved diagnostic techniques there are still problems with diagnosis of the organism that result from failure to recover the protozoan from the prepuce and penis due to errors in sample

collection technique as well as shipment of the collected sample to the lab in suboptimal transport conditions. The application of new diagnostic techniques used in human investigations of *T. vaginalis* could allow for increased detection of bulls infected with *T. foetus* by eliminating sampling error by detection of the organism in a more uniform semen sample.

This study encompassed the collection of both pre-seminal and seminal samples from known *T. foetus* positive bulls via standard electroejaculation techniques. A standard preputial scraping was collected in concert with the aforementioned sample collection. Seminal and pre-seminal samples were tested for the presence of *T. foetus* utilizing PCR techniques following a centrifugation protocol. The results of these two samples were then be compared to PCR results of the standard preputial scrapings from the known positive bulls as well as compared to samples from known negative control bulls.

Chapter 2: Literature Review

Bovine Trichomoniasis

2.1 History

Bovine trichomoniasis, a true venereal disease of cattle caused by the flagellated protozoan *Tritrichomonas foetus (T. foetus)*, was first described in France in the late 1800s (5). The research on this disease lagged until the mid-1930s because of the simultaneous emergence of brucellosis. Emmerson was the first to diagnose trichomoniasis in the United States cattle population in 1932 (6). The disease was further described in the late 1930s to mid-1950s by researchers such as Bartlett, Abelein, and Morgan (2). The disease has since been diagnosed and described by multiple scientists across the globe (7-10). Trichomoniasis has recently received increased attention as more states apply regulations to inter- and intrastate movement of bulls in regard to *T. foetus* testing. There is no current standardization of regulations amongst states for *T. foetus* testing, but efforts are focus towards more continuity across the United States.

2.2 Taxonomy

Trichomonads are categorized in the Phylum Parabasalia and the order Trichomonadida (11). Parabasalids are a robust group of flagellates that are characterized by having hydrogenosomes (modified mitochondria), a typical arrangement of mastigont

(set of kinetosomes and their associated appendages), a parabasal apparatus (a Golgi body attached to striated fibers), and nuclear division by a closed pleuromitosis with an extranuclear spindle (12). Most members of this phylum have either a parasitic or commensal relationship with animals and reside in the lower gastrointestinal tract (12). Parabasalids play an important role in certain ecosystems by enabling cellulose digestion in wood eating cockroaches and termites (13). The taxonomy for *T. foetus* was derived from work published by Adl et al. and Beugerolle et al. (13, 14). (Table 1)

Kingdom	Protista
Subkingdom	**Protozoa**
Phylum	**Parabasalia**
Class	**Trichomonada**
Order	**Trichomonadida**
Family	**Trichomonadidae**
Subfamily	**Tritrichomonadinae**
Genus	***Tritrichomonas***
Species	***Tritrichomonas foetus***

Table 1. Taxonomy of *Tritrichomonas foetus* (13, 14)

Tritrichomonas foetus is well documented as a pathogen of the reproductive tract of the bovid. Recently, both clinicians and researchers have focused on the protozoan as the etiologic agent of chronic, large bowel diarrhea of cats (15). There has been much debate over a similar trichomonad *Tritrichomonas suis (T. suis)* that resides on the nasal mucosa and gastrointestinal tract of swine. *T. foetus* and *T. suis* recently have been accepted as differing strains of the same species based on morphologic and molecular investigations (16, 17).

2.3 Economics

A significant economic burden is placed on a cattle operation after introduction of this disease into the herd through: 1) loss of calf crop from embryonic deaths and sporadic abortions; 2) loss of uniformity from a prolonged calving season; 3) decreased market weights of calves due to prolonged calving season; 4) increased feed costs as a result of a longer feeding period for light weight calves that are born late in the calving season; and 5) culling of open cows and infected bulls. An older report in 1958 by Fitzgerald showed a loss of $800 per infected bull that is diagnosed on the operation (18). Early estimates from the western United States reported in 1964 and 1986 showed losses in the tens of millions from the disease (19, 20). A study that investigated the economic loss in beef replacement heifers in Oklahoma reported $2.5 million total losses accrued in 1979 (21). Speer et al. reported an overall loss of $650 million annually in a 1989 study (22). This was based on a 5% calf loss due to trichomoniasis. The data from this study would equate to an even greater economic loss due to an increase in market value of

calves over time. An overall 5% loss in calf crop across the U.S. cattle population in 2015 would equal $1.5 billion in total losses according to USDA estimated cattle population numbers. These values are based from USDA estimated calf crop of 34.3 million head and a 450 pound calf bringing approximately $200.00/cwt. This would bring a gross return of $900 per calf.

A more recent report by Rae et al. (1989) evaluated the economic impact on individual ranches via a spread sheet simulation model (23). The report listed ranges of losses because of variation in production costs between operations. The model made predictions that included a 14 to 50% reduction in annual calf crop, a 5 to 12% reduction in the suckling/growing period, a 4 to 10% reduction in pounds of marketable calf crop at weaning, a 5 to 35% reduction in the return per cow confined with a fertile bull and a 12 to 30 day longer breeding season.

Although a single dollar amount cannot be placed on overall return of individual cows because of varied production costs a 5-35% reduction on return would be substantial considering a small profit margin in most production cycles (24).

A study reported by Villarroel et al. (2004) also used a spreadsheet simulation to estimate economic loss on individual operations based on a 300 cow herd with no control strategies applied. This model reported a 23% reduction in income over the entire operation as a result of introduction of trichomoniasis (25). This model agreed with the findings of other studies. All of these studies demonstrate that trichomoniasis has had a major negative effect on the overall profitability of the U.S. cattle industry.

2.4 Prevalence

Multiple studies have examined the prevalence of bovine trichomoniasis across the United States. Often, these reports have focused on a particular region or state. Diagnostic techniques that lack sensitivity due to single sampling in bulls has led to questionable validity of reported values. (26). Johnson reported a 7% prevalence in range bulls in the western United States (20). In a state-wide study in California, BonDurant et al. reported 15.8% of herds that were surveyed had at least one positive bull and an overall prevalence rate of 4.1% of all bulls tested (27). Rae et al. (2006) reported 28.8% of herds surveyed had at least one positive bull and an overall prevalence rate of 6% of all bulls tested (28). This study was performed across the state of Florida where *T. foetus* has been endemic for many years. This study also demonstrated increased prevalence rate of the disease in large herds (over 500 cows, 53.9%) compared to medium sized herds (100-499 cows, 10%) (28).

Abattoir studies performed in Colorado and Nebraska demonstrated much lower prevalence of only 0.172% as diagnosed by culture (29). The difference in prevalence compared to other studies that reported a higher incidence could be a regional effect of differing management schemes or a result of the cyclic nature of the organism. Another possible explanation of the low prevalence reported in this study was a lack of sensitivity of a single culture (26).

More recent prevalence studies from Alabama (9), Texas (30), and Wyoming (31) found prevalence rates of 0.27%, 3.7% and 2.17% respectively.

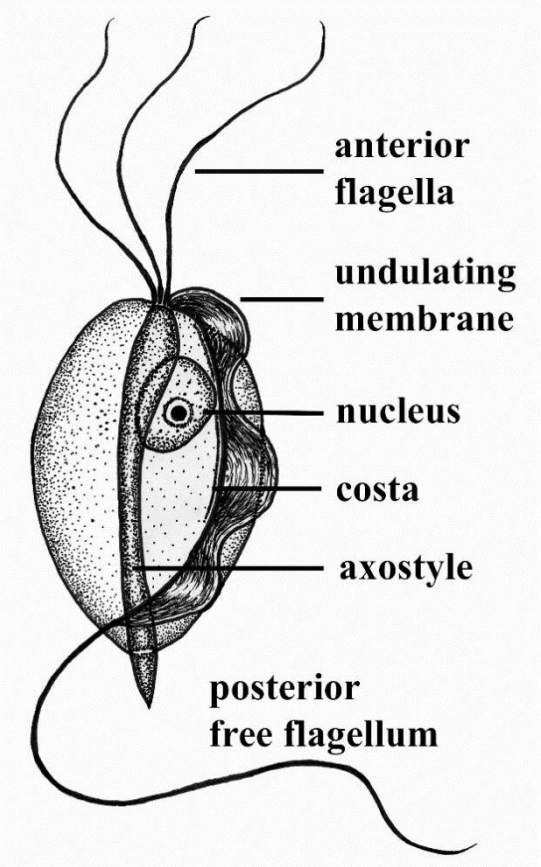
2.5 Morphology

T. foetus exists as both a trophozoite and pseudocyst form (32) but lacks a true cyst form that is evident in only a few members of the class Trichomonada (33). The trophozoite is a pyriform (tear-dropped) shaped, flagellated protozoan with a single nucleus (2). The cranial portion has a rounded shape in contrast to the caudal part being pointed (6). This form makes up most of the normal population of cells that have been studied (32) and is slightly larger than the head of a bovine spermatozoa at 10-15 x 5-10 µm (2, 34, 35). The organism possesses three characteristic anterior flagella and one posterior flagella (Illustration 1) that is an extension of the undulating membrane. The undulating membrane produces three to five characteristic waves which gives *T. foetus* a distinguishing characteristic compared to other trichomonads (34). The membrane rests on the lateral aspect of the trophozoite and provides the rolling, jerky movements that are also a distinguishing characteristic of *T. foetus*. The posterior flagellum is an extension of the undulating membrane (6). Reproduction of the trophozoite form of *T. foetus* is achieved asexually by cryptopleuromitosis, a special type of closed mitosis, characteristic of the trophozoite form that results in two identical daughter cells (36).

The trophozoite or motile form can easily undergo a morphologic transformation by internalizing its flagella, creating a rounded pseudocyst. This conformation can be induced in the presence of adverse conditions (32, 33, 37). It is deemed a pseudocyst because of the rounding up, compaction of the structure, and internalization of flagella, with the lack of a the true protective cyst wall (38). It is unknown what role the rounded pseudocyst form plays in the overall pathogenesis, but it has been shown to adhere to

vaginal epithelial cells to a greater degree than the trophozoite form (32). A recent study by Pereira-Neves et al. reported that the pseudocyst form was more prevalent than the trophozoite in the preputial cavity of the bull (39). These are very different findings than the pseudocyst form that was once only thought to be simply a pre-apoptotic cell type (40-42). Reproduction in the pseudocyst form has been described as a budding or shizongony-like division in which numerous daughter cells are formed and released as newly formed flagellated trophozoites (36). The induction and reversal of the organism into the non-motile, rounded pseudocyst form can happen rapidly (37). The emergence of several trophozoites from the pseudocyst has led many to question the role that the non-motile form plays in the overall pathogenesis of this organism. Perhaps, this is a survival mechanism that is part of the normal life cycle in the face of adverse conditions. Much is to be elucidated in future studies.

Illustration 1. Anatomy of *Tritrichomonas foetus* (Daly R. Bovine Trichomoniasis. The Range Beef Cow Symposium XIX2005. p. 123-32.)



2.6 Pathogenesis in the Male

T. foetus is an obligate parasite of the bovine reproductive tract. Like for most venereal diseases, the male bovid is an asymptomatic carrier of the disease (34, 35, 43). The parasite thrives in the microaerophilic environment provided by the preputial cavity. Here, the organism localizes along the epithelium of the penis and prepuce. The preputial secretions and smegma provide an environment in which the organism can efficiently propagate (1). There are a few isolated reports of the organism being discovered in the distal urethra of the bull (1, 2). Parsonson et al. discovered *T. foetus* trophozoites in the distal urethra at necropsy in four of 15 naturally infected bulls. There are also reports of the parasite's presence, although rare, in the epididymis and seminal vesicles (2). Discovery of *T. foetus* in the distal urethra and accessory sex glands, while rare, has led to questions regarding the pathogenesis and life cycle of this complex protozoan. The organism has not been shown to be locally invasive in the epithelium of the urogenital tract of the bull (43). Sperm morphology and libido are not affected by the presence of the parasite (43, 44). However, progressive motility of bull sperm has been demonstrated to be reduced by the cytotoxic secretions produced by *T. foetus* (45).

The age of the animal seems to play an important role in the development of a chronic infection compared to a transient infection in the bull. Transient infections have been reported in bulls less than three years of age, while bulls greater than three years old are more likely to be chronically infected. Younger bulls can spread the disease to uninfected cows if they breed within hours to days following initial infection prior to clearing the organism. There are also reports that suggest the organism was cleared from

the preputial cavity and penis within twenty minutes of initial inoculation in bulls less than four years old (46, 47). The understanding behind young bulls clearing the organism and older bulls being chronically infected with the disease was that bulls develop deeper crypts as they matured past 3-4 years of age. These crypts were thought to provide an enhanced microaerophilic environment that leads to proliferation of the organism (43, 44, 48, 49). A recent histologic study of the bovine preputial and penile epithelium refuted the early premise that bulls develop deeper crypts as they age and reported no marked difference in the depth of epithelial folds in mature bulls (50). The means by which bulls develop chronic infections still remains unknown.

Bulls become infected mechanically during coitus with an infected cow. This is more likely to be only a transient infection in young bulls less than three years of age (43). Although young bulls seem to be more resistant to developing a chronic infection, this does not occur in all cases and practitioners cannot be assured that those bulls will clear the infection. BonDurant et al. confirmed this in a California prevalence study documenting that 2% of 3 year old bulls tested positive for *T. foetus* in 57 natural service herds (27). During this initial infection, it has been discovered that young bulls can transmit the organism to uninfected cows through coitus in hours to days following initial infection (43). Clark et al. reported that young bulls can clear the organism within minutes of breeding an infected female (47). However, a bull that becomes infected under natural service conditions should be considered positive until proven otherwise.

2.7 Pathogenesis in the Female

The flagellated protist is most commonly mechanically transmitted from the penis of the bull into the caudal reproductive tract of the female during coitus (34). Parsonson reported that up to 95% of naïve females become infected when naturally exposed to an infected bull (51). Contaminated artificial insemination equipment and semen from bulls that have not been tested under the strict disease preventative guidelines of Certified Semen Services (CSS) could also be a source of infection. The organism was demonstrated to survive temperatures that are commonly implemented to cryopreserve bovine semen (2). The ability to survive cryopreservation stresses the possibility of disease dissemination through contaminated semen and mandates the necessity of strict testing protocols. The trophozoite form is motile and is capable of establishing an infection throughout the entire female reproductive tract within 2 weeks from the initial infection (52). The trichomonads are able to breach protective barriers and populate the uterus while the cervix is relaxed and under the influence of estradiol during the follicular phase of the estrus cycle (52). The parasite has been documented to cause an inflammatory response throughout the entire reproductive tract and is thought to interfere with conception and establishment of pregnancy (53). This report has been refuted by other studies, and it is now generally accepted that conception is not impeded by the infection (54, 55). Infections can lead to reproductive loss in the form of embryonic death, abortion, fetal maceration, and post-coital pyometra (56). The losses are most commonly seen after pregnancy recognition (days 15-17 of the estrous cycle) leading to a delayed return to estrus (52). The mechanism of the death of the embryo or fetus is still

undefined. Pregnancy losses are most commonly prior to five months of gestation (52). Post-coital pyometra is another distinguishing feature of this disease process. It is only diagnosed in a small percentage of females (< 5%). It is not considered pathognomonic but should alert a clinician to the possibility of trichomoniasis in herds using natural service (52). Cows infected with *T. foetus* have been shown to spontaneously clear the organism within two to four months (35). A small percentage (< 1%) of females are thought to become chronic carriers for undefined reasons and can carry a pregnancy to term while still infected with the organism (57).

Cows are capable of mounting an effective immune response against *T. foetus* (58). Humoral rather than cell-mediated immune responses are important as host defense mechanisms because of the non-invasive, extracellular nature of *T. foetus* (35). This type of response is evident throughout the reproductive tract following initial infection, but clearance of the organism is quite variable. Humoral responses evident in cows are absent in bulls.

Infections can result in an overall pregnancy loss of up to 50% in some herds (23). Losses with respect to low pregnancy rates are often not that dramatic in herds with a long breeding season, but dramatic economic effects are imposed on the industry. The organism can persist in genital secretions for as long as 13-28 weeks (59). A cow could become infected early in the breeding season and lose the pregnancy. She would then experience a few weeks to months of infertility while the immune system clears the organism. She then could conceive again late in the breeding season. Although this cow would produce a calf and would not be a total loss for the operation, the loss in weaning

weight, loss of uniformity in the calf crop, and increased costs associated with an extended feeding period are still a considerable financial burden.

