Evaluation of the accessory sex glands of the bull for the presence of *Tritrichomonas foetus*

by

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Abstract

A bull infected with the venereal protozoan *Tritrichomonas foetus* can become a chronic carrier, remaining infected for life. It has been theorized that as a bull ages he develops deep folds or crypts in the epithelium covering the penis and prepuce, creating an environment in which the parasite can establish long-term residence. No evidence-based reports have been found to support this assumption. *Trichomonas vaginalis,* the venereal protozoan of humans, will infect men and can establish a similar chronic carrier state following ascension of the urethra and infection of the prostate. This study was designed to determine if *T. foetus* could act similarly to *T. vaginalis* and ascend the urethra and infect the accessory sex glands of the bull.

Twenty two bulls naturally infected with *T. foetus* were followed to an abattoir to harvest tissues from the full complement of accessory sex glands. Each gland was identified and two tissue samples from each gland were placed in Diamond's media. One tissue sample remained in media and was observed daily according to routine *T. foetus* culture methods. Tissue cultures were observed over four days yielding no obvious parasite growth or motility. The second tissue sample from each organ was portioned and macerated to prepare for DNA extraction. PCR was performed using gel electrophoresis. A single prostate sample was identified as positive using this PCR protocol. Using comparative DNA sequencing, the positive sample was verified to be *T. foetus.*

Although further research must be conducted to validate results from this study, it appears that *T. foetus* may behave similarly to *T. vaginalis* in ascension of the urogenital tract and invasion of the prostate of its male host.

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Chapter 1

Introduction

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular flagellated protozoan *Tritrichomonas foetus* which is considered an obligate parasite of the female reproductive tract and the epidermal surface of the bull penis and prepuce (Peter, 1997). Infected bulls are asymptomatic carriers of *T. foetus* and efficiently transmit the organism to a female during coitus (Parsonson *et al.*, 1974). Infected females may experience vaginitis, cervicitis, and endometritis which may result in embryonic death, abortion, fetal maceration, infertility, or pyometra (Clark *et al.*, 1983b; Fitzgerald, 1986; Mickelsen *et al.*, 1986; Parsonson *et al.*, 1976). Decreased reproductive efficiency makes trichomoniasis an extremely costly disease for the cattle industry.

It has been commonly accepted that young bulls exposed to this organism may be able to resist infection or experience only a transient infection, while older bulls with similar sexual exposure usually become chronically infected (Roberts, 1986). It is theorized that as bulls age they develop deep folds or crypts in the epithelial covering of the penis and prepuce, creating an environment in which the parasite can establish long term residence (Peter, 1997), but no published reports have been found to support this assumption. Literature suggests that *T. foetus* may colonize the distal urethra of bulls (Parsonson *et al.*, 1974), but infection of anatomical

locations other than the surface of the bull penis or prepuce has not been thoroughly investigated with modern diagnostics.

Trichomonas vaginalis is a sexually transmitted protozoan of humans, with a life cycle and disease pathophysiology similar to that of *T. foetus* in cattle. *T. vaginalis* commonly resides in the male urethra but is also known to infect the prostate of infected men (Gardner *et al.*, 1986). Infection of this accessory sex gland appears to be important for transmission of disease and maintenance of chronic infections in men (Gardner *et al.*, 1986).

This project used contemporary culture techniques and a *T. foetus* specific polymerase chain reaction assay to evaluate the bulbourethral glands, prostate, vesicular glands, and ampullae of naturally infected bulls for the presence of *T. foetus*. The presence or absence of *T. foetus* in the accessory sex glands is important to understanding the pathophysiology of chronic infection in bulls and could have implications in future treatment protocols or prevention strategies.

Chapter 2

Literature Review

I. Trichomoniasis in Cattle

History

The venereal protozoan *Tritrichomonas foetus* was first described in France in 1888 (Morgan, 1944a). Unfortunately, the discovery of this organism closely coincided with the discovery of brucellosis in 1897 and little interest was given to *T. foetus* until the 1920's when research resumed in Germany (Roberts, 1986). In 1932, Emmerson described the first reported case of bovine trichomoniasis in the United States (U. S.) in the state of Pennsylvania (Emmerson, 1932). Since that time, trichomoniasis has been reported throughout the U. S. and around the world. Recently, recognition of the economic losses associated with the introduction of venereal trichomoniasis into cow herds has resulted in the adoption of regulations governing interstate, and often intrastate, movement of bulls for the majority of the continental U. S.

Morphology and Life Cycle

Tritrichomonas means "three-haired single-celled protozoan", which accurately depicts some of the morphological characteristics used to distinguish this organism from other protozoa as described by Taylor and colleagues (Taylor *et al.*, 1994). *Tritrichomonas foetus* has a single nucleus and four flagella. The trophozoite form is pear-shaped with a rounded anterior end and a

pointed posterior end. Three flagella are located on the anterior end, while the fourth trails backward as an extension of the undulating membrane (Roberts, 1986). The undulating membrane is located on one side of the organism and has three to five waves, giving the organism a characteristic vibrating movement. Its size can vary from 10 to 25 μm in length and 5 to 10 μm in width. Trophozoites undergo asexual reproduction by a special type of closed mitosis called cryptopleuromitosis where the nuclear envelope persists and the mitotic spindle is extranuclear. The eventual division produces two identical daughter cells (Pereira-Neves & Benchimol, 2009).

Trichomonads can exist as trophozoites, cysts, and pseudocysts depending on the individual species (Pereira-Neves *et al.*, 2003). The term cyst describes a life form created by an invagination of external organelles to form a compact, round, non-motile life stage in which changes in the exterior integrity of the cell plasma membrane create a protective barrier. *T. foetus* does not exhibit a cystic life form. The pseudocyst form develops following an invagination of external organelles that does not change the composition of the plasma membrane, thus the "pseudo" designation. Invaginated forms have often been considered to be degenerative life forms induced as a response to a stressful environment. However, *T. foetus* pseudocysts can revert to trophozoite form when favorable conditions return (Granger *et al.*, 2000).

T. foetus pseudocysts undergo asexual reproduction by a budding or "shizongony-like" division, a process in which numerous daughter trophozoites are formed within the pseudocyst. The polymastigont pseudocyst eventually releases each newly formed daughter cell and can return to trophozoite form (Pereira-Neves & Benchimol, 2009). The exact role of the pseudocyst

form for *T. foetus* remains unclear, but the increased fecundity of pseudocysts compared to trophozoites suggests that pseudocyst formation may be a part of the normal life cycle. A recent report indicates the pseudocyst form actually appears more commonly than the trophozoite stage in preputial secretions from naturally infected bulls (Pereira-Neves *et al.*, 2011).

Disease Pathogenesis in the Bull

Tritrichomonas foetus is an obligate, extracellular parasite of the bovine reproductive tract. (Peter, 1997). The preputial cavity of the bull provides an ideal microaerophilic environment where it localizes in the smegma or secretions of the epithelial lining of the bull penis and prepuce and may enter the external urethral orifice (Parsonson *et al.*, 1974; Peter, 1997). The organism does not invade the epithelium nor typically invoke an effective immune response in the bull (Peter, 1997). *T. foetus* infection results in no penile or preputial pathology and does not affect semen quality or libido (Morgan, 1944b; Peter, 1997). Therefore, an infected bull acts only as an asymptomatic carrier. Disease transmission occurs when an infected bull breeds a non-infected cow, or a non-infected bull breeds an infected female.

The period for which a bull remains infected is a subject of great interest. Transient infections which spontaneously clear without treatment and a chronic carrier state in which permanent infection results are the two commonly accepted outcomes following infection of the bull. *T. foetus* infections in bulls less than 3-4 years of age are purportedly more likely to be transient. Younger bulls reportedly only transmit *T. foetus* if sexual contact with a non-infected cow occurs within minutes to days following breeding of an infected female (Peter, 1997). Some studies indicate that clearance in a young bull is possible within minutes after breeding an infected female (Clark *et al.*, 1977; Morgan, 1944b). Therefore, transmission of *T. foetus* by a

young transiently infected bull has been described as a passive, mechanical transmission, compared to transmission associated with a chronically infected older bull. However, a 1985 report indicated that 21.7% of the 3 year old breeding bulls in a natural service herd were infected with *T. foetus* for at least 2 months after the last exposure to females (Skirrow *et al.*, 1985). BonDurant and associates found 2% of bulls less than or equal to 3 years of age to be positive for *T. foetus* when surveying 57 California beef herds (BonDurant *et al.*, 1990). Bulls were withheld from females for 2 weeks or more prior to sampling, convincing the authors that these bulls were truly infected and not just mechanical vectors of the organism. Therefore, any bull exposed to *T. foetus* in a natural breeding situation should be considered capable of becoming chronically infected, regardless of age.

Long term infection and the carrier state has often been related to the depth of the epithelial crypts found on the penile and preputial surfaces. Traditional thought is that older bulls have deeper epithelial crypts which provide the appropriate microaerophilic environment required for establishment of chronic infections (Anderson *et al.*, 1994; BonDurant & Honigberg, 1994; Honigberg, 1978; Peter, 1997). However, a recent study of the histological architecture of the penile and preputial epidermal surface of the bull fails to support this assumption. This study argues against the existence of structures that could be characterized as crypts in the penile and preputial epithelium and suggests that there is no difference in the depth or distribution of microscopic epithelial folds between young and older bulls (Strickland, 2010).

Another theory contributing to chronic infections involves ascending infections of the protozoan to other locations within the bull urogenital tract. Feiling reported finding trichomonads in the ejaculate of bulls with chronic infections (Feiling, 1935 as referenced in

Hammond and Bartlett, 1943). Kust suggested that organisms in these bulls must be present in the urinary apparatus from which they would be carried out with the ejaculate (Kust, 1935, as referenced in Hammond and Bartlett, 1943) and later reported finding trichomonads sporadically in the urethra (Kust, 1936, as referenced in Hammond and Bartlett, 1943). Andrews and Miller found trichomonads in pre-ejaculatory secretions obtained from rectal massage of the ampullae and vesicular glands of infected bulls (Andrews & Miller, 1938). Futamura reported finding the organism in the ampullae of an infected bull (Futamura, 1935). Karlson and Boyd found *T. foetus* in a vesicular gland of a bull determined to have bilateral orchitis and seminal vesiculitis (Karlson & Boyd, 1941). Cameron and colleagues identified an organism they believed to be *T. foetus* in the epididymis of an infected bull (Cameron *et al.*, 1933). In 1943, Hammond and Bartlett concluded that retrograde *T. foetus* infections of the ampullae, seminal vesicles and epididymides only occurs in unusual cases (Hammond & Bartlett, 1943).

Using direct microscopic observation, Albein was unable to demonstrate trichomonads in any of the internal reproductive organs of several bulls known to transmit trichomoniasis to cows (Albein, 1932 as referenced in Hammond and Bartlett, 1946) and later concluded that trichomonads occur only in the preputial cavity of bulls but may colonize the urethra near the distal orifice for short periods of time (Albein, 1941 as referenced in Hammond and Bartlett, 1943). Parsonson and associates reported that *T. foetus* would grow vigorously in a mixture of bull urine and seminal vesicle fluid (Parsonson *et al.*, 1974). However, the same investigators failed to culture *T. foetus* from samples taken from the epididymides, ampullae, seminal vesicles, pelvic urethra, and testes of fifteen naturally infected bulls (Parsonson *et al.*, 1974). Rhyan and colleagues failed to demonstrate the presence of *T. foetus* when examining the epididymides, prostate and seminal vesicles of bulls using immunohistochemical staining (Rhyan *et al.*, 1999).

Several of the reportedly infected bulls failed to test positive on routine preputial cultures at time of slaughter and the age of each bull used in this study was not reported.

Regardless, the chronic carrier state associated with *T. foetus* infections in bulls rarely clears and details regarding the pathophysiology and maintenance of chronic infection in the mature bull remain unknown.