2.8 Treatment

There are many factors that make trichomoniasis a significant challenge for the U.S. cattle industry to overcome, but one of the biggest is that there are no effective treatments for food-producing animals approved by the Food and Drug Administration (43). Imidazole derivatives like metronidazole, ipronidazole, and dimetridazole have shown to be efficacious and are used in other parts of the world to treat infections (60-63). Kimsey et al. reported 100% efficacy with treatment of bulls with dimetridazole orally at a dose of 50 mg/kg every 24 hours for 5 days duration of therapy (26). Topical protozoacidal compounds that include acriflavine, diminazene aceturate, trypaflavine (Bovoflavin Salve®), chlorhexidine, and nitrofurazone have all been used with variable results. The topical compounds have been difficult to apply and labor intensive with multiple applications required (52).

There are *in vitro* studies currently being performed in hopes of development of a safe and efficacious treatment option (64). However, the delay in research, development and Food and Drug Administration approval emphasizes the importance of sound management practices that include strict biosecurity, diagnostic testing of males, and vaccination of herds at risk for the disease.

2.9 Vaccination

Prevention and control of *T. foetus* requires intensive management schemes and part of that involves the implementation of biologicals in areas where this disease is endemic. Immunity following natural infections is short-lived for trichomoniasis with females becoming susceptible within one year and thus the following breeding season (65). Pathogen-specific antibodies of both isotypes of IgA and IgG_1 have been recovered within 6 weeks following natural infections in the female (58). IgA has not been shown to kill the organism, but it does immobilize and prevent adherence to the mucosa (65). Complement killing and opsonization have been described to be mediated by IgG_1 (66, 67). The extracellular nature of the organism initiates the expected humoral response from the host and, thus, the short-term immunity (67).

A commercial whole cell bacterin (TrichGuard® Boehringer Ingelheim VETMEDICA, Inc., St. Joseph, MO) is available to aid in the control of trichomoniasis. This vaccine is the only product licensed by the United States Department of Agriculture (USDA) for the control of trichomoniasis. The label claims to reduce the shedding of *T. foetus* in healthy cattle and, thus, reduce the spread of the disease. Kvasnicka et al. (1992) performed a study in naïve heifers that exposed those females to infected bulls and additionally were inoculated with $1x10^7$ organisms intravaginally. The experimental group received a polyvalent bacterin containing *Campylobacter fetus, Leptospira canicola-gryippotyphosa-hardjo-icterohaemorrhagiae-pomona* and 5×10^7 killed *T. foetus* organisms per dose. The control group was denied prophylaxis. Overall, the vaccinated group had a significantly higher pregnancy rate compared to the unvaccinated

(61% vs. 31%). The vaccine was shown to lack full protection but significantly improved the outcome of the challenge. It was suggested to at least provide partial protection. It is difficult to draw solid conclusions from this one study that stems from a lack of information of normal pregnancy rates from unchallenged females from this area (54). There are other reports that demonstrate efficacy of the vaccine in females (68-70), but studies showing similar results in bulls are lacking. The author has found several anecdotal reports from producers that there is efficacy with TrichGuard® (Boehringer Ingelheim VETMEDICA, Inc., St. Joseph, MO) in bulls, but data from controlled studies refute this idea. Vaccine trials in naturally infected and experimentally infected bulls are warranted in the future.

Whether a herd of cattle should be vaccinated with TrichGuard® (Boehringer Ingelheim VETMEDICA, Inc., St. Joseph, MO) was addressed in a study by Villarroel et al (25). The authors employed a mathematical formula that included several variables such as prevalence of the disease in that area, testing of the bulls, herd size, shared pasture, ages of bulls, and value of weaned calves to help determine if the vaccine should be part of the herd health protocol (25). Vaccination should be considered in endemic areas as a part of the control and prevention of trichomoniasis since no efficacious, legal treatments are available to veterinarians in the United States.

2.10 Prevention

With no available treatments and because trichomoniasis is such a costly disease to the U.S. cattle industry, the importance of prevention must be stressed to producers.

Herds that are at low risk in non-endemic areas that have well-managed fences and sound biosecurity may only need to keep good records and monitor calving distribution (35). Management practices that reduce the risk of the introduction of trichomoniasis into herds in endemic areas and particular in areas with shared grazing rights should include the following. It is important to become familiar with all neighboring cattle operations and elucidate if *T. foetus* is endemic in the area surrounding that operation. It is imperative that fences be maintained so that there is not a breach in biosecurity with neighboring cattle comingling on the operation. Management strategies against *T. foetus* may not have been implemented in those neighboring cattle and could compromise all strategies put in place by the producer. A pasture rotation scheme should be developed so that there is limited fence line contact with neighboring herds. This will limit exposure of cycling females to neighboring bulls that might encourage attempts at mounting behavior across fences that are not in sufficient working order (24). In addition, producers should avoid public grazing that allows for a significant increase in both males and females becoming infected through coitus (71). Producers should purchase virgin bulls and heifers from reputable producers that offer excellent health records along with purchase receipts. Although virgin bulls, they should still undergo testing for *T. foetus* during a 3-4 week isolation period after arrival out of an abundance of caution. It is recommended to perform three consecutive negative cultures or gel PCR tests run one week apart to ensure bulls are truly negative (72). Producers should also breed and maintain purchased replacements in a separate pasture away from the resident herd (34) and maintain a young bull battery that is ≤ 3 years of age. This will lower the risk of the development of chronic carriers amongst males (1, 73). Culling open females in the herd has been shown

to increase calving percentage in large beef operations (73). If this scheme is not financially feasible to the owner, open cows with no palpable pathology in the reproductive tract should be sequestered in a pasture away from pregnant cows (35). They should clear the infection prior to the next breeding season. It is imperative that artificial insemination or exclusive bulls be used on this separate high-risk group. Test all bulls prior to the breeding season with a culture or PCR. It has been recommended that, in an ideal situation, three negative tests be performed one week apart to allow for multiplication of the organism in order to ensure a negative test (24). All females in endemic areas should be vaccinated with the commercial product TrichGuard® (Boehringer Ingelheim VETMEDICA, Inc., St. Joseph, MO) following label directions; two initial immunizations 2-4 weeks apart and with the second injection administered no less than 4 weeks prior to the breeding season (35). In order to reduce propagation of *T. foetus* and to monitor reproductive efficiency that will identify a reproductive pathogen entry into the herd, producers should implement a controlled breeding season of 60-90 days. When using artificial insemination, females should only be bred with semen from sires that have been collected and stored under strict testing set forth by Certified Semen Services (43).

2.11 Diagnosis

The identification of viable *T. foetus* organisms via bright-field microscopy has long been considered the standard for diagnosing trichomoniasis in diagnostic samples from both male and female cattle. The characteristic morphological features previously

described and demonstrated in Illustration 1 plus the characteristic jerky, rolling motility displayed by *T. foetus* trophozoites are critical for diagnosticians to correctly identify the organism. Preputial smegma samples are the diagnostic specimen of choice in the bull while cervicovaginal mucus (CVM) and pyometritic fluids are the most common diagnostic samples from females. The presence of non-pathogenic trichomonads that are commensals of the bovine gastrointestinal tract that create false positive results and the long turnaround time for culture results have driven the development of molecular-based testing (74). The emergence of confirmatory molecular tests such as PCR have been an improvement in the diagnosis of *T. foetus*. However, a reported 62% of errors occur during sampling and handling prior to any diagnostic test performed (75). Thus, as Mukjufhi et al. suggested, a significant problem is getting the organism collected from the animal and to the laboratory (76).

2.12 Sampling Techniques in Males

The sample of choice in the bull has been preputial secretions and smegma. There have been several methods of sample collection described in the literature which include the following: 1) a cotton swab method (77); 2) preputial scraping (78); 3) wet pipette technique (26); 4) preputial lavage (77); 5) a metal brush technique (79, 80) ; and 6) washing of an artificial vagina (81). Despite the technique employed, the sample recovered is then prepared for direct observation (bright field microscopy), culture, or aliquots taken for molecular diagnostics.

The swab method described by Morgan involves absorption of preputial smegma and fluids by a cotton swap wrapped around the end of a metal rod (5). This could be compared to the same technique used to attain a sample for bacterial culture for other conditions. This method has long fallen out of favor because of a study performed by Fitzgerald et al. that described a significant reduction in the number of parasites recovered with the swab method compared to the dry pipette technique (77). The findings in this study suggested a 20% reduction in parasites recovered with the swab technique. A possible explanation for this is that the sample collection using a swab may not be aggressive enough to remove trichomonads from the epithelial surface of the preputial folds or crypts as previously described.

A dry pipette method (Illustration 2) has been the most popular method used in the United States because of convenience and efficiency in the field. The dry pipette technique involves vigorous scraping of the penis and prepuce along with concurrent aspiration with an attached 12 or 20 ml syringe as demonstrated in Illustration 2 (52). It is recommended to insert the pipette aseptically into the preputial cavity to avoid contamination by clipping preputial hairs, washing the preputial orifice, and using pipettes that are individually wrapped in plastic sleeves (19, 82). The wet pipette method involves infusion of buffered saline solution (e.g., phosphate buffered saline or Lactated Ringer's solution) via pipettes that are individually wrapped in plastic sleeves while the preputial orifice is sealed by the hand of the practitioner. Vigorous massage of the sheath is performed by an assistant and then the contents are aspirated from the preputial cavity in the same syringe used for infusion. The need for an assistant and decreased efficiency has led many practitioners towards the equally sensitive preputial scraping technique.

This is corroborated by Schönmann et al. who compared the dry pipette technique to the wet pipette technique and determined that there was no significant difference in sensitivities between the two methods (82). Samples from the dry pipette technique are immediately placed in the desired transport/culture medium. In contrast, the aspirated fluid from the wet technique often requires centrifugation, depending on the volume, prior to inoculation of the culture medium or direct evaluation via bright field microscopy. Preputial scrapping with aspiration has been demonstrated to be more traumatic to the penis and prepuce when compared to preputial lavage (83). Retropreputial abscesses could occur as a sequela to an excessively aggressive preputial scraping that could lead to the development of fibrosis between the elastic tissue and overlying skin with a poor prognosis for future reproductive function of that bull. Therefore, practitioners should use caution when using this method to attain a sample. The bull should be properly restrained in a properly working chute to facilitate safe sample collection and ensure safety for the veterinary team. Parker et al. demonstrated that sample collection is affected by the collection with the operator's dominant or non-dominant hand. The right handed practitioner was shown to be four times more likely to remove the organism from the bull when the sample was taken from the right side of the chute compared to the left side (84). This study illustrated that removing the organism from the bull is yet another diagnostic challenge to overcome.

Preputial lavage involves infusion of 100 to 200 ml of saline (85). External manipulation of the penis and prepuce is performed while the operator's hand seals the preputial orifice preventing evacuation of the fluid. The agitated fluid is collected in conical tubes that allow for centrifugation of the sample prior to inoculation of the

preferred culture medium (77). Centrifugation is necessary when such large fluid volumes dilute the sample.

The metal brush technique involved the use of a 70 cm rod with a 13-cm cylinder with shallow grooves perpendicular to the long axis of the brush. The grooves were hypothesized to aid in collection of trichomonads from the epithelial surface (80). Tedesco et al. (1979) reported increased sensitivity compared to dry pipette method with direct examination via bright field microscopy (79). However, this study and one other reported no significant difference between the metal brush and dry pipette techniques when the sample was cultured following collection (80). The metal brush required sterilization following a single use and was deemed impractical for field use.

It has been recommended that bulls undergo one to two weeks of sexual rest prior to collection of samples for testing (43). This practice allows for multiplication of the organism within the preputial cavity during sexual rest. An accurate diagnosis can be squandered when bulls are tested while with cows as demonstrated by Clark et al. (1983) (53).

Several new sample collection devices have emerged recently. TRICHIT™ (Morris Livestock Products, Delavan, WI) is similar to an AI infusion pipette. A plastic cup attached at one end of the pipette is hypothesized to increase trichomonad recovery while minimizing blood contamination (24). The Pizzle Stick (Lane Manufacturing Inc., Denver, CO) has been one of the latest collection devices to come onto the market. The 24 inch plastic pipette features a corrugated tip with multiple holes drilled into the tip to allow for aspiration of smegma and preputial fluids. The opposite end features a flexible plastic adaptor that allows for ease of connection to a slip tip syringe. The designer of this

device emphasized improved sample collection with less trauma to the prepuce and penis. This is important as studies revealed that blood can be inhibitory to molecular diagnostics that are now regularly performed as both confirmatory and primary tests for trichomoniasis (76).

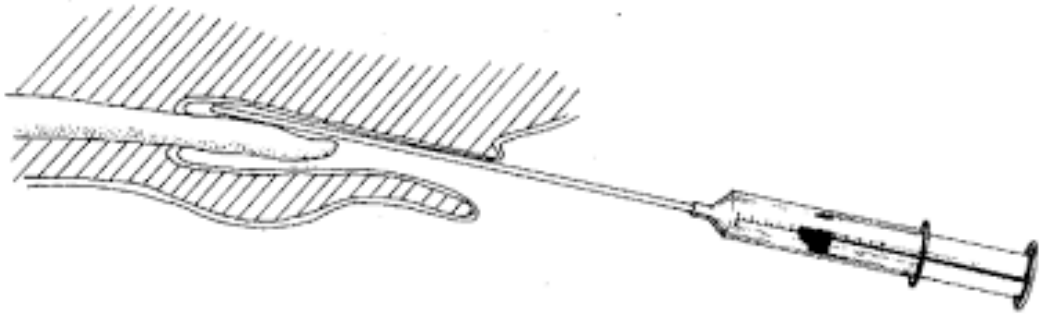


Illustration 2. Dry pipette technique for routine sample collection *Tritrichomonas foetus* (BonDurant, R.H. Diagnosis, Treatment and Control of Bovine Trichomoniasis. Compendium on Continuing Education for Veterinarians, 7(3): March 1985, S179-S187.)

2.13 Sampling Techniques in the Female

Sampling of females has not been the focus for most practitioners attempting to diagnose *T. foetus*. This is because of overall lack of success in discovering *T. foetus* from the female reproductive tract because of the female's inherent ability to clear the organism within a few weeks to months of infection. The cultures of smegma and preputial fluids have shown to have a higher sensitivity compared to culture of cervicovaginal mucus (CVM) from the female. Skirrow et al. (1988) demonstrated that cultures of CVM from the female only have a sensitivity of 58 to 75% (86). The reason for overall lack of sensitivity as compared to similar testing modalities in the bull is not fully elucidated, but some have suggested that a smaller number of organisms as a result of the significant immune response mounted by the female (35). Pyometras represent excellent sources of the organism in infected herds (52). This sample is usually teeming with *T. foetus* organisms and can be readily used to be identify the organism directly on a wet mount preparation without culture (35). The method most commonly described in the cow is the dry pipette technique similar to the method described in the bull (35, 43). An AI pipette that is protected in a plastic sheath is aseptically advanced into the cranial vaginal/cervical os area of the caudal reproductive tract. Cervicovaginal mucus or uterine discharges are aspirated and placed in culture medium as previously described in the bull. In the case of pyometra, the practitioner must traverse the cervix and aspirate intra-uterine fluids.

Additional samples that could be of diagnostic value are placental and fetal tissues from an abortion. Fetal tissues that have been shown to be infected with *T. foetus* are fetal

lung, spleen, and abomasal contents (43). Trichomonads have commonly been discovered in airways of aborted fetuses with post-mortem diagnoses of bronchopneumonia. *T. foetus* also has the propensity to diffusely invade the chorionic stroma of the fetal-placental unit (87-89). The discovery of the fetal-placental unit following an abortion is often limited in the field and even in research settings.

2.14 Sample Handling

Improper sample handling and delayed transport to the laboratory have been a major impedance of accurate diagnosis of *T. foetus*. Studies have examined various factors that can affect test outcome that include holding temperature and time delay until processing. It has been discovered that extremes in temperatures can affect the outcome of a culture. Bryan et al. reported that *T. foetus* contained in both Diamonds medium and InPouch ™ TF culture pouch systems (BioMed Diagnostics, Inc., White City, OR) at 22°C or 37°C were positive at four days post-inoculation (90). This study also revealed that all cultures were deemed negative by three hours post-inoculation when exposed to –20°C temperatures (90). The standardization of cultivation of *T. foetus* at 37°C was supported by this study and others that have shown *T. foetus* viability and multiplication is supported well at this temperature (91, 92). This is important to note so that provisions can be made in the field and during transport in areas where the ambient temperatures are low. Davidson et al. investigated the effect of high ambient temperature during transport on the outcome of both culture and real time PCR. InPouch™ TF systems were utilized for transport media and incubated at 37°C, 46.1°C, and 54.4°C for 1,3,6, and 24 hours.

The samples held at 46.1°C remained positive upon microscopic evaluation for only 3 hours and for only one hour when held at 54.4 °C. All samples were positive for all temperature ranges and duration when real time PCR was the testing modality (93). The ambient temperatures in some areas of the southern United States can produce similar temperatures during transport in carrier vehicles and PCR should be considered in such areas. It is generally recommended that samples be shipped to the laboratory in an expedient manner. Diagnostic sensitivities have shown an inverse relationship with transport times when molecular diagnostic modalities are employed (76). It is generally accepted that diagnosis of trichomoniasis is affected by many variables such as time from collection to the laboratory, transport medium, and ambient temperatures the sample is exposed to during transport. Samples should be placed in modified Diamonds media or InPouch ™ TF culture pouches immediately following collection and shipped to the laboratory within 1 to 2 days (10). These samples ideally should be placed upright in an incubator that is protected from light while still in the field. By placing the pouches upright, *T. foetus* organisms are permitted to settle down at the bottom of the pouch away from any residual air pockets that remain from the upper chamber of the InPouch ™ TF system.

2.15 Direct Microscopy

Direct microscopic evaluation can be diagnostic for *T. foetus*. This method involves immediate bright field microscopy of preputial fluids, preputial lavage, and preputial smegma from males and CVM or pyometritic fluids from the females. Survey

of the wet mount at 100 X magnification for motility is the initial step that is followed by confirmatory morphologic diagnosis (3 anterior flagella, 1 posterior flagella, axostyle, and an undulating membrane) at least at 400 X magnification. This method lacks the sensitivity of cultivation of the organism and has fallen out of favor for this reason (82, 91, 92, 94). Bartlett et al. suspected the lack of sensitivity with direct observation was because of a characteristically small population of trichomonads residing in the prepuce of infected bulls (95). The exception to this is an aspirate from a post-coital pyometra. In the author's experience and in other reports, this sample from the female is teeming with organisms (52). Direct microscopic examination necessitates a time-consuming detailed exam of a large sample size. It is therefore necessary to increase the number of protozoa by culturing.

2.16 *In vitro* Culture

The characteristically small population of trichomonads in the prepuce of infected bulls necessitates the cultivation of organisms for a consistently accurate laboratory diagnosis (96). There are several transport media that have been used over time for cultivation of this organism. Todorovic et al. (1967) compared 13 milk culture mediums that also contained antibiotics to suppress contaminant growth and determined that milk cultures were overall not as efficient at isolating the organism compared to inoculation in the control modified Plastridge medium (97). Others that have been described include Kupferberg medium and broth, Claussen's medium, Sutherland medium, and trypticase-yeast extract-maltose (TYM) medium. For many years TYM was the industry standard,

but later gave way to Diamond's medium that was produced by the addition of 1% agar to TYM. The agar trapped most of the contaminants near the top of the 10 ml tube and provided an anaerobic environment for the microaerophilic organism to thrive in close to the bottom (35). The industry has evolved towards the use of either a modified Diamond's media (98) or the commercially available InPouch ™ TF culture pouch.

InPouch ™ TF has gained popularity because it has a long storage time of one year at room temperature compared to Diamond's media which expires after 2 to 3 weeks under refrigeration. The pouch is constructed from thick plastic that is a barrier to oxygen, unlikely to break in field settings, and convenient for transport. The pouch features a two chambered system that allows for inoculation of the upper compartment prior to forcing the contents into the lower chamber. The proprietary enrichment medium has shown no significant difference in sensitivity when compared to Diamond's medium in field studies (82, 91, 99). InPouch ™ TF should be placed upright during incubation to allow trichomonads to settle to the bottom of the pouch away from any residual oxygen that remains after rolling the upper chamber down. The pouch is sealed with built in wire tabs that are similar to tabs on a Whirl-pak® sampling bag (Sigma-Aldrich Co. LLC., St. Louis, MO). The bull must be positively identified preferably with a permanent form of identification like a tattoo, brand, or nine character United States Department of Agriculture (USDA) silver National Uniform Eartagging Systems (NUES) tag or fifteen character "840" tag. The ranch/farm name, date, animal identification, and veterinarian should all be documented on each individual pouch or tube in the case of Diamond's medium. Another advantage to the InPouch ™ TF is that the culture can be examined daily directly in the pouch with the application of the accompanying slide over the

bottom of the pouch and placed on the stage of the microscope. Aliquots can be taken from the pouch by laboratory personnel for molecular diagnostics and most labs currently prefer shipment of the samples in the InPouch ™ TF system. It is still warranted to have a relationship with a lab that is comfortable working with *T. foetus* to avoid shipment and testing errors. The drawback to this system is that there is considerable expense compared to Diamond's medium and this can be a deterrent to some operations testing for *T. foetus*.

Samples are generally cultured at 37°C for one week and examined directly under bright- field microscopy every 24 to 48 hours during that time or until a positive diagnosis is made. A positive diagnosis can only be made by visualization of the viable, motile organism that exhibits distinct morphologic characteristics of *T. foetus* (35). The fact that this organism contains multiple lysosomal enzymes that lead to rapid destruction of the structure may explain why false negative reports occur in 10 to 20 bulls out of 100 bulls infected (35). The presence of enteric trichomonads has led to false positive results (74, 100, 101). This complicates conversations with producers that report a positive result in "known virgin" bulls and heifers. The confirmation of fecal trichomonads stressed the importance of sampling techniques and confirmatory molecular testing that is specific for *T. foetus*.

2.17 Molecular Diagnostics

Fecal contamination of the bovine urogenital tract is common and though this does not affect fertility of the bull during natural service it can present problems with diagnosis of *T. foetus* via direct identification of the organism on bright-field microscopy.

Most trichomonads live as non-pathogenic commensal organisms of the gastrointestinal tract of their host verses those that thrive in the urogenital tract (102). There are multiple reports confirming enteric trichomonads and similar flagellated protozoa that contaminate the urogenital tract such as *Monocercomonas ruminatum, Bodo spp., Spiromonas angusta, Cercomonas crassicauda, Polytoma uvella, Monas obliqua, Callimastix frontalis, Pseudotrichomonas* spp., and *Lembus pulillus* (101, 103-105). These flagellated protozoa have similar morphology and motility that can easily be mistaken for *T. foetus* on bright-field microscopy.

A confounding problem arose for practitioners when known "virgin" bulls were found positive for trichomoniasis on culture based systems. BonDurant et al. investigated three California herds with a total of 14 virgin bulls (100). Samples from these bulls were found to contain multiple flagellated protozoan using bright-field microscopy. The organisms showed a similar rolling, jerky movements to *T. foetus*. Staining techniques with Giemsa or Diff-Quick/iodine revealed an organism similar to *T. foetus,* although somewhat more rounded (less spindle-shaped) (100). Scanning electron microscopy (SEM) further revealed a protozoan with four anterior flagellum. Polymerase chain reaction (PCR) assay failed to amplify any products for *T. foetus* in the virgin bulls, but positive control isolates for *T. foetus* all yielded an amplicon of the expected size. The investigators suggested that the trichomonad was *Tetratrichomonas,* an enteric protozoa that were simply a non-pathogenic contaminant to the urogenital tract of these bulls, based upon SEM findings. This case highlighted the fact that cultures may produce false positive results due to the fact that *Tetratrichomonas sp*. and *T. foetus* are so similar that

they can only be distinguished by polymerase chain reactions or ultrastructural studies such as transmission electron microscopy or scanning electron microscopy (100)

Cobo et al. (2003) further highlighted this phenomenon following investigation of six 18-month-old virgin Angus bulls in Argentina that were all diagnosed positive for *T. foetus* by routine culture and observation via bright-field microscopy. All bulls had been housed together and had been separated from females since an early age. Due to the virgin status of the bulls the results were questioned, and the noted trichomonads underwent ultrastructural evaluation via SEM and transmission electron microscopy (TEM) to further elucidate the exact species. Scanning electron microscopy demonstrated that the protozoa cells generally exhibited four anterior flagella of unequal length with undulating membranes spanning the entire length of the body and displaying three to five waves (74). DNA was extracted and polymerase chain reaction (PCR) was performed from an aliquot taken from cultures. The amplification products were seen only with primers TFR1 and TFR2 (specific to trichomonads), but not with TFR3 and TFR4 (specific to *Tritrichomonas foetus*) (74). It was determined by the investigators that the organisms were not *T. foetus*, but in fact were *Tetratrichomona buttreyi*. These bulls were incorrectly diagnosed as positive by the commonly used culture and light microscopy examination.

These reports and others emphasized the importance of confirmatory testing beyond the traditional culture and light microscopy. The problems of false positive bulls place undue financial constraints on producers that are forced to cull positive bulls because of the lack of legal, efficacious treatment options in the United States. The lack of desired sensitivity of a single culture (80%) leads to increased costs and problems with

the practice of collection of three samples one week apart to give confidence to producers of 99% sensitivity and that the bulls are in fact negative (26). DNA based assays were developed in 1991 to help address the aforementioned problems associated with the diagnosis of *T. foetus* (22).

The first assay that was developed was a DNA probe that was specific for sequences that only *T. foetus* expressed. The probe was used to detect the presence of nucleic acid sequences following extraction of nucleic acid material that was bound to a filter (22). The probe was later abandoned following results from field studies that demonstrated an overall lack of sensitivity compared to traditional culture methods (91). Polymerase chain reaction (PCR) based tests that were later developed to rapidly amplify and detect DNA sequences that were specific to *T. foetus* showed improved efficiency and maintained equal sensitivities of culture based systems (106-109).

Polymerase chain reaction (PCR), amplifies specific DNA sequences(i.e. amplicon) in vitro through cycles of enzymatic DNA synthesis (110). Once amplified, the DNA produced by PCR can be used in many different laboratory procedures. The technology first described by Kerry Mullis is sometimes called “molecular photocopying” (111) and has been utilized around the world in a number of laboratory and clinical techniques, including DNA fingerprinting, detection of bacteria or viruses, and diagnosis of multiple genetic disorders. Mullis was the recipient of the Nobel Prize in Chemistry for this innovative work in 1993.

To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, or separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes and “builds” two new strands of DNA, using the original

strands as templates (111). This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then, each of these strands can be used to create two new copies ad infinitum. The cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times. Starting with a minute sample of total DNA, it is possible to amplify any such region 1 billion-fold while leaving the rest of the genome at its original concentration (110). Amplified target sequences are then detected by electrophoresis. Automation of the cycling process is achieved via a thermocycler. The cycler raises and lowers the temperature of the reaction in discrete, pre-programmed steps. The process only takes a few hours to complete and offers that advantage over traditional culture methods that can take up to 7 days to complete.

In 1994, Ho et al. reported development of a specific DNA probe and amplification system to detect *T. foetus.* They hypothesized it would be a prudent alternative method for diagnosis (109). Isolates were collected from bulls from several geographical areas where trichomoniasis is documented to be endemic such as California, Idaho, Nevada, and Costa Rica. Samples were deemed negative by three negative cultures in control bulls. A 0.85kb *T. foetus* probe was developed by identifying conserved sequences among these isolates from the four geographical areas. This sequence was selected based on previous work that showed specificity to *T. foetus* and no cross-reactivity with bovine cell DNA or DNA from pathogenic and non-pathogenic bacteria which inhabit the bovine reproductive tract (112). This probe demonstrated detection capabilities of a minimum of 10^5 *T. foetus* organisms. A partial sequence of the probe was used to identify oligonucleotide primers TF1 and TF2 that were implemented

in polymerase chain reactions to amplify a 162 base pair product from *T. foetus* (109). Hybridization of an internal chemiluminescent *T. foetus* probe to Southern Blots of the amplified product was used to improve sensitivity. The application of this technology allowed the detection limit to be lowered to one organism in culture and ten organisms in inoculated smegma samples (109). In a test of field isolates, this testing method showed an overall sensitivity of 90.4 % when compared to 84.6% when using traditional culture methods. Another point of interest was that no false positives were discovered during the clinical trial (109). It was concluded that this molecular-based system could be used as an alternative method for diagnosis of trichomoniasis (109).

The implications of the early work with PCR invited increased attention to this area of diagnostics. Felleisen et al. (1997) described the comparative sequence analysis of the 5.8S rRNA gene and the flanking internal transcribed spacer regions ITS1 and ITS2 of various *T. foetus* isolates originating from different geographic regions (106). The sequences proved to be highly conserved in all strains analyzed. *Trichomonas vaginalis, Trichomonas gallinae, Trichomonas tenax,* and *Pentatrichomonas hominis* could be clearly discriminated from *T. foetus* on the basis of rRNA gene sequences (106). In a subsequent study, Felleisen et al. (1998) developed a PCR for the sensitive detection of *Tritrichomonas* spp. and a complementary DNA enzyme immunoassay (DEIA) based upon primers delineated from rRNA gene unit sequences (107). This sequence information was applied to the delineation of primers TFR3 and TFR4 for the development of a PCR amplification system. TFR3 is complementary to the ultimate 5' end of the 28S rRNA gene, and TFR4 is located at the border of the 18S rRNA gene and ITS1 (107). These sequences were both unique when compared with other trichomonad

species. Through a series of serial dilutions, this method could detect genomic DNA that equaled the weight of one *T. foetus* organism. However, a decreased sensitivity was discovered when the PCR required at least 50 organisms per milliliter of sample to yield a positive result in pooled preputial washings or vaginal mucus samples (107). Inhibitory substances present in the smegma sample may be responsible for the loss of sensitivity. This study highlighted a common problem encountered with previous PCR- based studies (109) in that the presence of non-specific amplicons are similar in size to the specific amplicons creating difficulty in distinguishing positive and negative results. To overcome this problem, investigators adapted the use of the uracil DNA glycosylase system as a further confirmation test for samples with unclear or doubtful results when additional amplification bands were present (109). Favorable results from this study led to the proposed use of the newly developed PCR as either a confirmatory test or possibly stand-alone diagnostic for detection of *T. foetus*.

Previously developed PCR assays continued to evolve and improve by using diagnostic size variants from within the internal transcribed spacer 1 (ITS1) region that is between the 18S rRNA and 5.8S rRNA subunits (113). A study reported by Grahn et al. emphasized that one problem that has plagued previous PCR assays is false negatives due to failed amplification. Newly developed pan-trichomonad PCR primers allow the amplification of numerous trichomonad species (*T. foetus, T. suis, T. vaginalis, Trichomonas gallinae, Pentatrichomonas hominis,* and *Tetratrichomonas* spp.) from a variety of hosts that can be identified on a single round of PCR if present (113). The results of this study and others demonstrated acceptable sensitivities and specificities

from PCR assays which has led many states to accept PCR as a sole testing modality for transportation permits of bulls both intra- and interstate (113, 114).

Efforts to control *T. foetus* focus on tests with higher sensitivity, reduced cost, and time efficacy for diagnostic assays. Economic input by producers for testing has been a major factor limiting the number of bulls tested in large herds where a single sample can cost the producer $20-35 per head plus the cost of the veterinarian obtaining the sample (115). This limits the number of tests the producer is willing to perform on bulls within the herd often only choosing to test a small subset of the population and thus limiting the detection rate within the herd. Garcia Guerra et al. (2014) investigated PCR performed on pooled trichomoniasis samples as a proposed solution to reduce costs of testing large bull batteries (116). Preputial samples were packaged individually in the field in the InPouch ™ TF system and pooled in the laboratory. Pools containing a single known positive bull based on culture and examination by bright-field microscopy were added to multiple negative samples to test the sensitivity as serial dilutions were made. It was determined that a single infected bull in a pool of 25 samples was detected with a sensitivity of 93.5% (95% CI, 79.3% to 98.2%) (116). The overall sensitivity of all dilutions from this study was reported to be 94% (116). The authors noted a concentration effect on the sensitivity as they determined that the probability of a positive result increased 3.7 times for every log increase in concentration of organisms. This study also demonstrated that each day of culture prior to taking an aliquot for real time PCR increased the odds of a negative result by 9.8 times (116). These deleterious effects of prolonged culturing on PCR are mainly due contamination of the culture pouch causing breakdown and destruction of the DNA within the protozoa leading to false negative results. The

sensitivities reported above differ from previous work by Garcia Guerra et al. in 2013 that reported a sensitivity of 83.6% (95% CI: 75.6 to 89.4%) on 110 pooled samples of 5 bulls each and a sensitivity of 77.3% (95% CI: 68.6 to 84.1%) on pooled samples of 10 bulls each (117). The authors reported sensitivities of 100% on pooled samples of 5 bulls when sampled once a week for three weeks. The preputial smegma samples were placed directly into phosphate buffered saline and were not cultured in media. This may account for the differences in sensitivity results between the two studies. Further investigation of sampling, culturing, and PCR assays for *T. foetus* are warranted to increase the sensitivity and reduce the number of bulls that are falsely determined to be negative by current culture and PCR standards.