Diagnostic Technique

Diagnosis of trichomoniasis in the bull has historically relied on microscopic identification of organisms in preputial smegma. Testing of cows is usually limited to circumstances when post-coital vaginal discharge or pyometra are present and in such cases vaginal or uterine samples are processed utilizing methods similar to those for preputial samples. Identification of *T. foetus* is performed either via direct examination of preputial smegma or after incubation of diagnostic specimens in selective culture media. The presence of fecal trichomonads may confound microscopic evaluation of specimens and yield false positive culture results. Therefore, research has focused on the development of molecular-based confirmatory assays to help eliminate false positive culture results (BonDurant *et al.*, 1999; Cobo *et al.*, 2003).

Specimen collection in the bull involves retrieving smegma and secretions from the preputial cavity and transporting the sample to a laboratory for positive identification of organisms. Sampling techniques for obtaining diagnostic specimens include: 1) a swab technique (Morgan, 1946); 2) a dry pipette technique (Peter, 1997; Schönmann *et al.*, 1994); 3) a wet pipette technique (Kimsey, 1986); and 4) the douche technique (Kimsey, 1986). Each

technique can be performed alone or in combination. Fitzgerald and associates compared the swab and pipette techniques and reported that the number of parasites recovered via the swab technique is only 20 % of the number of parasites recovered via pipette scraping (Fitzgerald *et al.*, 1952). The dry pipette technique is one of the most common sampling methods in the U. S. while the douche method is the preferred technique in Europe. The two methods are not statistically different (Schönmann *et al.*, 1994).

Regardless of technique used, 1-2 weeks of sexual rest prior to testing is generally recommended (Peter, 1997). Coitus can mechanically remove many of the organisms from the bull's penis and prepuce and sexual rest will allow time for organismal replication and increase the chance of recovery during sampling. Samples may then be prepared for direct microscopic evaluation, culture or molecular-based evaluation.

Direct microscopic examination of specimens for *Tritrichomonas foetus* can be diagnostic, but *in vitro* culture of preputial smegma in a selective nutrient medium for up to one week is a more sensitive method for the detection of *T. foetus* (Johnson, 1965; Skirrow & BonDurant, 1988; Tedesco *et al.*, 1979). Diamond's media (BonDurant, 1997; Diamond, 1957)or the commercially available InPouch™ TF (BioMed Diagnostics; White City, OR) are currently the most common media used to culture *T. foetus* in the United States. *In vitro* culture allows proliferation of *T. foetus* to more readily detectable numbers. Cultures are typically observed once daily for up to one week. Cultures in which trichomonads are not observed are considered negative. A negative culture has one of four meanings: (1) the bull is not infected with *T. foetus* at the time of sample collection, (2) the bull was positive but no organisms were recovered during sample collection, (3) the bull was positive, organisms were collected to

inoculate media, but were not properly identified during microscopic examination, or (4) the bull was positive, organisms were collected to inoculate media, but failed to replicate and were not properly identified during microscopic examination. All cultures containing organisms resembling *T. foetus* should be confirmed with appropriate molecular-based assays to avoid false-positive results due to fecal trichomonad contamination of culture media (BonDurant *et al.*, 1999; Campero *et al.*, 2003; Cobo *et al.*, 2003).

Alternatively, samples of smegma may be submitted directly for molecular-based evaluation. At present, polymerase chain reaction (PCR) is the test of choice for molecular based assays. Improved methods (Felleisen *et al.*, 1998; Ho *et al.*, 1994; McMillen & Lew, 2006; Riley *et al.*, 1995) and development of optimized primers (Fernandes *et al.*, 2008; Grahn *et al.*, 2005) have allowed PCR to become an important diagnostic tool for trichomoniasis. In contrast to *in vitro* culture, PCR can be performed in a matter of hours and may offer a more rapid test result. Another potential advantage PCR offers is that recovered organisms are not required to be viable for detection. A negative PCR has one of three meanings: (1) the sample is negative for the presence of *T. foetus*, (2) the sample is *T. foetus* negative and the PCR amplification failed, or (3) the sample is *T. foetus* positive but reported as negative because of a failed PCR amplification (Grahn *et al.*, 2005). False negatives can also occur when small numbers of organisms are present or preputial inhibitors present in the sample prevent detection of the organisms (Parker *et al.*, 2001). False positives are not as commonly reported with the use *T. foetus* specific primers (Fernandes *et al.*, 2008; Grahn *et al.*, 2005).

A single sample evaluated by culture or PCR may yield inconclusive results, as false negatives are reported with either method. Sensitivity and specificity of both culture and PCR

are maximized with sequential testing of samples, collected at weekly intervals and repeating the same test (BonDurant, 1985). Positive bulls are more accurately identified when samples are tested in parallel or combination, performing cultures and PCR on a single sample (Cobo *et al.*, 2007). A current recommendation is to submit three samples at one week intervals for parallel testing, maximizing sensitivity and specificity. A sexually active bull should not be considered free of trichomoniasis until three negative test results have been achieved (Anonymous, 2006; BonDurant, 1985).

	Result	Sensitivity (in series)
First test	Negative	80 %
Second test (one week later)	Negative	96 %
Third test (one week later)	Negative	99 %

Illustration 1: Sensitivity of *Tritrichomonas foetus* cultures (Anonymous, 2006)

Disease Management

Historically, the most successful treatment for bulls with trichomoniasis utilized systemic administration of nitroimidazole derivatives (Kimsey *et al.*, 1980; Skirrow *et al.*, 1985). However, the use of nitroimidazole derivatives is now illegal in food-producing animals in the U.S., and no effective alternatives are available (BonDurant, 1997). With no legal form of therapy available, disease intervention must be aimed at prevention and control (Peter, 1997).

Because trichomoniasis is a venereal disease, utilization of artificial insemination can be used to prevent infection and completely eliminate this disease from a herd. Preventing introduction of trichomoniasis into a herd requires intense management practices that involve implementing proper biosecurity such as buying animals from disease free herds, avoiding areas of common grazing for cattle, avoiding the practice of leasing bulls, maintaining quality perimeter fencing, and screening all new bull purchases. Routine screening of the bull battery, using younger bulls as often as possible, and culling open cows and positive bulls are also effective strategies for reduction of disease (BonDurant, 1997; Peter, 1997).

Vaccination against *T. foetus* has been reported to be effective in the cow (Anderson *et al.*, 1996; Herr *et al.*, 1991; Kvasnicka *et al.*, 1989). In the bull, most reports state that vaccination is of limited to no value (Bondurant, 2005; Herr *et al.*, 1991; Soto & Parma, 1989), but some reports indicate that vaccination against *T. foetus* may be protective (Clark *et al.*, 1983a; Clark *et al.*, 1984). Cobo and colleagues reported that immunoglobulins IgG_1, IgG_2, IgA, and IgE directed against *T. foetus* appeared in the preputial secretions of bulls vaccinated with *T. foetus* antigens. Immunoglobulins IgG_1 and IgG_2 directed against *T. foetus* were elevated in the serum and vaccinated bulls demonstrated resistance to *T. foetus* colonization (Cobo *et al.*, 2009).

With no legal form of treatment and limited available data supporting immunization of the bull, complete understanding of the pathophysiology of infection is critical for future development of treatment protocols and prevention strategies.

II. Trichomoniasis in Humans

History

The human venereal protozoan *Trichomonas vaginalis* was discovered in 1836 by the Frenchman Alfred Francois Donne.(Thornburn, 1974) He originally named the organism "Trico-monas" for its similarities with two other organisms known at the time as "Tricodes" and "Monas". Ehrenberg, a Professor of Protozoology in Berlin, later gave the organism the name which we know it by today. Ehrenberg gave full credit to Donne for discovery, but validated that the human vagina was the normal environment, thus "vaginalis" was added to the nomenclature. Although Donne discovered *T. vaginialis*, he did not necessarily understand the pathological significance of the organism because most patients were asymptomatic (Thornburn, 1974). In 1916, Hohne was the first to use the term "trichomoniasis" to describe the clinical condition caused by *T.* vaginalis (Harp & Chowdhury, 2011). The development of culture media in the 1940's allowed for more detailed study of the organism and its pathogenicity (Thomason & Gelbart, 1989).

Today, the World Health Organization estimates that human trichomoniasis accounts for approximately half of the world's cases of sexually transmitted diseases. Worldwide 160-180 million people are affected annually by trichomoniasis.(Harp & Chowdhury, 2011) Human trichomoniasis is considered a predisposing factor for contracting and transmitting Human Immunodeficiency Virus.(Schwebke & Burgess, 2004)

Morphology and Life Cycle

Trichomonas vaginalis is a flagellated protozoan that averages 10 μm in length and 7 μm in width but varies in shape (Honiberg & King, 1964). This organism has a typical pyriform shape when present in pure culture, although an amoeboid shape is often seen when attached to vaginal epithelial cells (Heath, 1981). Four flagella are present on the anterior end and the fifth flagellum exists on the posterior end as an extension of the undulating membrane. The undulating membrane, which is present along one side of the organism, and flagellum give the organism its distinct quivering motility (Honiberg & King, 1964). This motile life form is termed a trophozoite and undergoes asexual reproduction by cryptopleuromitosis (Pereira-Neves & Benchimol, 2009).

Although *T. vaginalis* is one of the most extensively researched trichomonads, little is understood about the organism's life cycle. Similar to *Tritrichomonas foetus*, a pseudocyst life form has been observed (Pereira-Neves & Benchimol, 2009). This life form was originally thought to be a degenerative life form but asexual reproduction by a budding process (Abonyi, 1995) or closed mitosis "shizongony-like" division (Pereira-Neves & Benchimol, 2009) has been demonstrated. It is not clear what role this life form may play in disease pathogenesis. *T. vaginalis* does not display a cystic life form.

Disease Pathogenesis in Men

Humans are the only natural host of *Trichomonas vaginalis.* The parasitic protozoan is described as extracellular and anaerobic, making the female vagina an ideal environment (Harp & Chowdhury, 2011). Pathogenesis in men has not been investigated as thoroughly as in women

but is known to cause urethritis, balanoposthitis, prostatitis, cystitis, and epidiymo-orchichitis and increases the risk of HIV transmission (Harp & Chowdhury, 2011). Krieger and associates reported that urethritis was present in half of the men with trichomoniasis as the sole urethral pathogen (Krieger *et al.*, 1993). However, 70% of men diagnosed with trichomoniasis are asymptomatic (Sood & Kapil, 2008). Trichomoniasis may negatively impact semen quality in some infected men (Gopalkrishnan *et al.*, 1990; Lloyd *et al.*, 2003). Venereal transmission is believed to be the only route of transmission.

T. vaginalis has been identified in semen, urethral discharge, urine, and prostatic secretions of infected men (Andreeva *et al.*, 1981) and women commonly treated for trichomoniasis were often re-infected upon returning to sexual activity with the same partner. For decades these observations led many to speculate that the prostate gland of men served as a reservoir for *T. vaginalis*. In 1986, Gardner and colleagues were the first to report positive identification of *T. vaginalis* within the prostate gland (Gardner *et al.*, 1986). Using an immunoperoxidase procedure, the organism was identified in the prostatic urethra, glandular lumina, submucosa, and stroma. Infection of the prostate gland is a result of the motile organism's ascension and exiting of the urethra. They suggested that higher portions of the urogenital tract, such as the kidneys, ureters, and seminal vesicles, should be investigated for the presence of *T. vaginalis*. At this time, trichomoniasis is believed to also play a role in benign prostatic hyperplasia (Mitteregger *et al.*, 2012) and suspected to serve as an etiology for prostatic neoplasia (Chen *et al.*, 2013; Stark *et al.*, 2009; Sutcliffe, 2010; Sutcliffe *et al.*, 2006).