2.18 Human Trichomoniasis

Alfred Francois Donné, a notable French scientist of the 19th century, first described a motile microorganism from the purulent discharges of women (118). The organism was later named Tricomonas after a colleague of Donné noted the organism's resemblance to other protozoans, Trichodes and Monas which were already discovered. As Donné studied more about vaginal mucus he found that this newly discovered motile organism survived in the vaginal mucus of women. Subsequently, he gave the organism its current name of *Trichomonas vaginalis (T. vaginalis)* (118). Procaccini, an Italian scientist, followed a group of Italian soldiers from 1934 to 1939 that suffered from a transient hematuria (119). He originally thought he had discovered a new ameoba that he called nephrouroameba. It was decided upon further evaluation that these new ameobae

were actually trichomonads. Clark et al. developed a culture based examination to enhance diagnostic capabilities in 1959 (120). Efficacious treatment options with the use of 5-Nitroimidazoles was discovered in the 1960s (121). It would not be until 2007 that a monumental scientific accomplishment occurred as the entire *T. vaginalis* genome was sequenced in a group effort by several agencies (122). The organism was found to have a rather large genome exceeding 160Mb.

Trichomonas vaginalis, an extracellular, flagellated protozoan, causes the important venereally transmitted disease of humans called trichomoniasis. The parasite has a worldwide distribution, and humans are the only natural host. *Trichomonas tenax* and *hominis* also may infect humans but are non-pathogenic species. Significant prevalence is reported in areas of low socio-economic populations with 160-180 million people affected each year with approximately 8 to 10 million of those people residing within the United States (123). The World Health Organization (WHO) list *T. vaginalis* as the most prevalent non-viral sexually transmitted organism in humans (124). Despite having a significant prevalence within the human population this protist received comparably little attention from the scientific community until information emerged suggesting this organism is a risk factor for human immunodeficiency virus (HIV) and cervical neoplasia. The development of better diagnostics has led to better epidemiologic evaluations of the disease.

Trichomonas vaginalis is a pyriform to sometimes amoeboid-shaped organism that in the trophozoite form is approximately 9-23 x 7 μm (125). The structure has a characteristic four anterior flagella and a single posterior flagellum that originates from the blepharoplast. These characteristic structures have facilitated the classic method of

diagnosis of the disease by wet mount preparation. The organism's internal organelles include a single nucleus, axostyle, costa, pelta, and hydrogenosome (Illustration 3) similar to the internal makeup of *T. foetus*. Hydrogenosomes catalyze carbohydrates via a fermentation process that serve as the organisms main energy source (125). The organism reproduces via longitudinal binary fission equal to *T. foetus*. Also similar to *T. foetus*, the organism has no known true cyst form, but recent reports have discovered a pseudocyst life form (36). The role this life form plays in the life cycle or adaptability of the organism in different environments is still unclear.

Trichomonas vaginalis has been commonly isolated from the vagina, urethra, and Skeen's glands of the female urogenital tract (126). Reports suggest that a third to one half of infections in women are asymptomatic (125). Clinical signs reported from gynecological clinics are as follows: mild to severe vaginitis, green/brown to frothy malodorous vaginal discharge, pruritus, strawberry cervix, edema, erythema, urinary tract infections, dysuria, and pelvic pain (127-129). Furthermore, negative effects on pregnancies can occur in the form of premature delivery and low birth weights (125, 130). The disease is considered a venereal pathogen with non-venereal routes reported as rare. It is unlike *T. foetus* in that the organism is not invasive to the fetus during gestation. The erosive nature of this organism can lead to cervical neoplasia and has been shown to be a co-factor of acquiring HIV (131). The emerging information associated with the public health risks described has led to increased attention and focus from the scientific community.

The disease has not been studied in males to the extent that it has in women. Men, like the bull, are usually asymptomatic carriers of the organism with only a reported 30%

displaying clinical signs (132). However, unlike the bull, the human male can clear the infection without treatment usually within two weeks to four months following infection (133). The organism can populate the urethra, prostate, and epididymis of the male urogenital tract (134). Clinical signs that are occasionally the primary finding associated with trichomoniasis in males are urethritis, prostatitis, balanoposthitis, cystitis, and epididymitis (129). Studies suggest that males, like females, are at an increased risk for contracting HIV if concurrently infected with *T. vaginalis* when exposed to the virus. There are also several studies that report an increased risk of developing prostate cancer in males that are infected with *T. vaginalis* (135). The reduced occurrence of outward signs of disease in males compared to females has been hypothesized to be due to the oxidizing nature of fluids within the urogenital tract that are inhibitory to pathogenic secretions of the organism (132).

Diagnosis of trichomoniasis in women has most commonly relied upon identification of the organism directly on preparations of saline mixed with vaginal secretions. Observation of the characteristic pyriform shaped trichomonad with jerky, rolling motility has been considered 100% specific for *T. vaginalis*. However, the sensitivity is lagging behind with reported ranges of 44-68% (136-138). Wet-mount sensitivity is affected by transport temperatures (< 22°C), transport times of greater than 30 minutes, and experience of the laboratory technician. The true advantages to this method of testing are the ability of in-house results to be reported rapidly and the relatively inexpensive nature of the test.

Trichomonads can also be identified on traditional Papanicolaou (Pap) smears during routine gynecological examinations (139). The lack of sensitivity and specificity

has precluded this test as a viable option for diagnosis of trichomoniasis. Recently a liquid-based Pap test emerged that boasts an improved sensitivity/specificity for the organism of 60-96% and 98-100%, respectively (140). The fact that results are not reported for several days can be a drawback to this procedure.

Multiple diagnostic samples can be taken from men for the diagnosis of *T. vaginalis* including urethral swabs, evaluation of urine sediment following collection, and evaluation of semen samples. The above described direct microscope evaluation techniques when applied to the male for the diagnosis of *T. vaginalis* utilizing the traditional samples of urethral swabs or urine sediment have poor sensitivity and therefore are not viable options for diagnosis in men (3). Certainly direct evaluation of *T. vaginalis* for diagnosis has left something to be desired, and the search for additional means of diagnosis continues.

The medical community has agreed that culture of *T. vaginalis* has demonstrated superior sensitivities compared to direct microscopy with Diamond's media and InPouch TV test (BioMed Diagnostics; White City, OR) as the medias of choice to cultivate the organism. Like *T. foetus*, specimens (vaginal swabs, urethral swabs, urine sediment, and semen) should immediately be inoculated and cultured at 37°C for up to a week. Vaginal secretions are the preferred specimen in women as urine has demonstrated a reduced sensitivity (137). Kaydos-Daniels et al. (2004) demonstrated urethral swabs, urine sediment, and semen together correctly diagnosed the most cases (141). The media allows for multiplication of the organism and an increased chance of detecting the motile trophozoites. This is particularly important in males where the concentration of organisms is considerably decreased compared to the female (3). Still, the sensitivity of

the test for both females and males is far from ideal at reported ranges of 44 to 75% and 40 to 56%, respectively (3, 137). The delay in reporting results (up to one week) and requirement for trained personnel are also limitations of the culture system for diagnosis.

Diagnostic tests designed for the detection of *T. vaginalis* antigens or nucleic acids have emerged. OSOM Trichomonas Rapid Test (Sekisui Diagnostics, Lexington, MA) is a United States Food and Drug Administration (FDA) approved kit that incorporates antibodies on a test strip specific for *T. vaginalis*. The strip forms a blue line when antigens from the organism bind to the antibody. This test requires no special equipment and offers rapid results within 30 minutes (139). Affirm VPIII (Becton Dickinson, Sparks, MD) is also a United States FDA approved test for trichomoniasis. This is a non-amplified nucleic acid probe hybridization test for *T. vaginalis, Gardnerella vaginalis*, and *Candida albicans* detection (139). Oligonucleotide probes are designed to detect nucleic acids specific to these three organisms. This test requires special equipment and is completed in approximately one hour. Although these testing measures demonstrate superior sensitivities to direct microscopy, the sensitivity ranging from 40 to 95% is similar to previously described culture methodology (142, 143). The apparent equivocal sensitivity with a shorter time to results compared to culture makes these methods appealing especially in areas where limited diagnostic ability is available, sample transport is an issue, or there is limited ability for patient follow-up. However, these tests have only been validated in women that are showing clinical signs of disease. Asymptomatic women and men along with symptomatic men have yet to be validated by these newer modalities.

Molecular based testing strategies have evolved to improve the diagnosis of *T. vaginalis* in both men and women. Polymerase chain reaction and transcription-mediated amplification (TMA) show markedly improved sensitivity and specificity compared to culture. These tests are based on amplification of nucleic acids from the target organism. Lawing et al. (2000) demonstrated the use of PCR for diagnosing *T. vaginalis* from vaginal secretions was significantly improved compared to culture. The PCR had an overall sensitivity of 89% and specificity of 97% (144). Polymerase chain reaction run on first catch urine samples from women have not shown the same sensitivity as vaginal secretions (144).

Hobbs et al. was the first to evaluate a PCR detection assay for detection of *T. vaginalis* from urethral swab samples of a population of Malawian men (145). The results of the PCR were compared to traditional wet-mount microscopy and culture techniques. PCR performed with a sensitivity of 82% (95% CI: 0.66-0.92) and specificity of 95% (95% CI: 0.91-0.97) compared to wet-mount microscopy and culture (145). Schwebke et al. (2002) later performed a study on 300 men attending a sexually transmitted disease clinic for the first time. These men were screened for *T. vaginalis* by culture and PCR of both urine and urethral swabs. The study demonstrated overall an increase in detection by PCR compared to culture with PCR positively identifying 17% of screened subjects compared to 5% with culture. This study also highlighted a greater number of positive samples on PCR analysis of urine compared to urethral swabs (146). These results highlighted a gender difference as studies demonstrated that urine from women was less sensitive on PCR compared to vaginal secretions. Kaydos-Daniels et al. later validated a PCR–ELISA technique run on first catch urine samples in a population of Malawian men.

The cross-sectional study showed the PCR-ELISA technique performed with a sensitivity of 92.7% and adjusted specificity of 95.2% compared to culture of urethral swabs or urine sediment (147). The authors concluded that this technique was a useful and validated alternative to culture of urethral swabs or urine sediment. The urine-based PCR-ELISA was also performed in some 3000 women attending sexually transmitted disease (STD) clinics. Kaydos-Daniels et al. (2003) determined that using a digoxigenin-labeled ELISA for detection of amplified *T. vaginalis* DNA from urine, the sensitivity and specificity of the PCR was 90.8 and 93.4%, respectively, compared to wet mount or culture from vaginal swabs (148). Hobbs et al. (2006) investigated the most recent male partners of women that were diagnosed with *T. vaginalis* (3). They discovered among these 287 male sexual partners of women with trichomoniasis who submitted at least one specimen for culture, *T. vaginalis* was detected in 47 (16.4%) men. However, when these samples were analyzed by PCR four times more infections were identified (3). In a similar study, *T. vaginalis* was detected in 201 (71.8%) of 280 male partners who submitted at least one specimen (urine, urethral swab, and/or semen) for PCR (3). Urine samples demonstrated a higher sensitivity (69.3%) compared to semen samples (53.8%) on PCR. Polymerase chain reaction identified 35 of 65 semen samples as positive. Overall, this study showed that detection of *T. vaginalis* in men was improved by testing multiple urogenital samples with PCR techniques described (3). These techniques show promise for increased detection of *T. vaginalis* from non-invasive specimens such as semen collection and the possibility of larger studies examining the true impact on public health worldwide. This is especially important as more studies suggest an association of *T. vaginalis* with HIV infections, cervical neoplasia, and prostatic neoplasia.

Nucleic Acid Amplification Tests (NAATs) have been the latest diagnostics developed to detect *T. vaginalis* in genital specimens. These tests report high sensitivities and specificities in the range of 92-100% (137, 149). APTIMA assay® (Hologic Inc., San Diego, CA) was the first of these tests to become approved through the United States FDA. It demonstrated a high sensitivity for detection of *T. vaginalis* in women's urine and endocervical and vaginal swabs (137). These technologies are promising but currently have limited clinical use because of significant costs, the need for highly trained personnel, and expensive instrumentation.

The use of 5-nitroimidazoles has been a significant part of the overall control of trichomoniasis in human populations. Metronidazole belongs to this class of drugs and has been a staple of therapy since its beginnings in the late 1960s. It causes cell death of *T. vaginalis* organisms via nitrogen radicals that bind and disrupt the organismal DNA (125). Metronidazole has historically shown great efficacy against *T. vaginalis* until recently when metronidazole resistant isolates have been reported in up to 5% of cases (150). This led human medical professionals to increase the dose and duration of therapy to overcome these challenges. This has also increased the risk of side effects associated with prolonged exposure to a drug class that has been reported to be carcinogenic in mice (151, 152). Reports of the carcinogenic effect in animals specifically turkeys led the United States FDA to ban this class of drugs in food producing animals over concerns for human health. Human health professionals have been careful with dosage and period of exposure in pregnant women and women that are breast feeding because of unproven hypotheses of negative outcomes on the fetus or neonate (125). Tinidazole, a second generation nitroimadazole compound, has been implemented as the therapy of choice in

resistant cases. It has twice the half-life of metronidazole and has shown a better volume of distribution in urogenital tract of males, as well (125). Metronidazole and Tinidazole are the only approved drugs for treatment of human trichomoniasis in the United States. Similar to cattle, attempts to develop immunity with vaccination in women have proven ineffective as similar to *T. foetus*, *T. vaginalis* failed to induce long term immunity as 6-12 months after infection neither *T. vaginalis*-specific antibodies nor memory B cells were present in the circulation. Two vaccines have made it to human clinical trials, but lack of efficacy caused further investigation to cease (133). Other control strategies in humans include screening for other sexually transmitted diseases in patients with diagnosed trichomoniasis and screening followed by treatment of partners of confirmed trichomoniasis cases.

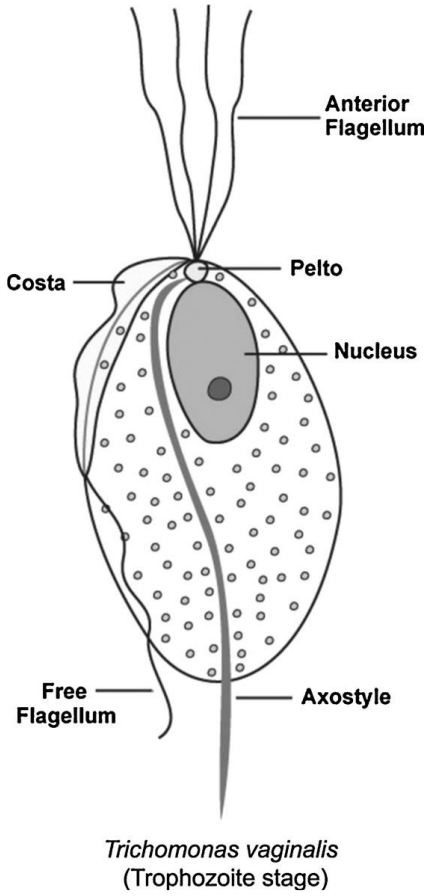


Illustration 3. *Trichomonas vaginalis* trophozoite form and internal organelles (Harp DF, Chowdhury I. Trichomoniasis: evaluation to execution. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2011;157(1):3-9.)

Chapter 3: Journal Article

Detection of *Tritrichomonas foetus* in Bovine Semen by PCR Amplification

Submitted to Theriogenology

Abstract

Bovine trichomoniasis caused by *Tritrichomonas foetus*, is a true venereal disease of cattle that is spread only through coitus. The objective of the study was to determine if a *T. foetus* infection could be detected in pre-seminal fluid and a seminal sample using polymerase chain reaction (PCR) collected from known positive bulls. Diagnostics have recently improved with the emergence of a more sensitive and specific PCR techniques. This is an improvement from traditional culture methods, but better diagnostics and collection methods are still needed given the serious consequences of inaccurate diagnosis.

Mature beef bulls (n=20) of various breeds from several south Florida ranches that were previously diagnosed to be positive for *T. foetus* by routine culture and real-time PCR on preputial smegma at an external state diagnostic laboratory were used for this study. These bulls underwent routine electroejaculation, and a dry preputial scraping sample was collected from each bull using a 52.5 cm infusion pipette with a flex adaptor and a 20 ml syringe. The samples collected from urethral emissions were fractionated into a pre-seminal sample and seminal sample based on gross appearance. The bulls all achieved an erection and extended completely outside of the sheath. The preputial sample was immediately suspended in 2 ml of Trypticase- Yeast Extract- Maltose Medium

(TYM) with agar (Diamond's media). The samples were transported to the laboratory in a commercial incubator at 37º C. Samples from each bull were centrifuged at 4000g for 9 minutes at room temperature and the resulting pellet was used for DNA isolation prior to processing for conventional PCR. Overall, 13 of 20 bulls were positive by traditional preputial scraping resulting in a test sensitivity of 0.65 (95% CI: 0.41-0.84). Four of these 13 positive bulls were also positive on the pre-seminal sample. One bull tested positive on the pre-seminal sample, but was negative on preputial scraping. The test sensitivity and specificity for detection of *T. foetus* by conventional PCR in pre-seminal fluid was 0.30 (95% CI: 0.1-0.61) and 0.86 (95% CI: 0.42-0.99), respectively. None of the semen samples were found to be positive for *T. foetus*. The combined sensitivity for the preputial scrape and pre-seminal fluid in this study was [1 - (1- 0.308) x (1- 0.65) = .758], suggesting that the diagnosis of *T. foetus* in infected bulls can be improved by additionally testing pre-seminal fluid during routine collection of a preputial scrape.

3.1 Introduction

Bovine trichomoniasis caused by *Tritrichomonas foetus (T. foetus),* is a true venereal disease of cattle that is spread only through coitus (153). This disease results in significant economic loss due to early embryonic death, abortions, and reduced weaning weights of calves born late in the calving season to cows that could not be bred because of infection early in the breeding season (19, 51, 53). The bull is an asymptomatic carrier of the disease (89). *T. foetus* persists on the prepuce and penis of the bull and has been isolated on rare occasions from the distal urethra via culture (1, 2). The gold standard for detection of *T. foetus* in the United States has been culture of a dry scraping of the prepuce in Diamond's media. The sensitivity of a single culture has varied widely in peer-reviewed studies from samples collected in the field ranging from 72% to 92% (25, 154-156). Specificity was long thought to have been 100% upon identification of the motile protozoan on bright field microscopy. However, a recent study highlights the possibilities of false positive due to misidentification of similar non-pathogenic trichomonads that originate from the gastrointestinal tract (100). It is generally accepted that the single culture carries a specificity of 95.24% based on work with naturally infected bulls (155). Cobo et al. (2007) investigated sensitivity and specificity of culture and PCR tests run in parallel and found that sensitivity was increased to 78% compared to 67.8% and 65.9% on the culture and PCR, respectively (114). Furthermore, three PCR performed on consecutive weeks demonstrated similar sensitivity and specificity (Se 85%, Sp 95.4%) to the standard six culture regime (Se 86.7%, Sp 97.5%) implemented by the artificial insemination industry (114). The implementation of PCR assays could

reduce economic inputs of testing while maintaining acceptable detection levels and greater efficiency than current protocols.

Diagnostics have recently improved with the emergence of more sensitive and specific PCR techniques. This reduces the number of false positive results from the identification of fecal trichomonads in culture and the false negatives that occur are due to unfavorable culture conditions for the trichomonad to grow, respectively. The challenge from a diagnostic perspective is multifactorial. Most of the test inaccuracies result from failure to recover the protozoan from the prepuce due to errors in sample collection technique and suboptimal transport conditions (84, 93). For example, Parker noted in 2003, that the operator's dominant hand and the side on which the collection is taken will affect the success in finding a positive bull (84). This is just one example of the difficulties faced by the veterinarian in trying to diagnosis these *T. foetus*. These problems highlight the need to find improved sampling methods that reliably produce highly sensitive and specific results.

Trichomonas vaginalis (T. vaginalis), the most common non-viral, venereally transmitted pathogen of humans, is similar in its pathogenesis to *T. foetus* and is known to cause urethritis and prostatitis in men (134). Recent studies in men infected with *T. vaginalis* demonstrated that PCR testing of centrifuged samples of semen or urine had increased sensitivity for detection of *T. vaginalis* as compared to culture of urine and urethral wet-mount scrapings (3). Hobbs et al. (2006) reported that infections were detected four times more often (16.4% compared to 71.8%) when PCR of semen or urine was compared to culture of semen, urine, or urethral swabs in a study evaluating male

partners of women diagnosed with *T. vaginalis* (3). This study was the first to report use of a PCR assay to discover *T. vaginalis* in the semen of men.

The implementation of novel diagnostic approaches from human medicine may allow improved detection of bulls infected with *T. foetus*. Pre-seminal fluid and semen are easily collected samples that could reduce sampling errors as practitioners could routinely procure a uniform sample for submission. Furthermore, these samples offer the benefit of causing less trauma as compared to traditional preputial scraping methods that could trauma that could lead to retropreputial abscess formation after sample collection.

3.2 Materials and Methods

3.2.1 Animals and Treatments

Bulls enrolled in this study were privately owned by several ranches in South Florida. A mixture of bull breeds representing *Bos taurus, Bos indicus* and *Bos indicus* crosses were utilized. All animals were previously diagnosed positive for *T. foetus* on a preputial scraping via quantitative polymerase chain reaction (qPCR) at accredited veterinary diagnostic laboratories. The bulls originated from multiple private ranches and were comingled at one location in South Central Florida. Each bull underwent a routine breeding soundness examination in accordance to the Society of Theriogenology published guidelines (157).

3.2.2 Sample Collection

Prior to electroejaculation, a preputial scraping was performed on each bull using a 53.34 cm infusion pipette (Infusette ® Tube with Adapters, Continental Plastic Corp. Delavan, WI, USA) and a non-sterile clean, 20 mL slip-tip syringe (Becton, Dickinson and Company Franklin Lakes, NJ, USA). The bull's prepuce was cleaned from organic debris with a paper towel and preputial hairs were trimmed prior to the insertion of the pipette into the preputial orifice to reduce contamination of the sample. Once inserted into the lumen of the prepuce, the pipette was advanced until it came into contact with the preputial reflection. At that time, the pipette was retracted 2-3 cm and a searching motion was commenced for less than 1 minute while applying negative pressure to the pipette via syringe to aspirate preputial smegma into the lumen of the pipette. The preputial sample was immediately suspended in 7.5 ml of Trypticase-Yeast Extract-Maltose Medium (TYM) with agar (Diamond's media). Disposable plastic collection cones and nitrile gloves were utilized and disposed of between each bull to prevent contamination of samples.

Following the preputial scraping, pre-seminal and seminal samples were collected with a Lane Pulsator IV Electroejaculator (Lane Manufacturing, Denver, CO, USA). A preset program function using a rectal probe that was 60 mm in diameter (Upright Weighted Bull Probe, Lane Manufacturing) facilitated the collection. During electroejaculation, all bulls achieved erection and full penile extension. Both a 0.5 ml pre-seminal and 0.5 ml seminal sample were collected individually based on gross appearance of the ejaculate with pre-seminal fluid being judged as clear fluid and seminal

fluid as cloudy ejaculate. Individual collection devices were assigned to pre-seminal and seminal samples to prevent contamination. The pre-seminal and seminal fluid were held at 37ºC via portable incubator (Koolatron ™, Brantford, Ontario, Canada) until arrival at Auburn University's College of Veterinary Medicine approximately nine hours after collection. Upon arrival, samples were stored at -20°C in a standard laboratory freezer. Samples were later thawed at 37ºC in a water bath and underwent centrifugation at 4000 g for 9 minutes at room temperature. The resulting pellet was used for DNA isolation prior to processing for conventional PCR. The pellet was stored at -20°C following removal of the supernatant to allow for further testing. Standard measures were taken to prevent contamination which included separate workspaces were maintained for specimen processing, PCR, and post-PCR work, and the use of sterile, disposable laboratory supplies.

3.2.3 Polymerase Chain Reaction (PCR)

DNA from standard preputial scrapings, pre-seminal samples, and seminal samples were extracted using QIAamp DNA Mini Kit (QIAGEN, Germantown, MD, USA) for blood and bodily fluids using spin protocol according to the manufacturer's instructions. NanoDrop Spectrophotometry (Thermo Scientific, Willmington, DE, USA) was utilized to validate the concentration of the extracted DNA products. A Polymerase Chain Reaction (PCR) assay was performed as previously described (106) (107). The PCR assay amplified the 5.8S rRNA gene and ITS1 and ITS2 regions of trichomonads by using the primers TFR-3 (5'-CGGGTCTTCCTATATGAGACAGAACC-3') and TFR-4

(5'-CCTGCCGTTGGATCAGTTTCGTTAA-3'), which annealed to the 5'-end of the 28S rRNA and to the junction of the 18s ITS1 region, respectively. Amplification was performed in an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany). The PCR reactions were performed with 1 x Taq buffer (Invitrogen, USA), 2.5 mM $MgCl_2$, 0.2 mM of each dNTPS, 1µM of specific primers, 1µl of the sample and 1.25 U of Taq DNA polymerase (Invitrogen, USA) in a final reaction volume of 50 µl. Initially, the sample was held at a 3-minute incubation at 94°C. Following the incubation, the assay was run through 30 cycles of denaturing (94°C, 30 s), annealing (58°C, 20 s) and extension (72°C, 30 s). In addition, a final step of extension (72°C, 20 s) was performed and the products held at 4°C.

The amplified products were electrophoresed on a 2% agarose gel for 90 minutes at 70 volts. The 2% agarose gel was prepared by heating 2g pure agarose in 100ml TBE buffer (Table 1) until dissolved and then cooled to 60°C. Next, 5ml 20X MOPS (BP308 Fisher Scientific, Fair Lawn, NJ, USA) running buffer and 17ml of 37% formaldehyde (12.3 M) were added and the gel poured using a comb to form wells. The RNA sample was prepared by adding ethidium bromide (E-7637, Sigma-Aldrich, and St. Louis, Missouri, USA) to the formaldehyde load dye (AM8556, Life Technologies, Carlsbad, California, USA). A *T. Foetus* clone CDTF-3 was utilized as a positive control. The internal negative control was an extracted human genomic DNA.

3.3 Statistical Analysis

For analysis, the 20 bulls naturally infected with *T. foetus* were considered the positive control group with previous positive diagnosis at validated diagnostic laboratories.

Sensitivity was defined as the probability of the test being positive among bulls that were naturally infected and previously tested positive for *T. foetus* [Se: true positives/(true positives + false negatives)]. Specificity was calculated for the pre-seminal sample using the reference values from the traditional preputial scraping for the true positive bulls [Sp: true negatives/(true negative + false positive)]. VassarStats, an online statistical calculator was used to calculate the results.

3.4 Results

Overall, 13 of 20 bulls were positive by traditional preputial scraping resulting in a test sensitivity of 65% (95% CI: 0.41-0.84). Four of these 13 positive bulls were also positive on the pre-seminal sample and one bull was positive on pre-seminal sample but not the preputial scraping sample. The test sensitivity and specificity for detection of *T. foetus* by conventional PCR in pre-seminal fluid was 30% (95% CI: 0.1-0.61) and 86% (95% CI: 0.42-0.99), respectively. None of the semen samples were found to be positive for *T. foetus* (Table 2). The combined sensitivity for the pre-seminal and preputial scrape fluid in this study was 75.8% [1 - (1- 0.308) x (1- 0.65) = 0.758].

Bull I.D.	Pre-seminal	Seminal	Preputial Scrape
1	Negative	Negative	**Positive**
2	Negative	Negative	**Positive**
3	Negative	Negative	Negative
4	Negative	Negative	**Positive**
5	Negative	Negative	**Positive**
6	**Positive**	Negative	**Positive**
7	Negative	Negative	Negative
8	**Positive**	Negative	**Positive**
9	Negative	Negative	Negative
10	Negative	Negative	**Positive**
11	**Positive**	Negative	Negative
12	Negative	Negative	Negative
13	Negative	Negative	**Positive**
14	**Positive**	Negative	**Positive**
15	**Positive**	Negative	**Positive**
16	Negative	Negative	**Positive**
17	Negative	Negative	Negative
18	Negative	Negative	**Positive**
19	Negative	Negative	Negative
20	Negative	Negative	**Positive**

Table 2: Sensitivity and specificity for detection of *T. foetus* by conventional PCR in pre-seminal fluid results