It has been hypothesized that the oxidative nature of the male reproductive tract may decrease certain pathogenic factors of the protozoan (Alderete & Provenzano, 1997). Zinc,

usually present in high concentrations in prostatic fluid of men, was demonstrated to be cytotoxic to certain strains of *T. vaginalis* (Krieger & Rein, 1982). The vagina, however, exhibits a reduced environment which may allow for increased activation of pathogenic mechanisms (Alderete & Provenzano, 1997). These differences may explain why women tend to amount a stronger immune response and the majority of men remain asymptomatic when infected with *T. vaginalis*.

Diagnostic Technique

Generalized symptoms of human trichomoniasis prevent diagnosis based on pathognomonic signs. Available diagnostic tests range from old to new and affordable to expensive, with varying reliability and accuracy across the testing spectrum. Thus, convenient, affordable, reliable, and accurate tests play a prominent role in disease diagnosis. Despite modern diagnostics, diagnosis of trichomoniasis in men is still considered difficult and a major impediment to the control of *T. vaginalis.* For the purpose of this review, discussed diagnostic techniques will be limited to those relevant for diagnosis of infection in men.

Historically, diagnosis of trichomoniasis in men was limited to direct observation of the organism in urine, manually expressed prostatic secretions, or semen (Trussell, 1947). Samples must be collected and observed immediately for optimal results and require high organismal burden for appropriate diagnosis. A variety of staining techniques were utilized by some, but direct visualization and wet mounts were more sensitive and specific. In the 1940's samples were more commonly submitted for culture, using placenta agar and Powell's medium, but this technique was usually limited to patients who tested negative under direct observation and was most often utilized during organismal research (Trussell, 1947).

Improvements in culture media over the years led culture to be considered the "gold standard" (Garber, 2005; Huppert, 2012). Yeast-tripticase-maltose based media were created by Sprince and Kupferberg in the late 1940's (Trussell, 1947) and modifications gave way to the preferred Diamond's medium. Today, a modified Diamond's medium is commercially available as the InPouch™ TV (BioMed Diagnostics; White City, OR), a two chambered plastic container designed specifically to culture *T. vaginalis*. A major limitation to culture technique is the turnaround time of approximately one week for results (Trussell, 1947). The preferred sample for inoculation is a urethral swab, collected before the patient voids urine for the first time after sleeping (Garber, 2005). Compared to single sample submissions, sensitivity of *T. vaginalis* cultures increases when multiple cultures are performed on a single sample. Culture sensitivity is further enhanced by combining two samples from a single patient and performing a single culture (Huppert, 2012). Others report that a urethral swab in combination with urine sedimentation offers the best diagnostic sample when culturing men (Krieger *et al.*, 1993).

Currently, polymerase chain reaction testing (PCR) is a popular diagnostic tool. Schwebke and Lawing report that PCR is a far superior technique compared to culture for routine urethral swab and urine sedimentation samples (Schwebke & Lawing, 2002). Kaydos-Daniels and associates validated a technique for PCR of free catch urine samples (Kaydos-Daniels *et al.*, 2003). The authors demonstrated that PCR testing of urine samples of infected men offered higher sensitivity and specificity than cultures of urethral swabs and urine sedimentation. They also add that the incidence of detection could increase using this technique because more men may be willing to submit urine samples than be subjected to the discomfort of urethral swabs utilized in sample retrieval for culture. Semen samples are screened with PCR for

the presence of *T. vaginalis* and other pathogens known to cause sexually transmitted infections. This process is limited to certain laboratories which receive and handle samples from semen donors used in assisted conception techniques; no numerical data is available for the accuracy of these test results (Peeling & Embree, 2005).

The nucleic acid amplification test (NAAT) is also listed as a technique to diagnose trichomoniasis in men. The test is commercially available but not yet approved by the U. S. Food and Drug Administration. This test would offer rapid, in-house results with the highest sensitivity, but NAAT is currently limited to research and not used in a clinical setting (Huppert, 2012).

Disease Management

Treatment of trichomoniasis was simply based on trial and error (Trussell, 1947) until the development of nitroimidazoles in the 1960's (Harp & Chowdhury, 2011). Many therapies were created and used to treat infected women, most of which were topical and produced inconsistent results (Trussell, 1947). Little historical information is available for the treatment of men. The use of systemic nitroimidazoles eliminated the need for topical therapy.

Metronidazole is the drug of choice for treatment of trichomoniasis. Women are administered a single 2g oral dose, and the cure rate is 97% (Lossick, 1980). Other dosing regimens are available, but the single dose offers an equivalent cure rate, better compliance, and fewer side effects. Men are commonly treated with the single dose regimen, but because older studies reported a 40% failure rate with single dose therapy, men are often treated with a multi-dose regimen (Huppert, 2012). It is an accepted practice for clinicians to diagnose

trichomoniasis in women and prescribe therapeutics for the patient and her sexual partner, again making single dose therapy the most widely used and compliant treatment protocol (Huppert, 2012).

It is estimated that approximately 2.5 to 5% of cases of *T. vaginalis* exhibit some level of resistance to metronidazole (Schmid *et al.*, 2001). In such cases, the dose of metronidazole can be increased and given over several days (Lossick *et al.*, 1986). Some refractory cases may be treated with tinidazole, a nitroimidazole chemically related to metronidazole that was recently licensed for treatment of trichomoniasis in the U. S (Schwebke & Burgess, 2004). Tinidazole offers a reliable alternative to metronidazole because it has twice the plasma half-life, patients exhibit fewer side effects, and clinical efficacy is equal for the treatment of trichomoniasis (Sawyer *et al.*, 1976).

Because *T. vaginalis* is highly transmissible, standard prevention practices for sexually transmitted diseases, such as monogamy and consistent condom use, should be employed (Huppert, 2012). Trichomoniasis is most prevalent in impoverished communities and strategies that increase available healthcare and education could reduce disease transmission. Medical professionals should strongly encourage positive patients to notify and educate all sexual partners. Improved diagnostics and screening guidelines for asymptomatic patients are critical to prevention and control of this global and easily treatable disease (Huppert, 2012).

III. Anatomy and Physiology of the Bull Accessory Sex Glands

The bull has a full complement of accessory sex glands: paired ampullae, paired vesicular glands, a unique prostate, and paired bulbourethral glands (Budras & Habel, 2003; Dyce *et al.*, 2002). Not all mammalian males have the full complement of accessory sex organs, thus each gland can have a different primary role among different species. Male ruminants and the stallion are the only domestic species to have a full complement of accessory sex organs. However, the glands are fully developed only in the bull and gland physiology and anatomy differ between the two species (Budras & Habel, 2003). There is some debate if each of these organs should be referred to as a "gland". According to Mann, each of the accessory sex organs in the bull has glandular tissue and secretory activity, thus accessory sex gland is the proper terminology (Mann, 1964). He also contends that the secretory activity of each accessory sex gland is androgen dependent with peak physiologic activity limited to intact males. The following provides a review of the anatomy and physiology of the accessory sex glands of the bull.

Ampullae

The ductus deferens is the ascending, tubular, soft tissue structure designed to carry spermatozoa from the testicle to the urethra. In the bull, the terminal 10-12 centimeters (cm) of each ductus deferens enlarges to approximately 1 cm in diameter to form the ampullae (Dyce *et al.*, 2002). This enlargement of the ductus deferens is due to dramatic glandular proliferation within the mucosa (Dyce *et al.*, 2002). The ampullae serve as a temporary storage site for spermatozoa, and in normal bulls the number of sperm in the ampullae is sufficient for at least one normal ejaculation after recent castration or vasectomy (Barth, 2007; Wrobel, 1998). The ampullae enter the cranial aspect of the urethra in an area called the colliculus seminalis (Budras

& Habel, 2003), also called the seminal colliculus. Conflicting reports exist as to whether the ampullae enter the colliculus seminalis via a common duct with the vesicular gland (Budras & Habel, 2003; Dyce *et al.*, 2002; Wrobel, 1998) or have their own excretory orifice (Constantinescu, 2007; Kainer *et al.*, 1969; Senger, 2012). One group of authors describe the excretory orifices in detail, adamant that the openings are separate (Bagshaw & Ladds, 1974). They describe the location of the ampullary opening as dorsal, ventral, or intermediate to the opening of the vesicular gland depending on the bull. Furthermore, certain bulls have a mixture of the relationship of the excretory openings of the ampulla and vesicular gland. The excretory orifice of the ampulla and vesicular gland on one side of the body can have a certain configuration, and the openings of the two contralateral glands may have a different configuration.

The lumen of the ampullae are lined by cells that range from tall columnar to cuboidal which have apical, bleb-like protrusions suggestive of secretory activity (Wrobel, 1998). Spherical or polyhedral basal cells are irregularly distributed between the columnar cells. The glandular epithelium is rich in glycogen and both columnar cells and basal cells commonly contain lipid droplets. The submucosa contains simple, branched, tubuloalveolor glands and is rich in smooth muscle cells. The tunica muscularis consists of variably arranged smooth muscle bundles surrounded by the highly vascularized loose connective tissue of the tunica adventitia (Wrobel, 1998).

When separated from spermatozoa, the ampullar secretion is a yellowish-colored fluid containing measurable amounts of ergothioneine and fructose (Mann, 1964). Ergothioneine is a naturally occurring, plant derived amino-acid found in many mammalian cells and tissues, most

abundantly present in erythrocytes, bone marrow, liver, kidney, seminal fluid and the lens and cornea of the eyes (Cheah & Halliwell, 2012). Ergothioneine acts as an antioxidant and cytoprotectant in tissues repeatedly subjected to oxidative stress (Cheah & Halliwell, 2012). Fructose is a reducing and yeast-fermentable sugar which ejaculated spermatozoa utilize as their primary energy source via anaerobic fructolysis (Mann, 1964). The vesicular glands are the main source of fructose, but the ampullae of the bull also produce this sugar in small amounts (Mann, 1964).

Pathology of the ampullae is not common in the bull but certain conditions do exist. Ampullitis, inflammation or infection of the ampullae, is considered an extension of disease associated with primary conditions such as orchitis, epididymitis, or vesiculitis (Roberts, 1986). Thus, ampullitis can occur due to ascension or dissention of infectious organisms within the urogenital tract. Congenital anomalies include segmental aplasia, fusion of an ampulla with the ipsilateral vesicular gland, and paramesonephric cysts or uterus masculinus in the urogenital fold between the ampullae (Bagshaw & Ladds, 1974; Roberts, 1986). Accumulation of senescent sperm in the ampullae can also occur. This condition can be corrected with frequent ejaculations, but is considered a permanent condition because accumulation reoccurs after sexual rest (Barth, 2007).

Vesicular Glands

Vesicular glands were originally named seminal vesicles because the fluid contained within the glands highly resembles that of ejaculated semen and early anatomists imagined that these organs were actual reservoirs for spermatozoa (Senger, 2012). Vesicular glands serve only as a reservoir for their own secretions, not spermatozoa. The paired glands lie lateral to the

ampullae (Dyce *et al.*, 2002) and dorsocranial to the pelvic urethra (Dyce *et al.*, 2002; Senger, 2012). The vesicular glands are the largest accessory sex gland in the bull. They are lobulated and irregular in shape and measure approximately 10-15 cm in length by 3-5 cm in width and depth (Dyce *et al.*, 2002). The glandular surface is uneven (Constantinescu, 2007) and asymmetry often exists between the pair of glands (Dyce *et al.*, 2002). The consistency of the glands vary and tend to become more firm with age (Dyce *et al.*, 2002).

The epithelium of the glands are pseudostratified, composed of tall columnar cells and small, round basal cells (Wrobel, 1998). The secretory columnar cells contain small lipid droplets, glycogen, and occasionally have apical projections. The basal cells often contain large lipid droplets. The submucosa contains highly vascularized loose connective tissue which is continuous with the dense connective tissue trabeculae, subdividing the organ into lobes and lobules. Interlobular septa are derived from the tunica muscularis which surrounds the entire organ (Wrobel, 1998). Intralobular secretory ducts drain the coiled tubular portions of the gland and coalesce to form a main secretory duct that enters the pelvic urethra at the colliculus seminalis (Mann, 1964; Wrobel, 1998). As stated previously, some texts indicate that the vesicular glands enter the urethra separate from the ampullae (Bagshaw & Ladds, 1974; Constantinescu, 2007; Senger, 2012), while others contend that the glands share a common excretory orifice (Budras & Habel, 2003; Dyce *et al.*, 2002; Wrobel, 1998). The tunica serosa or tunica adventitia, a capsule of dense irregular connective tissue with a few smooth muscle cells, covers the entire organ (Wrobel, 1998).

Vesicular gland fluid contributes the largest portion of the seminal plasma in the bull (Dyce *et al.*, 2002; Mann, 1964; Senger, 2012). Normal fluid is slightly yellow in color due to

riboflavins; the higher the concentration of flavins the deeper the color (Mann, 1964). High concentrations of flavins do not affect spermatozoa morphology and motility or fertility of the bull. The vesicular glands produce ascorbic acid, citric acid, uric acid, fructose, phosphorus, and ergothioneine. The primary products can be largely divided into reducing agents and sugars. Fructose is produced in large quantities for spermatozoa metabolism, and this gland serves as the main source of fructose production. Ergothioneine acts as an antioxidant and cytoprotectant. It also serves as a reducing agent along with uric acid, but ascorbic acid is considered the principle reducing agent of bovine seminal plasma. Citric acid is believed to play a role in the coagulation and liquefaction of semen, calcium binding capacity of semen, and maintaining the osmotic equilibrium in semen (Mann, 1964). Vesiculectomy, or removal of the vesicular glands, results in a reduction of semen volume, fructose levels, and citric acid levels (Hess *et al.*, 1960; Shah *et al.*, 1968), and a rise in seminal pH was reported in one study (Shah *et al.*, 1968).

Pathology of the vesicular glands includes congenital anomalies, such as hypoplasia, cysts, aplasia, duplication of the glands, and fusion with the ampullae (Bagshaw & Ladds, 1974; Roberts, 1986). Vesiculitis, however, is the most common condition associated with the vesicular glands of the bull (Roberts, 1986). Vesiculitis, inflammation or infection of the vesicular gland, can result from infectious agents ascending the urogenital tract, agents descending from the upper urinary or reproductive tracts, hematogenous dissemination, or direct invasion from local sources (Dargatz *et al.*, 1987).

Prostate

The prostate of the bull is unique in that it is comprised of two separate areas of organized tissue, the body and the disseminate part (Constantinescu, 2007). The body, or corpus

prostate (Senger, 2012; Wrobel, 1998), contains two lobes of compact tissue which push dorsally through the aponeurosis of the urethralis muscle on the dorsal surface of the most cranial portion of the urethra at the level of the colliculus seminalis (Dyce *et al.*, 2002; Wrobel, 1998). This configuration of tissue forms a 4 cm wide by 1 cm long palpable band of tissue (Dyce *et al.*, 2002) between the vesicular glands (Budras & Habel, 2003). A major duct from each lobe of the prostatic body opens into the urethra immediately caudal to the openings of the ipsilateral ampulla and vesicular gland, and 3 or 4 smaller openings flank each side of the colliculus seminalis (Kainer *et al.*, 1969).

The disseminate portion of the prostate begins caudal to the corpus prostate and extends caudally along the entire length of the pelvic urethra, diminishing as it travels caudally (Dyce *et al.*, 2002). The disseminate prostate is divided into lateral halves by a septum of longitudinal smooth muscle (Kainer *et al.*, 1969). Each lateral half is concentrated dorsal to the urethra but glandular tissue extends lateral and ventral, encircling the pelvic urethra. Openings for this portion of the prostate are roughly present in rows and are 2 to 5 millimeters apart. The rows are located along the dorsal and lateral urethra beginning just caudal to the seminal colliculus and terminate near the dorsal semilunar fold, an anatomical structure in the caudal pelvic urethra which creates the urethral recess of the bull (Kainer *et al.*, 1969).

Although the corpus prostate and disseminate prostate are considered two distinct collections of organized tissue, an indistinct continuity exists when moving caudally from the ventral aspect of the lobes of the body toward the disseminate prostate (Kainer *et al.*, 1969). Histologically, the prostate consists of a varying number of individual tubuloalveolar glands (Wrobel, 1998). The secretory tubules, alveoli, and intraglandular ducts are lined by simple

cuboidal or simple columnar epithelium with occasional basal cells. The simple epithelium changes to stratified columnar or transitional epithelium toward the terminal portions of the ducts. The duct system possesses saccular dilatations in which secretory material can be stored. Secretory portions and ducts of the gland are surrounded by loose connective tissue which contains smooth muscle cells. The prostate is covered by a capsule of dense irregular connective tissue which contains smooth muscle cells. The capsule of the disseminate prostate is covered by the urethralis muscle. The capsule of the corpus prostate is the most superficial layer. Large trabeculae originate from the capsule and separate the corpus and disseminate prostate into individual lobules (Wrobel, 1998).

The prostatic secretions of the bull contribute 4-6% of the total volume of the bull's ejaculate (Wrobel, 1998). Prostatic secretions are part of the colorless fraction that is observed prior to the sperm rich fraction. This fraction has a characteristic viscous nature, provided by a bound amino-sugar in the form of a mucopolysaccharide (Mann, 1964). Prostatic secretions neutralize seminal plasma and initiate active movement of the ejaculated spermatozoa (Wrobel, 1998). Several authors postulated that the pre-sperm fraction are contributions of both the prostate and bulbourethral glands (Kainer *et al.*, 1969; Seidel & Foote, 1970). When considering the contributions of both the prostate and bulbourethral glands, the fluid is termed urethral fluid and is identified by high concentrations of chloride ions (Seidel & Foote, 1970).

Disease of the prostate gland is rare (Roberts, 1986). Bagshaw and colleagues reported finding cystic lesions associated with the corpus prostate of one bull and aberrant tissue protruding into the urethral lumen of another bull that was histologically determined to be prostatic tissue (Bagshaw & Ladds, 1974). Prostatitis has been reported in the bull and is usually

associated with infection by *Brucella abortus* (Roberts, 1986). Melanosis, or aberrant deposition of melanin, has been reported in 4 bulls. Melanin deposits were discovered in the colliculus seminalis near the corpus prostate (Bagshaw & Ladds, 1974).

Bulbourethral Glands

The paired bulbourethral glands are walnut sized organs (Budras & Habel, 2003) that are dorsoventrally flattened or elliptical in shape (Budras & Habel, 2003; Constantinescu, 2007; Dyce *et al.*, 2002). The pair of glands lies close to one another along the midline dorsal to the urethra at the level of the ischial arch (Constantinescu, 2007; Dyce *et al.*, 2002). They are covered by the thick bulbospongiosus muscle (Dyce *et al.*, 2002) and cannot be detected with rectal palpation (Constantinescu, 2007). The excretory ducts of the glands open into the urethra via the urethral recess (Budras & Habel, 2003), also called the dorsal diverticulum (Dyce *et al.*, 2002).

The secretory portions of the tubuloalveolar gland are lined with a tall simple columnar epithelium and occasionally basal cells (Wrobel, 1998). Drainage occurs via collecting ducts lined with simple cuboidal or columnar epithelium which converge to create larger intraglandular ducts lined with a pseudostratified columnar epithelium. Intraglandular ducts eventually open into a single excretory duct for each gland. The interstitium of the gland is composed of loose connective tissue and a few smooth muscle fibers. The gland is covered by a dense fibroelastic capsule containing a variable amount of smooth muscle fibers. Dense irregular connective tissue along with smooth and striated muscle fibers extend from the capsule to create intraglandular trabeculae (Wrobel, 1998).

The mucous and proteinaceous secretory product of the bulbourethral glands clears the urethra prior to ejaculation and is the most important component in the first fraction of the ejaculate (Campero *et al.*, 1988). This product serves to neutralize the urethra and to lubricate both the urethra and the vagina (Wrobel, 1998). Contributions from the bulbourethral gland and prostate combine to comprise approximately 30% of the ejaculate (Seidel & Foote, 1970).

Pathological conditions associated with the bulbourethral glands are not common. Congenital anomalies such as aplasia, hypoplasia, and fusion of the two glands have been reported (Bagshaw & Ladds, 1974; Campero *et al.*, 1988). However, bulbourethral gland cysts and dilation of the excretory ducts are the most common abnormal findings (Bagshaw & Ladds, 1974; Campero *et al.*, 1988). Infectious adenitis has been reported in association with concurrent disease in other accessory sex glands or as a stand-alone condition, in which *Corynebacterium* species were cultured (Campero *et al.*, 1988). Inflammatory adenitis was often associated with the presence of calculi within the glands, and a single case of excretory duct impaction with a plant material has been reported (Campero *et al.*, 1988). A single case of bulbourethral gland melanosis has been reported (Bagshaw & Ladds, 1974).

Chapter 3

Evaluation of the accessory sex glands of the bull for the presence of *Tritrichomonas foetus*

I. Abstract

A bull infected with the venereal protozoan *Tritrichomonas foetus* can become a chronic carrier, remaining infected for life. It has been theorized that as bulls age they develop crypts in the epithelium covering the penis and prepuce, creating an environment in which the parasite can establish long-term residence. *Trichomonas vaginalis,* a venereal protozoan of humans, will infect men and can establish a similar chronic carrier state following ascension of the urethra and infection of the prostate. This study was designed to determine if *T. foetus* could act similarly to *T. vaginalis* and ascend the urethra and infect the accessory sex glands of the bull. Twenty two bulls naturally infected with *T. foetus* were followed to an abattoir to harvest the full complement of accessory sex glands. Each gland was identified and two tissue samples from each gland were placed in Diamond's media. One tissue sample remained in media and was observed daily for 4 days according to routine *T. foetus* culture methods, yielding no obvious parasite growth or motility. The second tissue sample from each gland was subjected to PCR and assessed with gel electrophoresis. A single prostate sample was identified as positive using this PCR protocol. Using comparative DNA sequencing, the positive sample was verified to be *T. foetus.* Although further research must be conducted to validate results from this study, it appears that *T. foetus*

may behave similarly to *T. vaginalis* in ascension of the urogenital tract and invasion of the prostate of its male host.

II. Introduction

Bovine trichomoniasis is a sexually transmitted disease caused by the extracellular flagellated protozoan *Tritrichomonas foetus* which is considered an obligate parasite of the female reproductive tract and the epidermal surface of the bull penis and prepuce (Peter, 1997). Infected bulls are asymptomatic carriers of *T. foetus* and efficiently transmit the organism to a female during coitus (Parsonson *et al.*, 1974). Infected females may experience vaginitis, cervicitis, and endometritis which may result in embryonic death, abortion, fetal maceration, infertility, or pyometra (Clark *et al.*, 1983b; Fitzgerald, 1986; Mickelsen *et al.*, 1986; Parsonson *et al.*, 1976). Decreased reproductive efficiency makes trichomoniasis an extremely costly disease for the cattle industry.

It has been commonly accepted that young bulls exposed to this organism resist infection or experience only a transient infection, while older bulls with similar sexual exposure usually become chronically infected (Roberts, 1986). It is theorized that as bulls age they develop deep folds or crypts in the epithelial covering of the penis and prepuce, creating an environment in which the parasite can establish long term residence (Peter, 1997), but no published reports have been found to support this assumption. Literature suggests that *T. foetus* may colonize the distal urethra of bulls (Parsonson *et al.*, 1974), but infection of anatomical locations other than the surface of the bull penis or prepuce has not been thoroughly investigated with modern diagnostics.