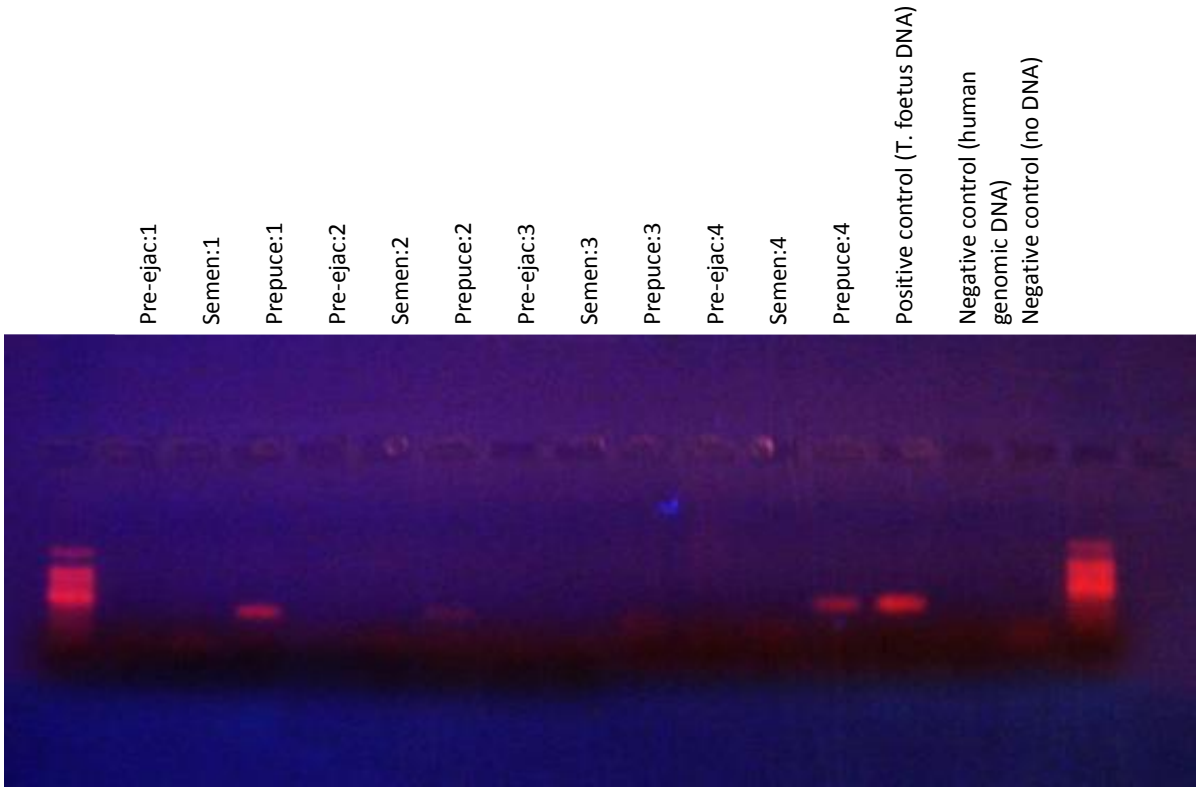


Illustration 4: *Tritrichomonas foetus* PCR gel bulls 1-4

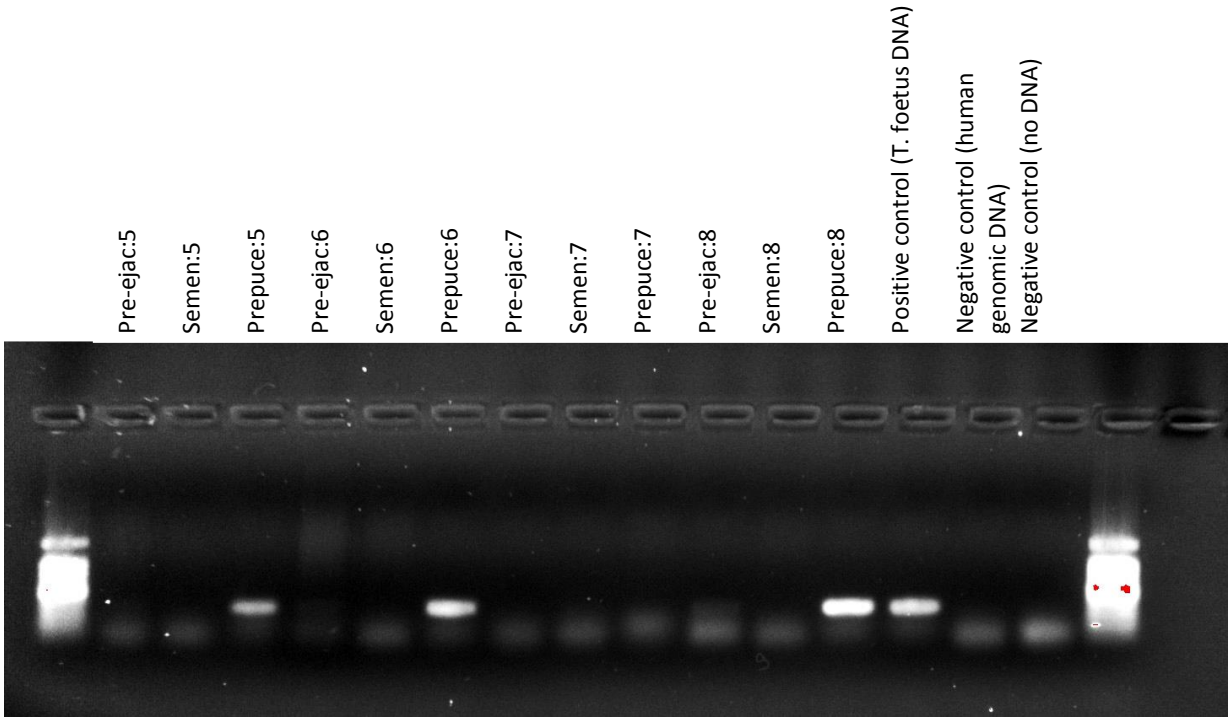


Illustration 5: *Tritrichomonas foetus* PCR gel bulls 5-8

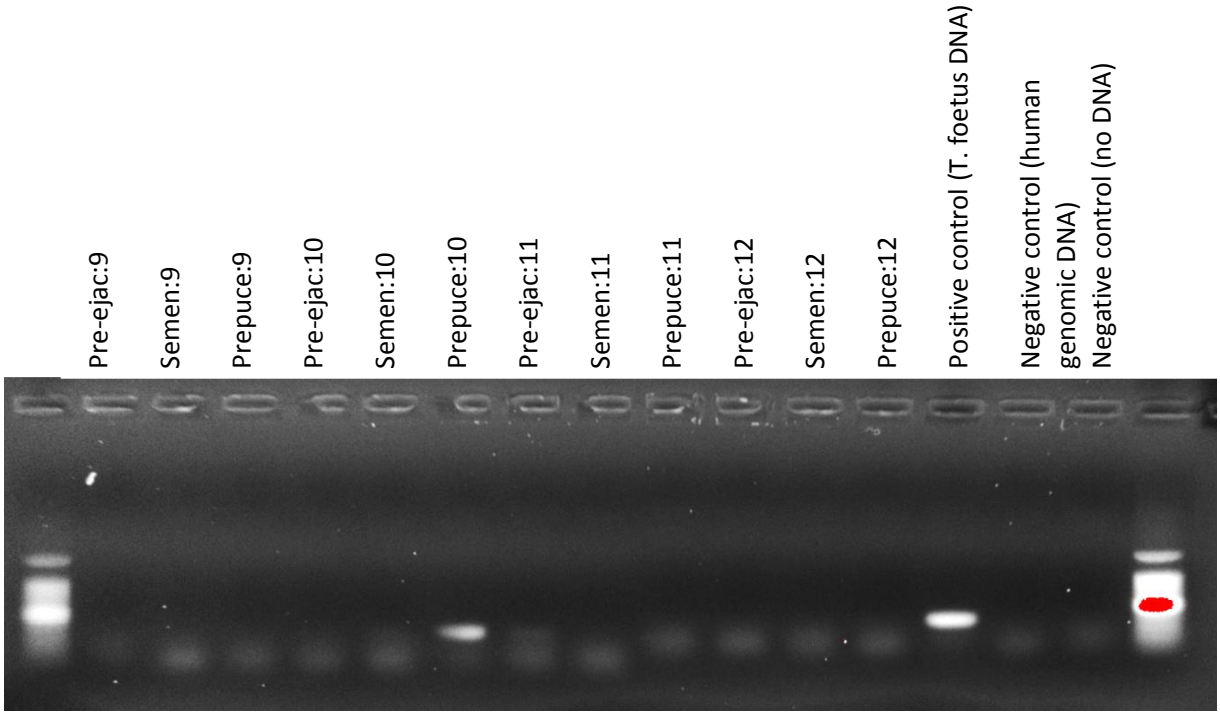


Illustration 6: *Tritrichomonas foetus* PCR gel bulls 9-12

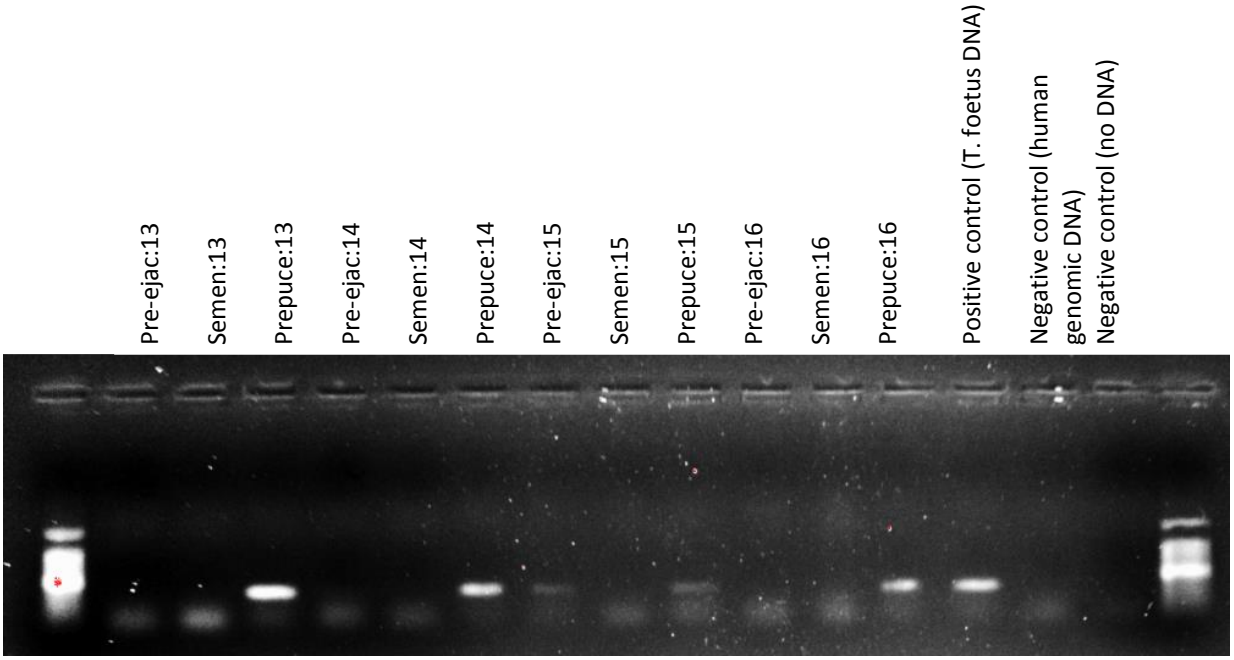


Illustration 7: *Tritrichomonas foetus* PCR gel bulls 13-16

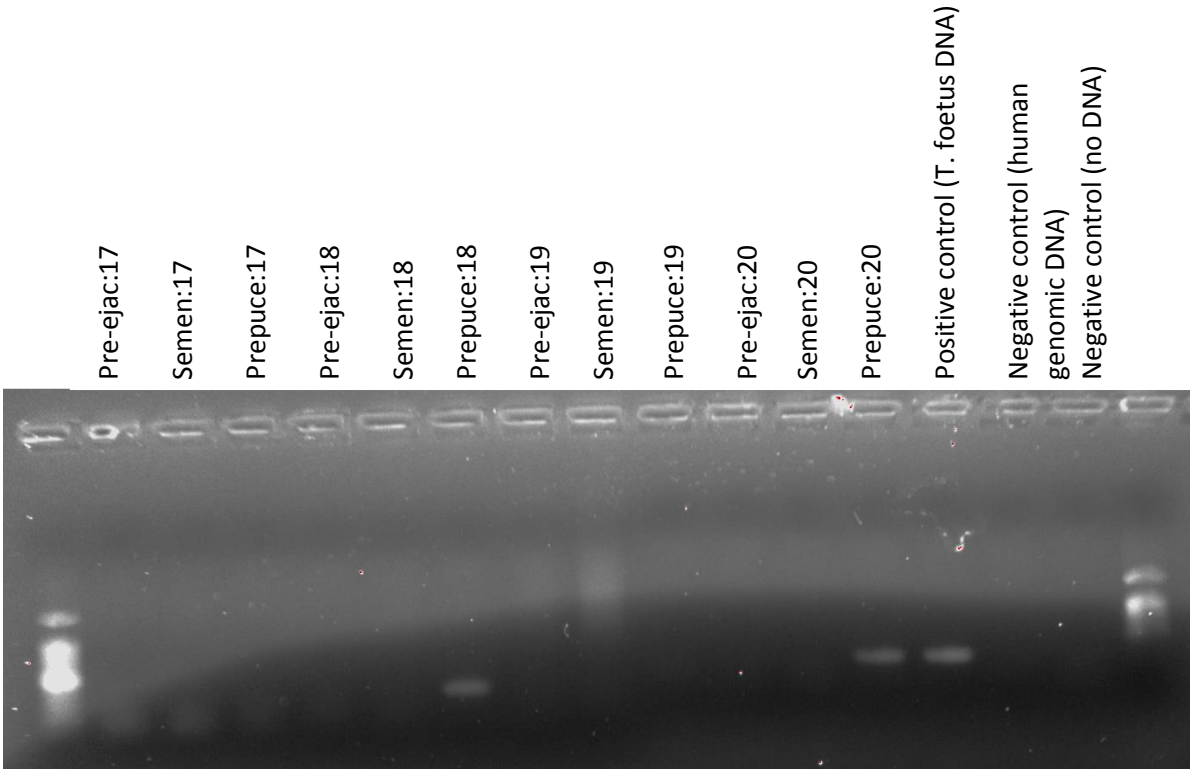


Illustration 8: *Tritrichomonas foetus* PCR gel bulls 17-20

3.5 Discussion

Bovine trichomoniasis can have devastating effects on the profitability of any cattle operation due to the losses from chronic infertility. The insidious nature of the disease results in an asymptomatic bull that will remain a carrier of the disease if not detected. In an ideal testing situation, bulls would undergo testing with culture or PCR techniques for three consecutive weeks with one to two weeks sexual rest to allow for parasite multiplication (43). This testing protocol all but eliminates infected bulls from falsely going undetected by current methods (72, 114). The major limitations to this protocol are increased labor and diagnostic costs to the owner. These factors combined with the fact that the current standard and most commonly performed diagnostic test of collecting a single preputial scraping has demonstrated sensitivities as low as 67.8% in one study evaluating both culture and PCR testing methods in tandem allows this disease to remain endemic in certain geographical regions (114). It has been suggested that there is a lack of standardization of how samples are collected, a decreased sensitivity of available diagnostic tests and an overall concern that preputial scrapings may induce significant trauma to the penis and/or prepuce. Therefore, the need for an easy, safe, and economical sampling method with improved sensitivity over the diagnostic tests currently available would eliminate the risk of false negatives.

In humans, *T. vaginalis* was correctly identified using PCR when using urine and seminal samples. PCR assays run on urine was the most sensitive in one study (69.8%; 95% CI, 64.2%-75.5%) compared to semen specimens (50.8%; 95% CI, 38.1%-63.6%) (158). However, this study demonstrated vastly different results than those published in

human literature. *Tritrichomonas foetus* was detected in 25% of the pre-seminal samples utilizing PCR methods and agreed with findings of the preputial scraping.

In contrast to the pre-seminal sample, *T. foetus* was undetectable in semen from all 20 bulls which was vastly different than what was reported in the human literature (50.8%; 95% CI, 38.1%-63.6%) (158). The difference in these results and the human literature may be a result of proteins present in the semen of the bovid. Seminalplasmin, an antimicrobial protein, present in the seminal plasma of the bovid has been demonstrated to be a powerful inhibitor of *E. coli* RNA polymerase (159). It is still possible that low numbers of organisms in the sample could have been below the detectable limits of the assay. Furthermore, reports vary on whether *T. foetus* actually transcends the entire urogenital tract of the bull or not. Laboratory techniques applied from investigations into the discovery of viral particles in bovine semen could serve as a tool for investigation with *T. foetus* in the future.

It is of interest that nine bulls which tested positive by preputial scraping were not found to have positive PCR pre-seminal samples. Another significant finding was Bull 11 that tested negative for *T. foetus* via a preputial scraping but tested positive in the pre-seminal sample. Furthermore, it should also be noted that 7 of the 20 bulls which were previously diagnosed with *T. foetus* tested negative by preputial scraping taken by an experienced practitioner and six of these bulls were negative on both pre-seminal and preputial samples. These findings illustrate the frustration of sampling for diagnosis of this organism and reinforce that the addition of a readily available pre-seminal sample can increase the sensitivity by reducing the number of false negative bulls. This increase

in sensitivity would aim in correctly diagnosing and removing bulls infected with *Tritrichomonas foetus* from the herd.

References

1. Parsonson IM, Clark BL, Dufty J. The pathogenesis of *Tritrichomonas foetus* infection in the bull. Australian Veterinary Journal. 1974;50(10):421-3.
2. Roberts SJ. Infectious diseases causing infertility in cows. In: Veterinary Obstetrics and Genital Diseases 2 ed. Ann Arbor, MI: Edward Bros., Inc; 1971. p. 391.
3. Hobbs MM, Lapple DM, Lawing LF, et al. Methods for detection of *Trichomonas vaginalis* in the male partners of infected women: implications for control of trichomoniasis. Journal of Clinical Microbiology. 2006;44(11):3994-9.
4. Certified Semen Services (CSS). Minimum requirements for disease control of semen produced for artificial insemination. Columbia, MO: National Association Animal Breeders (NAAB);. 2014.
5. Morgan BB. Bovine Trichomoniasis. Minneapolis, MN: Burgess Publishing Company; 1946.
6. Roberts SJ. Infectious diseases causing infertility in cows. In: Veterinary Obstetrics and Genital Diseases 3 ed. Ann Arbor, MI: Edward Bros., Inc; 1986. p. 447.
7. Erasmus JA, De Wet JA, Van der Merwe HE, et al. Bovine trichomoniasis in the north western Cape Province, western Transvaal and the Orange Free State. Journal of the South African Veterinary Association. 1989;60(1):51-2.

8. Riley DE, Wagner B, Polley L, et al. PCR-based study of conserved and variable DNA sequences of *Tritrichomonas foetus* isolates from Saskatchewan, Canada. Journal of Clinical Microbiology. 1995;33(5):1308-13.

9. Rodning SP, Wolfe DF, Carson RL, et al. Prevalence of *Tritrichomonas foetus* in several subpopulations of Alabama beef bulls. Theriogenology. 2008;69(2):212-7.

10. Yao C. Diagnosis of *Tritrichomonas foetus*-infected bulls, an ultimate approach to eradicate bovine trichomoniasis in US cattle. Journal of Medical Microbiology. 2013;62(Pt 1):1-9.

11. Kleina P, Bettim-Bandinelli J, Bonatto SL, et al. Molecular phylogeny of Trichomonadidae family inferred from ITS-1, 5.8S rRNA and ITS-2 sequences. International Journal for Parasitology. 2004;34(8):963-70.

12. Cepicka I, Hampl V, Kulda J. Critical taxonomic revision of Parabasalids with description of one new genus and three new species. Protist. 2010;161(3):400-33.

13. Brugerolle G, Lee J. Phylum parabasalia. In: An illustrated guide to the protozoa 2 ed. Lawrence, KS: Wiley-Blackwell 2000;2:1196-250.

14. Adl SM, Simpson AG, Farmer MA, et al. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. The Journal of Eukaryotic Microbiology. 2005;52(5):399-451.

15. Tolbert MK, Gookin J. *Tritrichomonas foetus*: a new agent of feline diarrhea. Compend Contin Educ Vet. 2009;31(8):374-81, 90; quiz 81.

16. Tachezy J, Tachezy R, Hampl V, et al. Cattle pathogen *tritrichomonas foetus* (Riedmuller, 1928) and pig commensal *Tritrichomonas suis* (Gruby & Delafond,

1843) belong to the same species. The Journal of Eukaryotic Microbiology. 2002;49(2):154-63.

17. Lun ZR, Chen XG, Zhu XQ, et al. Are *Tritrichomonas foetus* and *Tritrichomonas suis* synonyms? Trends in Parasitology. 2005;21(3):122-5.

18. Fitzgerald P, Johnson A, Thorne J, et al. Trichomoniasis in range cattle. Veterinary Medicine. 1958;53:249-52.

19. Fitzgerald PR. Bovine trichomoniasis. The Veterinary Clinics of North America Food Animal Practice. 1986;2(2):277-82.

20. Johnson AE. Incidence and Diagnosis of Trichomoniasis in Western Beef Bulls. Journal of the American Veterinary Medical Association. 1964;145:1007-10.

21. Wilson S, Kocan A, Gaudy E, et al. The prevalence of trichomoniasis in Oklahoma beef bulls. Bovine Practitioner. 1979;14:109-10.

22. Speer C, White M. Bovine trichomoniasis. Better diagnostics and control could save beef industry $650 million annually. Large Animal Veterinarian. 1991;46(1):18-20.

23. Rae DO. Impact of trichomoniasis on the cow-calf producer's profitability. Journal of the American Veterinary Medical Association. 1989;194(6):771-5.

24. Ondrak JD. *Tritrichomonas foetus* Prevention and Control in Cattle. The Veterinary Clinics of North America Food Animal Practice. 2016;32(2):411-23.

25. Villarroel A, Carpenter TE, BonDurant RH. Development of a simulation model to evaluate the effect of vaccination against *Tritrichomonas foetus* on reproductive efficiency in beef herds. American Journal Veterinary Research. 2004;65(6):770-5.

26. Kimsey PB, Darien BJ, Kendrick JW, et al. Bovine trichomoniasis: diagnosis and treatment. Journal of the American Veterinary Medical Association. 1980;177(7):616-9.

27. BonDurant RH, Anderson ML, Blanchard P, et al. Prevalence of trichomoniasis among California beef herds. Journal of the American Veterinary Medical Association. 1990;196(10):1590-3.

28. Rae DO, Crews JE, Greiner EC, et al. Epidemiology of *Tritrichomonas foetus* in beef bull populations in Florida. Theriogenology. 2004;61(4):605-18.

29. Grotelueschen DM, Cheney J, Hudson DB, et al. Bovine trichomoniasis: Results of a slaughter survey in Colorado and Nebraska. Theriogenology. 1994;42(1):165-71.

30. Szonyi B, Srinath I, Schwartz A, et al. Spatio-temporal epidemiology of *Tritrichomonas foetus* infection in Texas bulls based on state-wide diagnostic laboratory data. Veterinary Parasitology. 2012;186(3):450-5.

31. Yao C, Bardsley KD, Litzman EA, et al. *Tritrichomonas foetus* infection in beef bull populations in Wyoming. Journal of Bacteriology & Parasitology. 2012;2011.

32. Mariante RM, Lopes LC, Benchimol M. *Tritrichomonas foetus* pseudocysts adhere to vaginal epithelial cells in a contact-dependent manner. Parasitology Research. 2004;92(4):303-12.

33. Pereira-Neves A, Ribeiro KC, Benchimol M. Pseudocysts in trichomonads--new insights. Protist. 2003;154(3-4):313-29.

34. Rae DO, Crews JE. *Tritrichomonas foetus*. The Veterinary Clinics of North America Food Animal Practice. 2006;22(3):595-611.

35. BonDurant RH. Pathogenesis, diagnosis, and management of trichomoniasis in cattle. The Veterinary Clinics of North America Food Animal Practice. 1997;13(2):345-61.

36. Pereira-Neves A, Benchimol M. *Tritrichomonas foetus*: budding from multinucleated pseudocysts. Protist. 2009;160(4):536-51.

37. Granger BL, Warwood SJ, Benchimol M, et al. Transient invagination of flagella by *Tritrichomonas foetus*. Parasitology Research. 2000;86(9):699-709.

38. Benchimol M. Trichomonads under microscopy. Microscopy and Microanalysis. 2004;10(05):528-50.

39. Pereira-Neves A, Campero CM, Martinez A, et al. Identification of *Tritrichomonas foetus* pseudocysts in fresh preputial secretion samples from bulls. Veterinary Parasitology. 2011;175(1-2):1-8.

40. Honigberg, BM. Trichomonads found outside the urogenital tract of humans. In: Trichomonads Parasitic in Humans. Springer, NY: 1990. p. 342-393.

41. Mattern CF, Honigberg BM, Daniel WA. Fine-structural changes associated with pseudocyst formation in *Trichomitus batrachorum*. The Journal of Protozoology. 1973;20(2):222-9.

42. Samuels R. Studies of *Tritrichomonas batrachorum* 3. Abnormal mitosis and morphogenesis. Transactions of the American Microscopical Society, 1959;78(1):49-65.

43. Peter D. Bovine venereal diseases. In: Youngquist, RS, editor. Current Therapy in Large Animal Theriogenology. 1 ed. Philadelphia, PA: W.B. Saunders Co.; 1997. p. 355–363.

44. Anderson ML, Barr BC, Conrad PA. Protozoal causes of reproductive failure in domestic ruminants. The Veterinary Clinics of North America Food Animal Practice. 1994;10(3):439-61.

45. Ribeiro CM, Falleiros MB, Bicudo SD, et al. *Tritrichomonas fetus* extracellular products decrease progressive motility of bull sperm. Theriogenology. 2010;73(1):64-70.

46. Morgan B. Studies on the trichomonad carrier-cow problem. Journal of Animal Science. 1944;3:437.

47. Christensen HR, Clark BL, Parsonson IM. Incidence of *Tritrichomonas foetus* in young replacement bulls following introduction into an infected herd. Australian Veterinary Journal. 1977;53(3):132-4.

48. BonDurant R, Honigberg B. Trichomonads of veterinary importance. Parasitic Protozoa. 1994;2:111-88.

49. Honigberg BM. Trichomonads of veterinary importance. In: Kreier, JP, editor. Parasitic Protozoa, vol. II. New York, N.Y.: Academic Press; 1978.p. 163

50. Strickland LG, Edmondson MA, Maxwell HS, et al. Surface architectural anatomy of the penile and preputial epithelium of bulls. Clinical Theriogenology. 2014; 6:445-51.

51. Parsonson IM, Clark BL, Dufty JH. Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. Journal Comparative Pathology. 1976;86(1):59-66.

52. BonDurant, R.H. Diagnosis, treatment, and control of bovine trichomoniasis. Compendium on Continuing Education for the Practicing Veterinarian 1985;7: S179- S188.

53. Clark BL, Dufty JH, Parsonson IM. The effect of *Tritrichomonas foetus* infection on calving rates in beef cattle. Australian Veterinary Journal. 1983;60(3):71-4.

54. Kvasnicka WG, Hanks D, Huang JC, et al. Clinical evaluation of the efficacy of inoculating cattle with a vaccine containing *Tritrichomonas foetus.* American Journal of Veterinary Research. 1992;53(11):2023-7.

55. Abbitt B. Trichomoniasis in cattle. In: Morrow DA, editor. Current therapy in theriogenology diagnosis, treatment, and prevention of reproductive diseases in small and large animals. 1 ed. Philadelphia, PA: W.B. Saunders Co.; 1980. p. 482-488.

56. Yule A, Skirrow SZ, BonDurant RH. Bovine trichomoniasis. Parasitology Today 1989;5(12):373-7.

57. Skirrow S. Identification of trichomonad-carrier cows. Journal of the AmericanVeterinary Medical Association. 1987;191(5):553-4.

58. Skirrow SZ, BonDurant RH. Immunoglobulin isotype of specific antibodies in reproductive tract secretions and sera in *Tritrichomonas foetus*-infected heifers. American Journal Veterinary Research. 1990;51(4):645-53.

59. Skirrow SZ, BonDurant RH. Induced *Tritrichomonas foetus* infection in beef heifers. Journal of the American Veterinary Medical Association. 1990;196(6):885-9.

60. Dawson L. Diagnosis, prevention, and control of campylobacteriosis and trichomoniasis. Bovine Practitioner. 1986;21:180.

61. Ball L, Dargatz DA, Cheney JM, et al. Control of venereal disease in infected herds. The Veterinary Clinics of North America Food Animal Practice. 1987;3(3):561-74.

62. Gasparini G, Vaghi M, Tardani A. Treatment of bovine trichomoniasis with metronidazole (8823 RP). The Veterinary Record. 1963;75:940.

63. Skirrow SZ, Bondurant RH. Treatment of bovine trichomoniasis with ipronidazole. Australian Veterinary Journal. 1988;65(5):156.

64. Koziol JH. In vitro efficacy of anti-protozoal compounds against *Tritrichomonas foetus* (Masters Thesis): Auburn University; 2016.

65. Bondurant RH. Venereal diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. The Veterinary Clinics of North America Food Animal Practice. 2005;21(2):383-408.

66. Corbeil LB. Immunization and diagnosis in bovine reproductive tract infections. Advances in Veterinary Medicine. 1999;41:217-39.

67. Corbeil LB, Anderson ML, Corbeil RR, et al. Female reproductive tract immunity in bovine trichomoniasis. American Journal of Reproductive Immunology. New York, NY : 1998;39(3):189-98.

68. Anderson ML, BonDurant RH, Corbeil RR, et al. Immune and inflammatory responses to reproductive tract infection with *Tritrichomonas foetus* in immunized and control heifers. The Journal of Parasitology. 1996;82(4):594-600.

69. Kvasnicka WG, Taylor RE, Huang JC, et al. Investigations of the incidence of bovine trichomoniasis in nevada and of the efficacy of immunizing cattle with vaccines containing *Tritrichomonas foetus*. Theriogenology. 1989;31(5):963-71.

70. Herr S, Ribeiro LM, Claassen E, et al. A reduction in the duration of infection with *Tritrichomonas foetus* following vaccination in heifers and the failure to demonstrate a curative effect in infected bulls. Onderstepoort Journal Veterinary Research. 1991;58(1):41-5.

71. Gay JM, Ebel ED, Kearley WP. Commingled grazing as a risk factor for trichomonosis in beef herds. Journal of the American Veterinary Medical Association. 1996;209(3):643-6.

72. Ondrak JD, Keen JE, Rupp GP, et al. Repeated testing by use of culture and PCR assay to detect *Tritrichomonas foetus* carrier bulls in an infected Nebraska herd. Journal of the American Veterinary Medical Association. 2010;237(9):1068-73.

73. Clark BL, Parsonson IM, White MB, et al.. Control of trichomoniasis in a large herd of beef cattle. Australian Veterinary Journal. 1974;50(10):424-6.

74. Cobo ER, Campero CM, Mariante RM, et al. Ultrastructural study of a tetratrichomonad species isolated from prepucial smegma of virgin bulls. Veterinary Parasitology. 2003;117(3):195-211.

75. Plebani M. The detection and prevention of errors in laboratory medicine. Ann Clinical Biochemistry. 2010;47(Pt 2):101-10.

76. Mukhufhi N, Irons PC, Michel A, et al. Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: effects of sample collection method, storage and transport medium on the test. Theriogenology. 2003;60(7):1269-78.

77. Fitzgerald PR, Hammond DM, Miner ML, et al. Relative efficacy of various methods of obtaining preputial samples for diagnosis of trichomoniasis in bulls. American Journal Veterinary Research. 1952;13(49):452-7.

78. Sutka P, Katai PL. Rapid demonstration of bull trichomonadosis in unstained smear preparations from preputial scrapings. Acta Vet Acad Sci Hung. 1969;19(4):385-9.

79. Tedesco LF, Errico F, Del Baglivi LP. Diagnosis of *Tritrichomonas foetus* infection in bulls using two sampling methods and a transport medium. Australian Veterinary Journal. 1979;55(7):322-4.

80. Parker S, Campbell J, Ribble C, et al.. Comparison of two sampling tools for diagnosis of *Tritrichomonas foetus* in bulls and clinical interpretation of culture results. Journal of the American Veterinary Medical Association. 1999;215(2):231-5.

81. Gregory MW, Ellis B, Redwood DW. Comparison of sampling methods for the detection of *Tritrichomonas foetus* infection in bulls. The Veterinary Record. 1990;127(1):16.

82. Schonmann MJ, BonDurant RH, Gardner IA, et al.. Comparison of sampling and culture methods for the diagnosis of *Tritrichomonas foetus* infection in bulls. The Veterinary Record. 1994;134(24):620-2.

83. Sager H, Ferre I, Henning K, et al. Tritrichomonosis. Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control. 2007. p. 232.

84. Parker S, Campbell J, Ribble C, et al. Sample collection factors affect the sensitivity of the diagnostic test for *Tritrichomonas foetus* in bulls. Canadian Journal of Veterinary Research. 2003;67(2):138-41.

85 Kimsey PB. Bovine Trichomoniasis. In: Morrow DA, editor. Current therapy in theriogenology. 2 ed. Philadelphia, PA: W.B. Saunders Co.; 1986. p. 275-9.

86. Skirrow SZ, BonDurant RH. Bovine trichomoniasis. Veterinary Bulletin. 1988; 58, 591-603.

87. Rhyan JC, Stackhouse LL, Quinn WJ. Fetal and placental lesions in bovine abortion due to *Tritrichomonas foetus*. Veterinary Pathology. 1988;25(5):350-5.

88. Rhyan JC, Wilson KL, Burgess DE, et al. Immunohistochemical detection of *Tritrichomonas foetus* in formalin-fixed, paraffin-embedded sections of bovine placenta and fetal lung. Journal of Veterinary Diagnostic Investigation. 1995;7(1):98-101.

89. Rhyan JC, Wilson KL, Wagner B, et al. Demonstration of *Tritrichomonas foetus* in the external genitalia and of specific antibodies in preputial secretions of naturally infected bulls. Veterinary Pathology. 1999;36(5):406-11.

90. Bryan L, Campbell J, Gajadhar A. Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch TF media. The Veterinary Record. 1999;144(9):227-32.

91. Appell L, Mickelsen W, Thomas M, et al. A comparison of techniques used for the diagnosis of *Tritrichomonas foetus* infection in beef cattle. Agri Practice. 1993.

92. Borchardt K, Norman B, Thomas M,et al. Evaluation of a new culture method for diagnosing *Tritrichomonas foetus* infection. Veterinary Medicine. 1992;87(2).

93. Davidson JM, Ondrak JD, Anderson AA, et al. Evaluation of effects of high incubation temperatures on results of protozoal culture and real-time PCR testing for *Tritrichomonas foetus* inoculated in a commercially available self-contained culture media system. Journal of the American Veterinary Medical Association. 2011;239(12):1589-93.

94. Thomas M, Harmon W, White C. An improved method for the detection of *Tritrichomonas foetus* infection by culture in bulls. Agri-Practice. 1990;11(1):13-7.

95. Bartlett D, Hasson E, Teeter KG. Occurrence of *Trichomonas foetus* in preputial samples from infected bulls. Journal of the American Veterinary Medical Association. 1947;110(839):114.

96. Bartlett DE, Hasson EV, Teeter KG. Occurrence of *Trichomonas foetus* in preputial samples from infected bulls. Journal of the American Veterinary Medical Association. 1947;110(839):114-20.

97. Todorovic R, McNutt SH. Diagnosis of *Trichomonas foetus* infection in bulls. American Journal Veterinary Research. 1967;28(126):1581-90.

98. Diamond LS. The establishment of various trichomonads of animals and man in axenic cultures. The Journal of Parasitology. 1957;43(4):488-90.

99. Skirrow S, BonDurant R, Farley J, et al. Efficacy of ipronidazole against trichomoniasis in beef bulls. Journal of the American Veterinary Medical Association. 1985;187(4):405-7.

100. BonDurant R, Gajadhar A, Campero C, et al. Preliminary characterization of a *Tritrichomonas foetus*-like protozoan isolated from preputial smegma of virgin bulls. Bovine Practitioner. 1999;33(2):124-7.

101. Taylor M, Marshall R, Stack M. Morphological differentiation of *Tritrichomonas foetus* from other protozoa of the bovine reproductive tract. British Veterinary Journal. 1994;150(1):73-80.

102. Castella J, Munoz E, Ferrer D, et al.. Isolation of the trichomonad *Tetratrichomonas buttreyi* (Hibler et al., 1960) Honigberg, 1963 in bovine diarrhoeic faeces. Veterinary Parasitology. 1997;70(1-3):41-5.

103. Levine ND. Protozoan parasites of domestic animals and of man. In: Levine ND, editor. Protozoan Parasites of Domestic Animals and of Man. Minneapolis, MN: Burgess Pub Co.; 1961.

104. Morgan B, Noland L. Laboratory methods for differentiating *Trichomonas foetus* from other protozoa in the diagnosis of trichomoniasis in cattle. Journal American Veterinary Medicine Association. 1943;102:11-5.