Trichomonas vaginalis is a sexually transmitted protozoan of humans, with a life cycle and disease pathophysiology similar to that of *T. foetus* in cattle. *T. vaginalis* commonly resides in the male urethra but is also known to infect the prostate of infected men (Gardner *et al.*, 1986).

Infection of this accessory sex gland appears to be important for transmission of disease and maintenance of chronic infections in men (Gardner *et al.*, 1986).

This project used contemporary culture techniques and a *T. foetus* specific polymerase chain reaction assay to evaluate the bulbourethral glands, prostate, vesicular glands, and ampullae of naturally infected bulls for the presence of *T. foetus*. The presence or absence of *T. foetus* in the accessory sex glands is important to understanding the pathophysiology of chronic infection in bulls and could have implications in future treatment protocols or prevention strategies.

III. Materials and Methods

Bull Selection

Contact alliance was established with private practitioners, the Alabama State Extension Veterinarian, members of industry, and beef cattle farm producers across the Southeastern United States. These individuals identified herd bulls that were test positive to natural infection with *Tritrichomonas foetus* during routine herd screening. All information from contacts was revealed to our research group with full permission of the bull's owner or farm manager, and herd confidentiality was strictly maintained.

In order to eliminate transiently infected immature bulls, bulls included in this study were 3 years of age or older (Peter, 1997). Using samples collected by a licensed veterinarian, all bulls were identified as positive for preputial *T. foetus* infection using standard culture and/or molecular-based assays performed at an accredited diagnostic laboratory. Bulls were deemed positive with repeatable results when using single modality testing. For example, if a bull was tested using only culture or only polymerase chain reaction (PCR), the bull must have tested positive on two or more sequential cultures or molecular assays. Bulls were also identified as positive if a single sample was submitted and found to be positive on both culture and PCR. These parameters were designed to eliminate false positive bulls.

Using the assumption that a sampled tissue has a 10% chance of containing *T. foetus* as compared to a negative tissue, 20 bulls would provide a computed power >80% at alpha = 0.05. Sample size was determined based on power analysis as implemented in PROC POWER of SAS® 9.2 (Cary, NC, USA).

Tissue Collection and Transport

Twenty-two bulls (n = 22) identified as positive for *T. foetus* were followed to a large commercial abattoir located in the Southeastern United States. Following facility protocol, the penis, prepuce, and testicles were removed and discarded prior to removing the bull's hide or opening the abdominal cavity. The viscera and internal reproductive organs from each bull were removed by facility employees and made available to the licensed USDA facility inspector. Removal of the penis, prepuce, and hide prior to opening the peritoneal cavity eliminated surface contamination of the accessory sex glands. After inspection, the accessory sex organs were released to research team members. Each set of accessory sex glands was placed in an individual clean receptacle and labeled to correspond to the bull of origin using an assigned alphabetical letter ("A to "V").

Facility employees occasionally failed to remove the full complement of accessory sex organs from each bull, but the majority of the accessory sex glands were obtained from 20 of the 22 bulls (Table 1). Extreme mutilation of the accessory sex organs occurred during removal of the internal reproductive tract for 2 of the bulls, and resulted in collection of less than half of the available organs or gross fecal contamination of the remaining organs. These 2 bulls, identified as "O" and "V", were eliminated from the study, and final evaluation included 20 *T. foetus* positive bulls (n = 20).

Bull	Ampulla		Vesicular Gland		Prostate	Bulbourethral Gland	
	L	R	L	R		L	R
A	x	x	x	x	x	x	x
B	x	x	x	x	x	x	x
C	x	x	x	x	x	x	x
D	x	x	x	x	x	x	x
E	x	x	x	x			
F	x	x	x	x	x		
G	x	x	x	x	x	x	x
H	x	x	x	x	x	x	x
I	x	x	x	x	x	x	x
J	x	x	x	x		x	
K	x	x	x	x	x	x	x
L	x	x	x	x	x	x	
M	x	x	x	x	x		
N	x	x	x	x	x	x	x
P	x	x	x	x	x	x	x
Q	x	x	x	x	x	x	
R	x	x	x	x	x	x	x
S	x	x	x	x	x	x	x
T	x	x	x	x	x	x	x
U	x	x	x	x	x	x	x

Table 1: Summary of accessory sex glands recovered during slaughter from each bull (A-U)

The internal reproductive tracts from the remaining 20 study bulls were immediately transported outside of the abattoir for complete dissection in a constructed field necropsy collection site, which included portable refrigeration (4˚C) (ThermoTote Incubator; Scientific Device Laboratory, Des Plaines, IL, USA), portable tissue incubators (37˚C) (Koolatron™, Brantford, Ontario, Canada), and dissection tables with appropriate disposable fomite transmission barriers to prevent cross contamination between tissues and bulls. Each accessory sex gland was dissected free from the tract. Paired accessory sex glands were separated and treated as individual samples. Tissue dissection instruments and gloves were changed after handling each gland from each bull to prevent cross contamination. Each collected gland was equally divided into three smaller tissue sections. Two tissue sections were placed in separate 15 ml conical tubes containing 5 ml of Diamond's media; one sample was labeled for tissue culture and one for subsequent PCR. Yeast extract-maltose Diamond's media supplemented with fetal bovine serum (Diamond's Media Formulation: Appendix 1), the media of choice for culturing *T. foetus*, was used during this study (BonDurant, 1997; Diamond, 1957). When tissue size was a limiting factor, PCR was utilized as the test of choice for *T. foetus* isolation. Thus, a total of 127 tissue samples were collected for culture (Table 2) and 129 tissue samples were collected for testing by PCR (Table 3). The third tissue section from each gland was placed in 10% neutral buffered formalin and reserved for subsequent histopathologic evaluation if necessary to confirm *T. foetus* infection in selected tissues.

Bull	Ampulla		Vesicular Gland		Prostate	Bulbourethral Gland	
	L	R	L	R		L	R
A	x	x	x	x	x	x	x
B	x	x	x	x	x	x	x
C	x	x	x	x	x	x	x
D	x	x	x	x	x	x	x
E	x	x	x	x			
F	x	x	x	x	x		
G	x	x	x	x	x	x	x
H	x	x	x	x	x	x	x
I	x	x	x	x	x	x	x
J	x	x	x	x		x	
K	x	x	x	x	x	x	x
L	x	x	x	x	x	x	
M	x	x	x	x	x		
N		x	x	x	x	x	x
P	x	x	x	x	x	x	x
Q	x	x	x	x		x	
R	x	x	x	x	x	x	x
S	x	x	x	x	x	x	x
T	x	x	x	x	x	x	x
U	x	x	x	x	x	x	x

Table 2: Accessory sex gland tissues collected for standard culture in Diamond's media

Bull	Ampulla		Vesicular Gland		Prostate	Bulbourethral Gland	
	L	R	L	R		L	R
A	x	x	x	x	x	x	x
B	x	x	x	x	x	x	x
C	x	x	x	x	x	x	x
D	x	x	x	x	x	x	x
E	x	x	x	x			
F	x	x	x	x	x		
G	x	x	x	x	x	x	x
H	x	x	x	x	x	x	x
I	x	x	x	x	x	x	x
J	x	x	x	x		x	
K	x	x	x	x	x	x	x
L	x	x	x	x	x	x	
M	x	x	x	x	x		
N	x	x	x	x	x	x	x
P	x	x	x	x	x	x	x
Q	x	x	x	x	x	x	
R	x	x	x	x	x	x	x
S	x	x	x	x	x	x	x
T	x	x	x	x	x	x	x
U	x	x	x	x	x	x	x

Table 3: Accessory sex gland tissues collected for amplification by polymerase chain reaction

All samples placed in Diamond's media were transported in a portable incubator (Koolatron™, Brantford, Ontario, Canada) and maintained at 37˚C. Samples were returned to the laboratory at Auburn University's College of Veterinary Medicine (Auburn, AL, USA). Transport time was approximately 9 hours. Upon arrival, culture samples were removed from the portable incubator and immediately placed in a laboratory incubator maintained at 37˚C. PCR samples were placed in a standard laboratory freezer and maintained at -20˚C.

Culture Protocol

The culture samples remained in the original Diamond's media that tissue samples were added to at time of collection; containers were never opened and no new media was added during the culture process. All culture samples remained in the laboratory incubator except for brief observation with light microscopy for organismal motility and positive morphological identification of *T. foetus*. Prior to observation, the tube containing each sample was gently inverted to mix the sample. Each sample was observed once daily for 4 consecutive days with light microscopy at 100X for a minimum of 3 minutes. Cultures suspected as positive for trichomonads were subsequently examined at 400X for a minimum of 3 minutes. The development of turbidity in culture media associated with bacterial overgrowth prevented meaningful observation beyond day 4. All cultures were centrifuged at 2700 rpm for 5 minutes, the supernatant was carefully removed from the resultant pellet. The pellet was placed in a standard laboratory freezer and maintained at -20˚C, and the supernatant was discarded using standard decontamination procedures.

Polymerase Chain Reaction Protocol

Tissue samples corresponding to individual bulls were processed as individual sets, to further prevent cross contamination between the accessory sex glands of each bull in the study. Each sample was allowed to equilibrate to room temperature (21.1˚C), removed from the Diamond's media and 25 mg of glandular stroma was dissected free and placed in a 2.5 ml microcentrifuge tube for subsequent DNA extraction. New gloves and dissection blades were used for each sample and disposable covers were placed over the dissection surface between each sample to prevent cross contamination. Any tissue remaining after a dissection was returned to the proper container of Diamond's media and placed back in the -20˚C freezer for storage.

DNA from accessory sex gland stromal samples was extracted using QIAamp DNA Mini Kit (QIAGEN, Germantown, MD, USA) for solid tissues according to manufacturer's instructions. The DNA concentration in the extracted product was analyzed using a NanoDrop Spectrophotometer (Thermo Scientific, Willmington, DE, USA).

Specific primers designed for *T. foetus* amplification utilized during this project were obtained from Invitrogen™ Custom Primers (Life Technologies, Grand Island, NY, USA), and designated as TFR3 and TFR4. Sequences are provided in Illustration 2.

TFR3: 5′-CGGGTCTTCCTATATGAGACAGAAC-3′ (25 bases)

TFR4: 5′- CCTGCCGTTGGATCAGTTTCGTTAA -3′ (25 bases)

Illustration 2: *Tritrichomonas foetus* specific primers for DNA amplification by polymerase chain reaction

The *T. foetus* positive control was obtained from DNA isolated from an in-house *T. foetus* clone, *Tf*-CD3, which is stored and commonly employed as a positive control for experiments conducted in the Joiner Laboratory located in the Department of Pathobiology at Auburn University's College of Veterinary Medicine. Human male genomic DNA (Applied Biosystems®; Life Technologies, Grand Island, NY, USA) and PCR reaction mix containing all reagents as described below but lacking DNA templates served as dual negative controls.

PCR master mix for individual reactions contained each of the following: 5 µl of 10X PCR Buffer, 2 µl of 50 mM $MgCl_2$, 2 µl of 1:10 TFR3 primer dilution, 2 µl of 1:10 TFR4 primer dilution, 0.4 µl of 25mM dNTP mix, 28.5 µl of DEPC-treated Water, 0.2 µl of BSA, and 0.25 µl of Taq DNA polymerase (Invitrogen™; Life Technologies, Grand Island, NY, USA). Five (5) µl of DNA from each extracted tissue sample was added to the 40 µl PCR master mix of PCR reagents for amplification. One (1) µl each from the *T. foetus* positive control and the human male DNA negative control were added to positive and negative controls, respectively.

To prevent cross-contamination of test samples, all *T. foetus* positive control materials remained in the freezer until after the PCR reagents were inoculated with DNA from each tissue sample and placed in the thermal cycler for amplification. Inoculation of PCR reagents with positive controls was the last step performed prior to the amplification process.

The PCR was carried out using an Eppendorf Mastercycler® gradient Thermal Cycler (Hauppauge, NY, USA) under the following conditions: incubation at 94˚C for 3 minutes; 30 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 20 sec, and extension at 72˚C for 30 sec; followed by a final extension at 72˚C for 20 min. The final product was held at 4˚C.

Gel electrophoresis using 2% agarose gel prepared in Tris-boric acid-EDTA (TBE) incorporating ethidium bromide was used to visualize amplified *T. foetus* PCR products. Electrophoresis was performed at 60 volts for 1 hour.

Upon completion of PCR testing, all original nucleic acid extractions and processed inoculated PCR reagents were stored at -20˚C for future reference.

Gene Sequencing

The band from any positive sample was removed from the agar gel with sharp dissection along with the positive control band. Approximately 5-10 µl of extracted DNA from a positive sample and the positive control, and 10 µl each of undiluted TFR3 and TFR4 primers were required for sequencing of amplified PCR products. Each of these products was individually packaged and sent to Eurofins MWG Operon (Huntsville, AL, USA) for DNA sequencing. Sequence comparison between the positive sample and the positive control was performed to ensure that positive samples were homologous with DNA recovered from *T. foetus* organisms.

IV. Results

Culture

Active motility of trichomonad organisms was not detected throughout the observation process. The tissue sample began to degenerate within the media and overt tissue necrosis was obvious by day 2 and progressed rapidly over the next two days. Tissue necrosis impaired visualization within the media and on day 4 culture observation was suspended due to poor visibility. All samples were placed in a freezer at -20˚C for storage until project completion.

Polymerase Chain Reaction

A single, positive sample was identified by PCR. This sample was obtained from the prostate gland (PT) of the bull identified as "U", thus this sample was designate as "U-PT". The PCR amplification products obtained from U-PT and the positive *Tf*-CD3 control migrated equidistant during electrophoresis corresponding to a molecular weight of approximately 270 kilodaltons (kD) (Illustration 5). PCR amplification procedures were repeated using the originally extracted DNA from this single positive sample, U-PT; thus, confirming the amplification and identification of *T. foetus* in the prostate from this individual study bull.

The *Tf*-CD3 positive control produced an approximately 270 kD band on each gel, while all negative controls remained negative throughout the experiment.

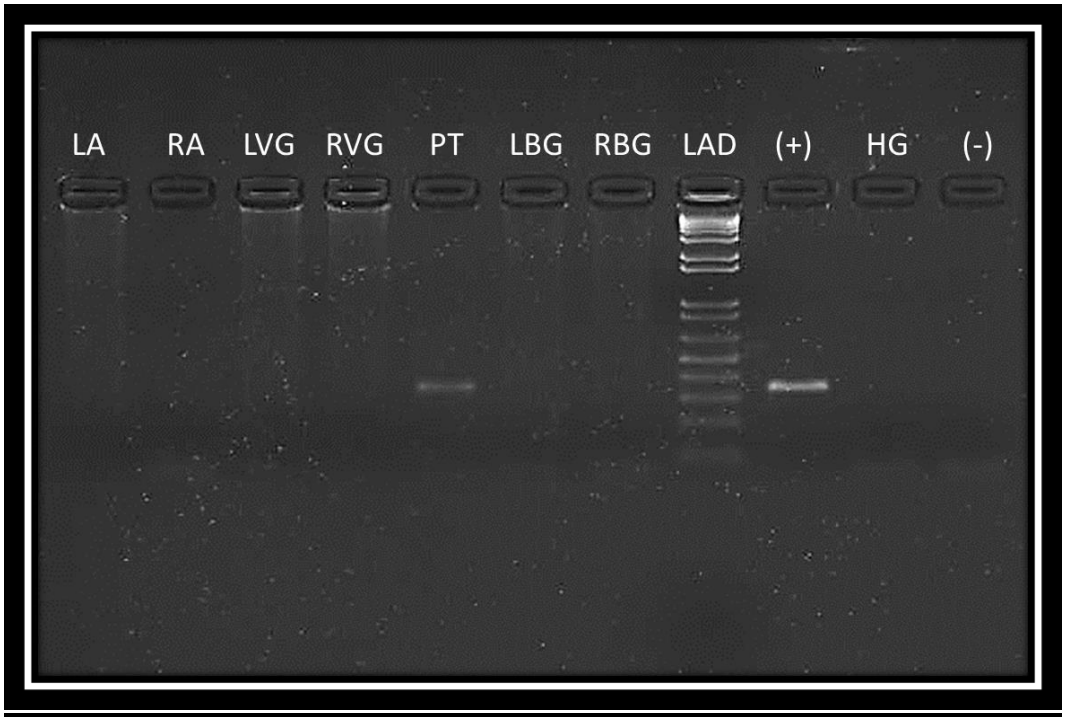

Illustration 3: Agarose gel electrophoresis, bull U accessory sex glands. *T. foetus* amplicons isolated from the prostate gland. Note amplified PCR product comigrated with the *Tf*-CD3 positive control sample. *T. foetus* was not identified in other accessory sex organs.

LA = Left Ampulla; RA = Right Ampulla; LVG = Left Vesicular Gland;
RVG = Right Vesicular Gland; PT = Prostate; LBG = Left Bulbourethral Gland
RBG = Right Bulbourethral Gland; LAD = Ladder; (+) = *T. foetus* Positive Control;
HG = Human Genomic Control; (-) = Negative Control

Statistical Analysis

This study determined that 1 out of 20 (5%) prostates were positive for *T. foetus* in bulls known to have preputial infections with *T. foetus*. Assuming the proportion of PCR positive prostate samples from bulls test positive for preputial infection with *T. foetus* is normally distributed, then a 95% confidence interval based on these results is 0.05 $^{+}/_{-}$ 0.0232 (0.0268, 0.0732).

Gene Sequencing

Gene sequencing of the single positive sample U-PT and the positive control were performed by Eurofins MWG Operon (Illustrations 6 and 7). The sequencing process analyzes a region of approximately 320 base pairs with a known genetic homology of approximately 99% between all *T. foetus* organisms. Gene sequencing comparisons revealed a 100% homology between U-PT and *Tf*-CD3, validating that the DNA recovered from the positive sample U-PT represented DNA extracted from *T. foetus* organisms.