105. Dufernez F, Walker RL, Noel C, et al. Morphological and molecular identification of non-*Tritrichomonas foetus* trichomonad protozoa from the bovine preputial cavity. The Journal of Eukaryotic Microbiology. 2007;54(2):161-8.

106. Felleisen RS. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology. 1997;115 (Pt 2):111-9.

107. Felleisen RS. Comparative genetic analysis of tritrichomonadid protozoa by the random amplified polymorphic DNA technique. Parasitology research. 1998;84(2):153-6.

108. Felleisen RS, Lambelet N, Bachmann P, et al. Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. Journal of Clinical Microbiology. 1998;36(2):513-9.

109. Ho MS, Conrad PA, Conrad PJ, et al. Detection of bovine trichomoniasis with a specific DNA probe and PCR amplification system. Journal Clinical Microbiology. 1994;32(1):98-104.

110. Olson MV. The human genome project. Proceedings of the National Academy of Sciences. 1993;90(10):4338-44.

111. National Human Genome Research Institute [updated 2015]. Available from: www.genone.gov.

112. Corbeil LB, Woodward W, Ward AC, et al. Bacterial interactions in bovine respiratory and reproductive infections. Journal of Clinical Microbiology. 1985;21(5):803-7.

113. Grahn RA, BonDurant RH, van Hoosear KA, et al. An improved molecular assay for *Tritrichomonas foetus*. Veterinary parasitology. 2005;127(1):33-41.

114. Cobo ER, Favetto PH, Lane VM, et al. Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with *Tritrichomonas foetus*. Theriogenology. 2007;68(6):853-60.

115. Kennedy JA, Pearl D, Tomky L, et al. Pooled polymerase chain reaction to detect *Tritrichomonas foetus* in beef bulls. Journal of veterinary diagnostic investigation

: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 2008;20(1):97-9.

116. Garcia Guerra A, Hill JE, Campbell J, et al. Use of pooled protozoal cultures of preputial scraping samples obtained from bulls for the detection of *Tritrichomonas foetus* by means of a real-time polymerase chain reaction assay. Journal of the American Veterinary Medical Association. 2014;244(3):352-6.

117. Garcia Guerra A, Hill JE, Waldner CL, et al. Sensitivity of a real-time polymerase chain reaction for *Tritrichomonas fetus* in direct individual and pooled preputial samples. Theriogenology. 2013;80(9):1097-103.

118. Thorburn AL. Alfred François Donné, 1801-1878, discoverer of *Trichomonas vaginalis* and of leukaemia. British Journal of Venereal Diseases. 1974;50(5):377.

119. Bellinghieri G, Santoro D, Mallamace A, et al. The discovery of nephrouroameba: was it real or not? American Journal Nephrology. 2002;22(2-3):266-70.

120. Clark DH, Solomons E. An evaluation of routine culture examinations for *Trichomonas vaginalis* and Candida. American Journal of Obstetrics and Gynecology. 1959;78:1314-9.

121. Harp DF, Chowdhury I. Trichomoniasis: evaluation to execution. European Journal of Obstetrics, Gynecology, and Reproductive Biology. 2011;157(1):3-9.

122. Carlton JM, Hirt RP, Silva JC, et al. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis.* Science. 2007;315(5809):207-12.

123. McClelland RS. *Trichomonas vaginalis* infection: can we afford to do nothing? Journal of Infectious Diseases. 2008;197(4):487-9.

124. World Health Organization. Department of Epidemic and Pandemic Alert and Response. Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates. Geneva. 2001.

125. Harp DF, Chowdhury I. Trichomoniasis: evaluation to execution. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2011;157(1):3-9.

126. Wolner-Hanssen P, Krieger JN, Stevens CE, et al. Clinical manifestations of vaginal trichomoniasis. The Journal of the American Medical Association. 1989;261(4):571-6.

127. Schwebke JR, Burgess D. Trichomoniasis. Clinical Microbiology Reviews. 2004;17(4):794-803.

128. Hawkes S, Santhya KG. Diverse realities: sexually transmitted infections and HIV in India. Sexually Transmitted Infections. 2002;78 Suppl 1:i31-9.

129. Lewis DA. Trichomoniasis. Medicine. 2010;38(6):291-3.

130. Hardy P, Nell EE, Spence M, et al. Prevalence of six sexually transmitted disease agents among pregnant inner-city adolescents and pregnancy outcome. The Lancet. 1984;324(8398):333-7.

131. Ryan CM, de Miguel N, Johnson PJ. *Trichomonas vaginalis*: current understanding of host-parasite interactions. Essays in Bochemistry. 2011;51:161-75.

132. Sood S, Kapil A. An update on *Trichomonas vaginalis*. Indian Journal of Sexually Transmitted Diseases and AIDS. 2008;29(1):7.

133. Chapwanya A, Usman AY, Irons PC. Comparative aspects of immunity and vaccination in human and bovine trichomoniasis: a review. Tropical Animal Health and Production. 2016;48(1):1-7.

134. Krieger JN, Jenny C, Verdon M, et al. Clinical manifestations of trichomoniasis in men. Annals of Internal Medicine. 1993;118(11):844-9.

135. Kissinger P. *Trichomonas vaginalis*: a review of epidemiologic, clinical and treatment issues. BMC Infectious Diseases. 2015;15(1):307.

136. Patil MJ, Nagamoti JM, Metgud SC. Diagnosis of *Trichomonas vaginalis* from vaginal specimens by wet mount microscopy, in pouch TV culture system, and PCR. Journal of Global Infectious Diseases. 2012;4(1):22.

137. Nye MB, Schwebke JR, et al. Comparison of APTIMA *Trichomonas vaginalis* transcription-mediated amplification to wet mount microscopy, culture, and polymerase chain reaction for diagnosis of trichomoniasis in men and women. American Journal of Obstetrics and Gynecology. 2009;200(2):188. e1-. e7.

138. Huppert JS, Mortensen JE, Reed JL, et al. Rapid antigen testing compares favorably with transcription-mediated amplification assay for the detection of *Trichomonas vaginalis* in young women. Clinical Infectious Diseases. 2007;45(2):194-8.

139. Hobbs MM, Seña AC. Modern diagnosis of *Trichomonas vaginalis* infection. Sexually Transmitted Infections. 2013;89(6):434-8.

140. Lara-Torre E, Pinkerton JS. Accuracy of detection of *Trichomonas vaginalis* organisms on a liquid-based Papanicolaou smear. American Journal of Obstetrics and Gynecology. 2003;188(2):354-6.

141. Kaydos-Daniels SC, Miller WC, Hoffman I, et al. The use of specimens from various genitourinary sites in men, to detect *Trichomonas vaginalis* infection. Journal of Infectious Diseases. 2004;189(10):1926-31.

142. Campbell L, Woods V, Lloyd T, et al. Evaluation of the OSOM Trichomonas rapid test versus wet preparation examination for detection of *Trichomonas vaginalis* vaginitis in specimens from women with a low prevalence of infection. Journal of Clinical Microbiology. 2008;46(10):3467-9.

143. Andrea SB, Chapin KC. Comparison of Aptima *Trichomonas vaginalis* transcription-mediated amplification assay and BD affirm VPIII for detection of *T. vaginalis* in symptomatic women: performance parameters and epidemiological implications. Journal of Clinical Microbiology. 2011;49(3):866-9.

144. Lawing LF, Hedges SR, Schwebke JR. Detection of trichomonosis in vaginal and urine specimens from women by culture and PCR. Journal of Clinical Microbiology. 2000;38(10):3585-8.

145. Hobbs MM, Kazembe P, Reed AW, et al. *Trichomonas vaginalis* as a cause of urethritis in Malawian men. Sexually Transmitted Diseases. 1999;26(7):381-7.

146. Schwebke JR, Lawing LF. Improved detection by DNA amplification of Trichomonas Vaginalis in males. Journal of Clinical Microbiology. 2002;40(10):3681-3.

147. Kaydos-Daniels SC, Miller WC, Hoffman I, et al. Validation of a urine-based PCR-enzyme-linked immunosorbent assay for use in clinical research settings to detect *Trichomonas vaginalis* in men. Journal of Clinical Microbiology. 2003;41(1):318-23.

148. Kaydos SC, Swygard H, Wise SL, et al. Development and validation of a PCR-based enzyme-linked immunosorbent assay with urine for use in clinical research settings to detect *Trichomonas vaginalis* in women. Journal of Clinical Microbiology. 2002;40(1):89-95.

149. Nathan B, Appiah J, Saunders P, et al. Microscopy outperformed in a comparison of five methods for detecting *Trichomonas vaginalis* in symptomatic women. International journal of STD & AIDS. 2014:0956462414534833.

150. Schmid G, Narcisi E, Mosure D, et al. Prevalence of metronidazole-resistant *Trichomonas vaginalis* in a gynecology clinic. The Journal of Reproductive Medicine. 2001;46(6):545-9.

151. Huff J, Eustis S, Haseman J. Occurrence and relevance of chemically induced benign neoplasms in long-term carcinogenicity studies. Cancer and Metastasis Reviews. 1989;8(1):1-21.

152. Rustia M, Shubik P. Induction of lung tumors and malignant lymphomas in mice by metronidazole. Journal of the National Cancer Institute. 1972;48(3):721-9.

153. Bartlett DE. *Trichomonas foetus* infection and bovine reproduction. Am J Vet Res. 1947;8(29):343-52.

154. Parker S, Campbell J, Gajadhar A. Comparison of the diagnostic sensitivity of a commercially available culture kit and a diagnostic culture test using Diamond's media for diagnosing *Tritrichomonas foetus* in bulls. Journal of Veterinary Diagnostic Investigation. 2003;15(5):460-5.

155. Perez A, Cobo E, Martinez A, et al. Bayesian estimation of *Tritrichomonas foetus* diagnostic test sensitivity and specificity in range beef bulls. Veterinary Parasitology. 2006;142(1-2):159-62.

156. BonDurant RH, Campero CM, Anderson ML, et al. Detection of *Tritrichomonas foetus* by polymerase chain reaction in cultured isolates, cervicovaginal mucus, and formalin-fixed tissues from infected heifers and fetuses. Journal of Veterinary Diagnostic Investigation. 2003;15(6):579-84.

157. Chenoweth P, Hopkins FM, Spitzer J, et al. Guidelines for using the bull breeding soundness evaluation form. Clinical Theriogenology. 2010;2(1):43-50.

158. Sena AC, Miller WC, Hobbs MM, et al. *Trichomonas vaginalis* infection in male sexual partners: implications for diagnosis, treatment, and prevention. Clinical Infectious Diseases. 2007;44(1):13-22.

159. Scheit K, Reddy E, Bhargava P. Seminal plasmin is a potent inhibitor of E. coli RNA polymerase in vitro. Nature. 1979; 279: 728-731.

APPENDICES

APPENDIX A:

The preset program function illustrating the incremental voltage increases of the Lane Pulsator IV Electroejaculator (Lane Manufacturing, Denver, CO. USA).

Step	RMS Voltage
1	1.85
2	3.87
3	4.91
4	7.76
5	9.97
6	12
7	14.2
8	16.5
9	18.7

APPENDIX B: *T. foetus* PCR Protocol

Reagent*	Amount/Sample (µl)	Total Amount (µl)
10 X PCR Buffer	5	80
50 mM MgCl	2	32
1:10 Primer TF3	2	32
1:10 Primer TF4	2	32
25 mM dNTP	0.4	6.4
PCR H_20	28.5	456
Taq Polymerase	0.25	4
BSA	0.2	3.2

*Master Mix

APPENDIX C: Stock Solutions, Base Media and Diamonds Media Preparation

Stock Solutions Required for Preparations for Diamond's Media

1.	Stock Solution	(in 100ml dH2O)
2.	K_2HPO_4	10 g
3.	KH_2PO_4	6 g
4.	NaCl	20 g
5.	Bovine Bile	7.5 g
6.	Cysteine HCL	20 g
7.	Ascorbic Acid	2 g
8.	$Fe(NH_4)$ Citrate (keep in dark)	2.2g
9.	20% Glucose (Autoclave)	20 g
10.	20% Maltose (Autoclave)	20 g

ALL STOCK SOLUTIONS MUST BE FILTER STERILIZED BEFORE ADDING TO MEDIA EXCEPT (K_2HPO_4/KH_2PO_4/NaCl) and 20% Sugars

Base Media Used for Preparation of Diamonds Media

1.	To a 500 ml bottle add:	
2.	K_2HPO_4/KH_2PO_4/NaCl	5.0 mLs
3.	Trypticase Peptone	10 g
4.	Yeast extract	5.0 g
5.	NaOH pellets	2.0 g
6.	Adjust pH to 7.0	
7.	q.s.	415 mLs
8.	Autoclave and label base media	
9.	Can store at room temperature	

Diamonds Media*

1.	Base Media	415 mL (prepared as outlined above)
2.	Sterile Cysteine	2.5 mL
3.	Sterile Ascorbic Acid	5.0 mL
4.	Sterile 20% Maltose	25 mL
5.	Sterile dH_2O	5.5 mL
6.	Fetal Bovine Serum	50 mL
7.	Pen/Strep	5.0 mL
8.	Store at 4˚C	

* Diamond's Media should be prepared immediately prior to utilization.

The Detection of Tritrichomonas foetus in Bovine Semen with Centrifugation and PCR

C. Armstrong[1], D. Wolfe[1], T. Passler[1], M. Edmondson[1], K. Joiner[1], R. Carson[1], S. Rodning[2]

[1]College of Veterinary Medicine Auburn University, AL 36849 USA

[2]Department of Animal Sciences, College of Agriculture, Auburn University, AL 36849 USA

INTRODUCTION

Bovine trichomoniasis caused by Tritrichomonas foetus, is a true venereal disease of cattle that is spread only through coitus. The objective of the study was to determine if a T. foetus infection could be detected in pre-seminal fluid and a seminal sample using polymerase chain reaction (PCR) collected from known positive bulls. Diagnostics have recently improved with the emergence of a more sensitive and specific PCR techniques. This is an improvement from traditional culture methods, but better diagnostics and collection methods are still needed given the serious consequences of inaccurate diagnosis.

METHODS

Mature beef bulls (n=20) of various breeds from several south Florida ranches that were previously diagnosed to be positive for T. foetus by routine culture and real-time PCR on preputial smegma at an external state diagnostic laboratory were used for this study. These bulls underwent routine electroejaculation, and a dry preputial scraping sample was collected from each bull using a 52.5 cm infusion pipette with a flex adaptor and a 20 ml syringe. The samples collected from urethral emissions were fractionated into a pre-seminal sample and seminal sample based on gross appearance. The bulls all achieved an erection and extended completely outside of the sheath. The preputial sample was immediately suspended in 2 ml of Trypticase- Yeast Extract- Maltose Medium (TYM) with agar (Diamond's media). The samples were transported to the laboratory in a commercial incubator at 37° C. Samples from each bull were centrifuged at 4000g for 9 minutes at room temperature and the resulting pellet was used for DNA isolation prior to processing for conventional PCR.

Bull	Pre-seminal	Seminal	Preputial Scrape
1	-	-	+
2	-	-	+
3	-	-	-
4	-	-	+
5	-	-	+
6	+	-	+
7	-	-	-
8	+	-	+
9	-	-	-
10	-	-	+
11	+	-	-
12	-	-	-
13	-	-	+
14	+	-	+
15	+	-	+
16	-	-	+
17	-	-	-
18	-	-	+
19	-	-	-
20	-	-	+

Preputial Scrape Sample Collection

Results from 5 bulls of 20 total that were positive on Pre-ejaculate samples from several South Florida Ranches

Conventional PCR Results

RESULTS

Overall, 13 of 20 bulls were positive by traditional preputial scraping resulting in a test sensitivity of 0.65 (95% CI: 0.41-0.84). Four of these 13 positive bulls were also positive on the pre-seminal sample. One bull tested positive on the pre-seminal sample, but was negative on preputial scraping. The test sensitivity and specificity for detection of T. foetus by conventional PCR in pre-seminal fluid was 0.30 (95% CI: 0.1-0.61) and, respectively. None of the semen samples were found to be positive for T. foetus. The combined sensitivity for the preputial scrape and pre-seminal fluid in this study was [1 - (1- 0.308) x (1- 0.65) = .758]

CONCLUSIONS

It is common for practitioners to only have an opportunity to test a battery of bulls once because of both logistical and financial constraints. The results of this study suggest that the diagnosis of T. foetus in infected bulls can be improved by additionally testing a pre-seminal fluid sample during routine collection of a preputial scrape.

Funding for this project was provided by the Theriogenology Foundation.

theriogenology foundation
The Future of Animal Reproduction