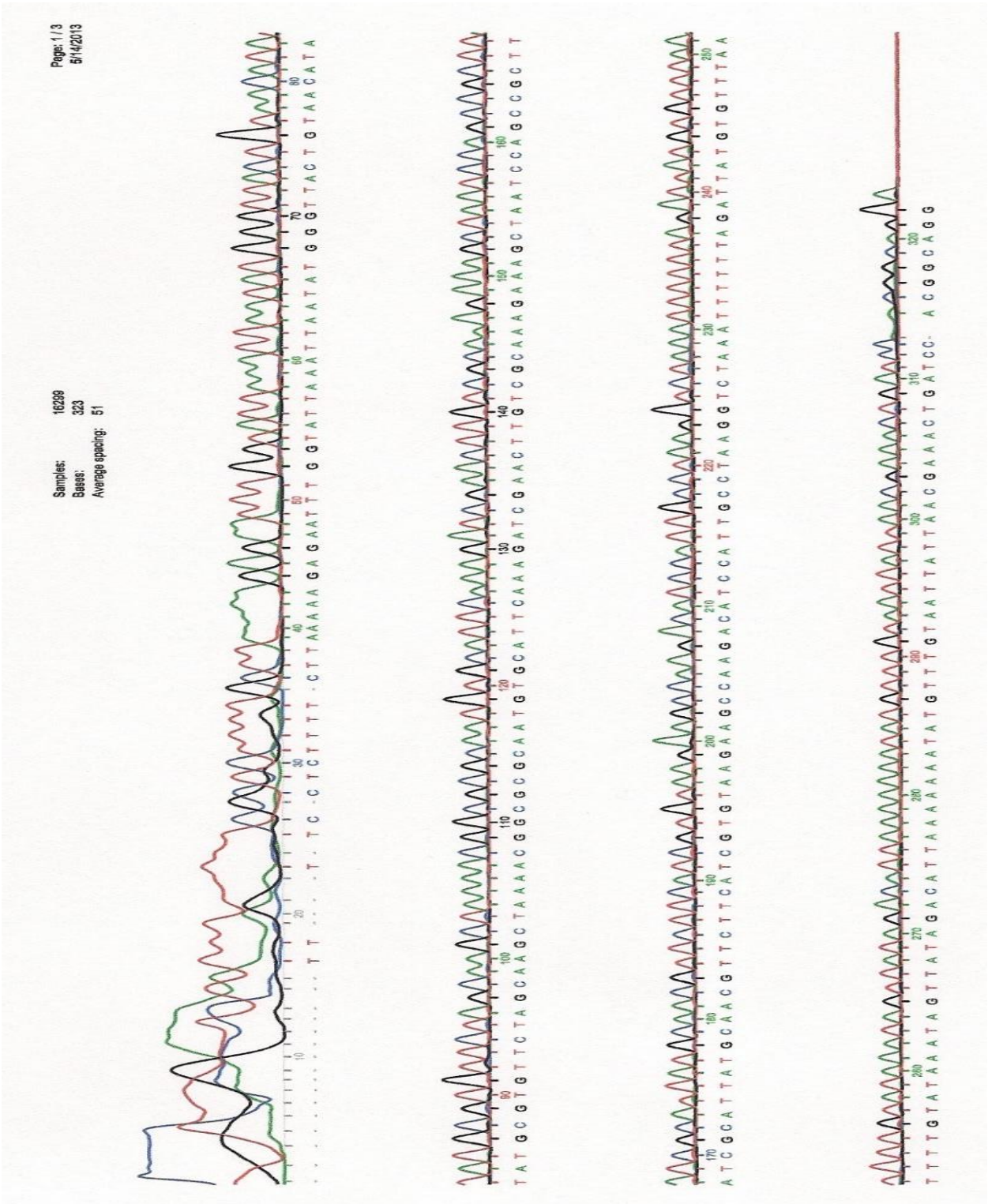

Illustration 6: U-PT Gene Sequencing

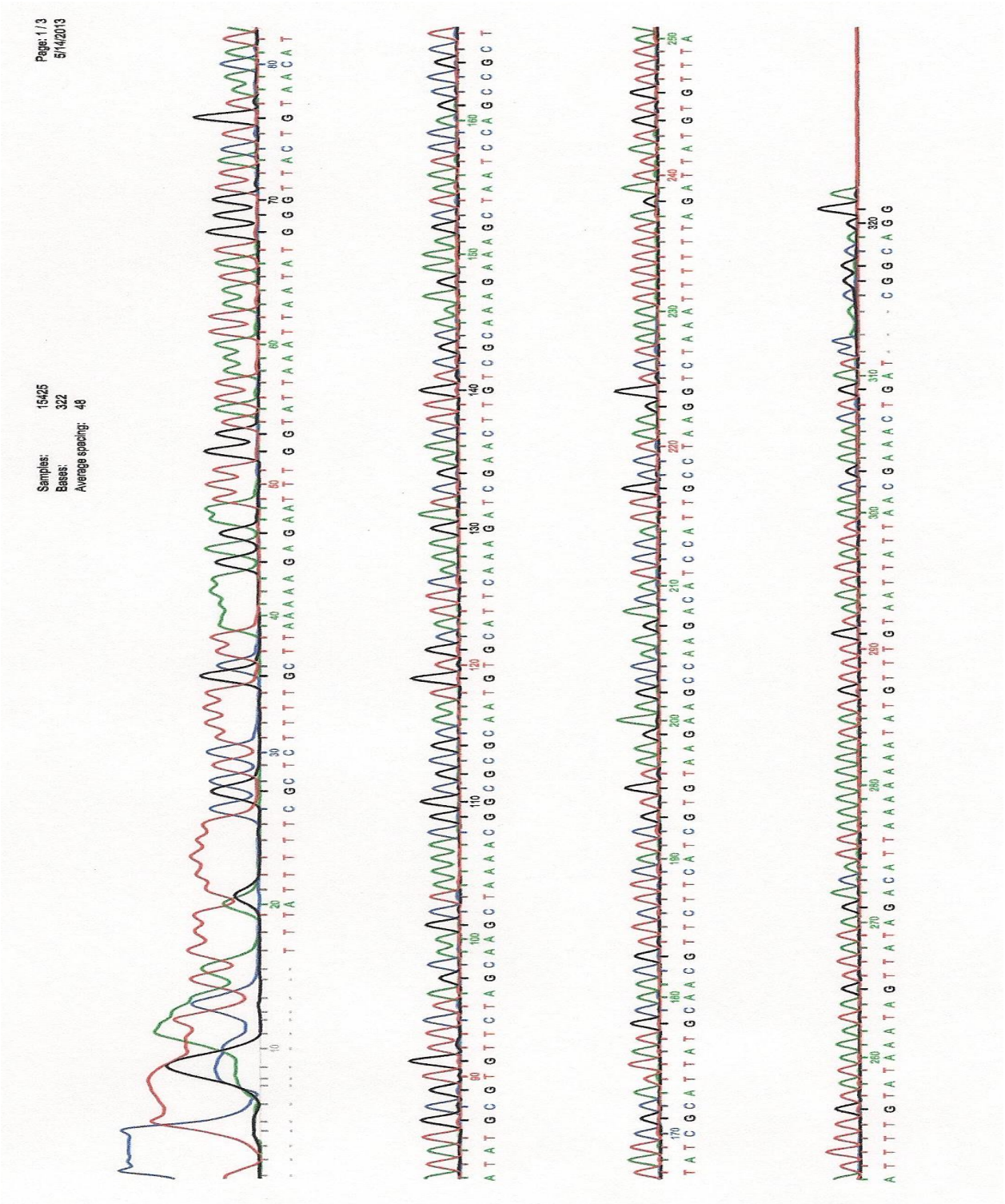

Illustration 7: *Tf*-CD3 Gene Sequencing

V. Discussion

Hammond and Bartlett concluded that in rare instances retrograde *Tritrichomonas foetus* infections of the ampullae, seminal vesicles, and epididymides may occur (Hammond & Bartlett, 1943). Although their conclusions are based on three isolated reports claiming a single case of infection within each of the respective organs, it does offer proof that ascension of *T. foetus* in the urogenital tract of the bull is possible. Further evidence exists with numerous reports of isolating *T. foetus* from the urethra of bulls (Kust, 1936 as referenced in Hammond and Bartlett, 1943; Albein, 1941 as referenced in Hammond and Bartlett, 1943; Parsonson, 1974).

This study was designed to revisit the concept of infection of the accessory sex glands of the bull by *T. foetus*. Positive findings could offer a potential explanation for chronic infection of bulls with *T. foetus*, as no factual or evidence-based explanation exists. The most commonly accepted explanations are theoretical assumptions and suggestions which have been perpetuated in the literature since the 1940's. Furthermore, confirmation of the presence of *T. foetus* in the accessory sex glands of the bull would direct future treatment protocols. Topical treatments have been implemented in the past, but knowledge of *T. foetus* infections beyond the surface of the penis and prepuce would dictate the necessity for systemic treatment. The objective of the study was to utilize current PCR protocols to evaluate the accessory sex glands of infected bulls for the presence of *T. foetus*. No known published reports are available using PCR to investigate this question.

The original project design called for collection of all seven accessory sex glands and a preputial sample from each infected bull. A preputial sample would serve as an internal positive control for infection of each bull, eliminating the use of false positive bulls. Unfortunately,

facilities necessary for collection of a preputial sample immediately prior to slaughter were unavailable and work flow inside the processing plant following slaughter did not allow harvesting of preputial samples. Although the removal of the penis, prepuce, and hide prior to opening the peritoneal cavity eliminated surface contamination of the accessory sex glands, future work should ensure the opportunity to prove preputial infection with *T. foetus* at the time of accessory sex gland evaluation.

A major limitation of this study is that sampling of the disseminate prostate was not included in the protocol; all prostate tissue retrieved and evaluated originated from the corpus prostate. The disseminate prostate contains a greater number of urethral openings and the glandular mass is larger than that of the corpus prostate, theoretically providing better opportunity for infection by *T. foetus*. An obvious protocol adaptation would be inclusion of the entire disseminate prostate, as the positive corpus prostate sample could be a minor representation of the potential for *T. foetus* to invade the prostate of the bull. Further protocol adaption would require processing the entirety of each gland, rather than a defined amount of glandular stroma, possibly increasing the odds of organismal detection.

This study determined that 1 out of 20 (5%) prostates were positive for *T. foetus* in bulls known to have preputial infections with *T. foetus*. The positive prostate sample was 100% homologous with the positive control and determined to represent *T. foetus* DNA when a *T. foetus* specific 320 base pair region was sequenced and compared. Sample contamination may have occurred despite precautionary measures, and only complete genomic sequencing of DNA from the positive prostate sample and the positive control could determine the nucleic acids to be homologues or from different individual organisms.

No published reports were available reporting infection of the bull prostate by *T. foetus.* Bulls and men both have a full complement of accessory sex glands and *Trichomonas vaginalis* is known to invade the prostate of men (Gardner *et al.*, 1986). The presence of a positive prostate sample in this bull may indicate that *T. foetus* infections in bulls can parallel *T. vaginalis* infections in men and become established in the prostate. Conversely, the prostate sample infected with *T. foetus* could represent further confirmation of a "rare instance of retrograde infection" suggested by Hammond and Bartlett in 1943. Further research is necessary to determine if the positive sample associated with the corpus prostate in this bull was an isolated finding.

References

Abonyi, A. (1995). Examination of nonflagellate and flagellate round forms of Trichomonas vaginalis by transmission electron microscopy. *Appl Parasitol*, **36**(4), 303-310.

Alderete, J. F. and Provenzano, D. (1997). The vagina has reducing environment sufficient for activation of Trichomonas vaginalis cysteine proteinases. *Genitourin Med*, **73**(4), 291-296.

Anderson, M. L., Barr, B. C. and Conrad, P. A. (1994). Protozoal causes of reproductive failure in domestic ruminants. *Vet Clin North Am Food Anim Pract*, **10**(3), 439-461.

Anderson, M. L., BonDurant, R. H., Corbeil, R. R. and Corbeil, L. B. (1996). Immune and inflammatory responses to reproductive tract infection with Tritrichomonas foetus in immunized and control heifers. *J Parasitol*, **82**(4), 594-600.

Andreeva, N., Tzvetkova, A., Gikov, D., Milchev, A., Hristeva, G., Uchikova, T., Haydushka, I. and Vachkova, I. (1981). Studies on the etiological role of Tr. vaginalis in chronic prostatitis, and efficiency of the diagnostic methods employed. *Folia Med (Plovdiv)*, **23**(2), 36-39.

Andrews, J. and Miller, F. W. (1938). *Trichomonas foetus* in Bulls. *Am J of Hyg*, **28**, 40-50.

Anonymous (2006). Bovine Trichomonsis: Essential Facts and Testing. Food Animal Reproduction and Herd Health Services, University of California Davis, http://ucanr.edu/sites/UCCE_LR/files/151788.pdf.

Bagshaw, P. A. and Ladds, P. W. (1974). A study of the accessory sex glands of bulls in abattoirs in Northern Australia. *Aust Vet J*, **50**(11), 489-495.

Barth, A. D. (2007). Sperm accumulation in the ampullae and cauda epididymides of bulls. *Anim Reprod Sci*, **102**(3-4), 238-246. doi: 10.1016/j.anireprosci.2006.11.005.

BonDurant, R. H. (1985). Diagnosis, treatment, and control of bovine trichomoniasis. *Compendium of Continuing Education for the Practicing Veterinarian,* **7**, S179-188.

BonDurant, R. H. (1997). Pathogenesis, diagnosis, and management of trichomoniasis in cattle. *Vet Clin North Am Food Anim Pract,* **13**(2), 345-361.

Bondurant, R. H. (2005). Venereal diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. *Vet Clin North Am Food Anim Pract,* **21**(2), 383-408. doi: 10.1016/j.cvfa.2005.03.002.

BonDurant, R. H., Anderson, M. L., Blanchard, P., Hird, D., Danaye-Elmi, C., Palmer, C., Sischo, W. M., Suther, D., Utterback, W. and Weigler, B. J. (1990). Prevalence of trichomoniasis among California beef herds. *J Am Vet Med Assoc,* **196**(10), 1590-1593.

BonDurant, R. H., Gajadhar, A., Campero, C. M., Johnson, E., Lun, Z. R., Nordhausen, R. W., Hoosear, K. A. V., Villanueva, M. R. and Walker, R. L. (1999). Preliminary characterization of a Tritrichomonas foetus-like protozoan isolated from preputial smegma of virgin bulls. In *Bov Pract*, Vol. 33 pp. 124-127.

BonDurant, R. H. and Honigberg, B. N. (1994). Trichomonads of Veterinary Importance. In *Parasitic Protozoa* (ed. Kreier, J. P.), pp. 111-188. Academic Press, San Diego, CA.

Budras, K.-D. and Habel, R. E. (2003). Male Genital Organs and Scrotum. In *Bovine Anatomy* pp. 92-93. Schlutersche GmbH & Co, Hannover, Germany.

Cameron, H. S., Fincher, M. G. and Gilman, H. L. (1933). Trichomonas Infection in Cattle. Preliminary Report. *Cornell Vet,* **23**, 297-303.

Campero, C. M., Ladds, P. W. and Thomas, A. D. (1988). Pathological findings in the bulbourethral glands of bulls. *Aust Vet J,* **65**(8), 241-244.

Campero, C. M., Rodriguez Dubra, C., Bolondi, A., Cacciato, C., Cobo, E., Perez, S., Odeon, A., Cipolla, A. and BonDurant, R. H. (2003). Two-step (culture and PCR) diagnostic approach for differentiation of non-T. foetus trichomonads from genitalia of virgin beef bulls in Argentina. *Vet Parasitol*, **112**(3), 167-175.

Cheah, I. K. and Halliwell, B. (2012). Ergothioneine; antioxidant potential, physiological function and role in disease. *Biochim Biophys Acta,* **1822**(5), 784-793. doi: 10.1016/j.bbadis.2011.09.017.

Chen, Y. C., Huang, Y. L., Platz, E. A., Alderete, J. F., Zheng, L., Rider, J. R., Kraft, P., Giovannucci, E. and Sutcliffe, S. (2013). Prospective study of effect modification by Toll-like receptor 4 variation on the association between Trichomonas vaginalis serostatus and prostate cancer. *Cancer Causes Control,* **24**(1), 175-180. doi: 10.1007/s10552-012-0103-y.

Clark, B. L., Dufty, J. H. and Parsonson, I. M. (1977). Studies on the transmission of Tritrichomonas foetus. *Aust Vet J,* **53**(4), 170-172.

Clark, B. L., Dufty, J. H. and Parsonson, I. M. (1983a). Immunisation of bulls against trichomoniasis. *Aust Vet J,* **60**(6), 178-179.

Clark, B. L., Dufty, J. H. and Parsonson, I. M. (1983b). The effect of Tritrichomonas foetus infection on calving rates in beef cattle. *Aust Vet J,* **60**(3), 71-74.

Clark, B. L., Emery, D. L. and Dufty, J. H. (1984). Therapeutic immunisation of bulls with the membranes and glycoproteins of Tritrichomonas foetus var. brisbane. *Aust Vet J,* **61**(2), 65-66.

Cobo, E. R., Campero, C. M., Mariante, R. M. and Benchimol, M. (2003). Ultrastructural study of a tetratrichomonad species isolated from prepucial smegma of virgin bulls. *Vet Parasitol,* **117**(3), 195-211.

Cobo, E. R., Corbeil, L. B., Gershwin, L. J. and BonDurant, R. H. (2009). Preputial cellular and antibody responses of bulls vaccinated and/or challenged with Tritrichomonas foetus. *Vaccine,* **28**(2), 361-370. doi: 10.1016/j.vaccine.2009.10.039.

Cobo, E. R., Favetto, P. H., Lane, V. M., Friend, A., VanHooser, K., Mitchell, J. and BonDurant, R. H. (2007). Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with Tritrichomonas foetus. *Theriogenology,* **68**(6), 853-860. doi: 10.1016/j.theriogenology.2007.06.019.

Constantinescu, G. H. (2007). The Genital Apparatus in the Ruminant. In *Comparative Reproductive Biology* (eds. Schatten, K., and Constantinescu, G. H.), pp. 33-48. Blackwell Publishing, Ames, IA.

Dargatz, D. A., Mortimer, R. G. and Ball, L. (1987). Vesicular adenitis of bulls: A review. *Theriogenology,* **28**(4), 513-521.

Diamond, L. S. (1957). The establishment of various trichomonads of animals and man in axenic cultures. *J Parasitol,* **43**(4), 488-490.

Dyce, K. M., Sack, W. O. and Wensig, C. J. G. (2002). The Pelvis and Reproductive Organs of Male Ruminants. In *Textbook of Veterinary Anatomy* pp. 715-717. Saunders of Elsevier, Philidelphia, PA.

Emmerson, M. (1932). Trichomoniasis in Cattle. *Journal of American Veterinary Medical Association,* **34**, 636-640.

Felleisen, R. S., Lambelet, N., Bachmann, P., Nicolet, J., Müller, N. and Gottstein, B. (1998). Detection of Tritrichomonas foetus by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. *J Clin Microbiol,* **36**(2), 513-519.

Fernandes, P. R., Da Silva, A. C., Gambarini, M. L. and Linhares, G. F. (2008). Primers design and optimization of PCR and nested-PCR assays for the specific detection of Tritrichomonas foetus. *Rev Bras Parasitol Vet,* **17**(3), 133-138.

Fitzgerald, P. R. (1986). Bovine trichomoniasis. *Vet Clin North Am Food Anim Pract,* **2**(2), 277-282.

Fitzgerald, P. R., Hammond, D. M., Miner, M. L. and Binns, W. (1952). Relative efficacy of various methods of obtaining preputial samples for diagnosis of trichomoniasis in bulls. *Am J Vet Res,* **13**(49), 452-457.

Futamura, H. (1935). Studies on Trichomonas Abortion in Cattle. *J Japan Soc Vet Sci,* **14**, 392-416.

Garber, G. E. (2005). The laboratory diagnosis of Trichomonas vaginalis. *Can J Infect Dis Med Microbiol,* **16**(1), 35-38.

Gardner, W. A., Culberson, D. E. and Bennett, B. D. (1986). Trichomonas vaginalis in the prostate gland. *Arch Pathol Lab Med,* **110**(5), 430-432.

Gopalkrishnan, K., Hinduja, I. N. and Kumar, T. C. (1990). Semen characteristics of asymptomatic males affected by Trichomonas vaginalis. *J In Vitro Fert Embryo Transf*, **7**(3), 165-167.

Grahn, R. A., BonDurant, R. H., van Hoosear, K. A., Walker, R. L. and Lyons, L. A. (2005). An improved molecular assay for Tritrichomonas foetus. *Vet Parasitol*, **127**(1), 33-41. doi: 10.1016/j.vetpar.2004.08.018.

Granger, B. L., Warwood, S. J., Benchimol, M. and De Souza, W. (2000). Transient invagination of flagella by Tritrichomonas foetus. *Parasitol Res*, **86**(9), 699-709.

Hammond, D. A. and Bartlett, D. E. (1943). The Distribution of Trichomonas foetus in the Preputial Cavity of Infected Bulls. *Am J of Vet Res*, **4**, 143-149.

Harp, D. F. and Chowdhury, I. (2011). Trichomoniasis: evaluation to execution. *Eur J Obstet Gynecol Reprod Biol*, **157**(1), 3-9. doi: 10.1016/j.ejogrb.2011.02.024.

Heath, J. P. (1981). Behaviour and pathogenicity of Trichomonas vaginalis in epithelial cell cultures: a study by light and scanning electron microscopy. *Br J Vener Dis*, **57**(2), 106-117.

Herr, S., Ribeiro, L. M., Claassen, E. and Myburgh, J. G. (1991). 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 J Vet Res*, **58**(1), 41-45.

Hess, E. A., Ludwick, T. M., Martig, R. C. and Ely, F. (1960). Influence of Seminal Vesiculectomy on Certain Physical and Biochemical Properties of Bovine Semen1,2. *Journal of dairy science*, **43**(2), 256-265.

Ho, M. S., Conrad, P. A., Conrad, P. J., LeFebvre, R. B., Perez, E. and BonDurant, R. H. (1994). Detection of bovine trichomoniasis with a specific DNA probe and PCR amplification system. *J Clin Microbiol*, **32**(1), 98-104.

Honiberg, B. M. and King, V. M. (1964). STRUCTURE OF TRICHOMONAS VAGINALIS DONN'E. *J Parasitol*, **50**, 345-364.

Honigberg, B. N. (1978). Trichomonads of Veterinary Importance: Tritrichomonas foetus. In *Parasitic Protozoa* (ed. Kreier, J. P.), pp. 207-273. Academic Press, New York, NY.

Huppert, J. S. (2012). Trichomoniasis. In *Netter's Infectious Diseases* pp. 303-310. Saunders of Elsevier Inc., Philadelphia, PA.

Johnson, A. E. (1965). The Diagnosis of Trichomoniasis in the Bull. *Proc US Livestock Sanitary Assoc,* **69**, 183-189.

Kainer, R. A., Faulkner, L. C. and Abdel-Raouf, M. (1969). Glands associated with the urethra of the bull. *Am J Vet Res,* **30**(6), 963-974.

Karlson, A. G. and Boyd, W. L. (1941). Orchitis and Seminal Vesiculitis Associated with *Trichomonas fetus* and *Corynebacterium pyogenes* Infection in a Bull. *Cornell Vet,* **31**, 311-313.

Kaydos-Daniels, S. C., Miller, W. C., Hoffman, I., Banda, T., Dzinyemba, W., Martinson, F., Cohen, M. S. and Hobbs, M. M. (2003). Validation of a urine-based PCR-enzyme-linked immunosorbent assay for use in clinical research settings to detect Trichomonas vaginalis in men. *J Clin Microbiol,* **41**(1), 318-323.

Kimsey, P. B. (1986). Bovine Trichomoniasis. In *Current Therapy in Theriogenology* (ed. Morrow, D. A.), pp. 275-279. W B Saunders Company, Philidelphia, PA.

Kimsey, P. B., Darien, B. J., Kendrick, J. W. and Franti, C. E. (1980). Bovine trichomoniasis: diagnosis and treatment. *J Am Vet Med Assoc,* **177**(7), 616-619.

Krieger, J. N. and Rein, M. F. (1982). Zinc sensitivity of Trichomonas vaginalis: in vitro studies and clinical implications. *J Infect Dis,* **146**(3), 341-345.

Krieger, J. N., Verdon, M., Siegel, N. and Holmes, K. K. (1993). Natural history of urogenital trichomoniasis in men. *J Urol,* **149**(6), 1455-1458.

Kvasnicka, W. G., Taylor, R. E., Huang, J. C., Hanks, D., Tronstad, R. J., Bosomworth, A. and Hall, M. R. (1989). Investigations of the incidence of bovine trichomoniasis in nevada and of the efficacy of immunizing cattle with vaccines containing Tritrichomonas foetus. *Theriogenology,* **31**(5), 963-971.

Lloyd, G., Case, J. R., De Frias, D. and Brannigan, R. E. (2003). Trichomonas vaginalis orchitis with associated severe oligoasthenoteratospermia and hypogonadism. *J Urol,* **170**(3), 924. doi: 10.1097/01.ju.0000080375.18547.cc.

Lossick, J. G. (1980). Single-dose metronidazole treatment for vaginal trichomoniasis. *Obstet Gynecol,* **56**(4), 508-510.

Lossick, J. G., Müller, M. and Gorrell, T. E. (1986). In vitro drug susceptibility and doses of metronidazole required for cure in cases of refractory vaginal trichomoniasis. *J Infect Dis,* **153**(5), 948-955.

Mann, T. (1964). *The biochemistry of semen and of the male reproductive tract,* Methuen; Wiley, London, New York.

McMillen, L. and Lew, A. E. (2006). Improved detection of Tritrichomonas foetus in bovine diagnostic specimens using a novel probe-based real time PCR assay. *Vet Parasitol,* **141**(3-4), 204-215. doi: 10.1016/j.vetpar.2006.06.012.

Mickelsen, W. D., Paisley, L. G. and Anderson, P. B. (1986). Survey of the prevalence and types of infertility in beef cows and heifers. *J Am Vet Med Assoc,* **189**(1), 51-54.

Mitteregger, D., Aberle, S. W., Makristathis, A., Walochnik, J., Brozek, W., Marberger, M. and Kramer, G. (2012). High detection rate of Trichomonas vaginalis in benign hyperplastic prostatic tissue. *Med Microbiol Immunol,* **201**(1), 113-116. doi: 10.1007/s00430-011-0205-2.

Morgan, B. B. (1944a). *Bovine trichomoniasis,* Burgess publishing co., Minneapolis, Minn.

Morgan, B. B. (1944b). Studies on the trichomonad carrier-cow problem. *J Anim Sci,* **3**, 437.

Morgan, B. B. (1946). *Bovine trichomoniasis,* Burgess publishing co., Minneapolis, Minn.

Parker, S., Lun, Z. R. and Gajadhar, A. (2001). Application of a PCR assay to enhance the detection and identification of Tritrichomonas foetus in cultured preputial samples. *J Vet Diagn Invest,* **13**(6), 508-513.

Parsonson, I. M., Clark, B. L. and Dufty, J. (1974). The pathogenesis of Tritrichomonas foetus infection in the bull. *Aust Vet J,* **50**(10), 421-423.

Parsonson, I. M., Clark, B. L. and Dufty, J. H. (1976). Early pathogenesis and pathology of Tritrichomonas foetus infection in virgin heifers. *J Comp Pathol,* **86**(1), 59-66.

Peeling, R. and Embree, J. (2005). Screening for sexually transmitted infection pathogens in semen samples. *Can J Infect Dis Med Microbiol,* **16**(2), 73-76.

Pereira-Neves, A. and Benchimol, M. (2009). Tritrichomonas foetus: budding from multinucleated pseudocysts. *Protist,* **160**(4), 536-551. doi: 10.1016/j.protis.2009.05.001.

Pereira-Neves, A., Campero, C. M., Martínez, A. and Benchimol, M. (2011). Identification of Tritrichomonas foetus pseudocysts in fresh preputial secretion samples from bulls. *Vet Parasitol,* **175**(1-2), 1-8. doi: 10.1016/j.vetpar.2010.10.007.

Pereira-Neves, A., Ribeiro, K. C. and Benchimol, M. (2003). Pseudocysts in trichomonads--new insights. *Protist,* **154**(3-4), 313-329. doi: 10.1078/143446103322454095.

Peter, D. (1997). Bovine Venereal Diseases. In *Current Therapy in Large Animal Theriogenology* (ed. Youngquist, R. S.), pp. 355-363. W. B. Saunders Company, Philadelphia, PA.

Rhyan, J. C., Wilson, K. L., Wagner, B., Anderson, M. L., BonDurant, R. H., Burgess, D. E., Mutwiri, G. K. and Corbeil, L. B. (1999). Demonstration of Tritrichomonas foetus in the external genitalia and of specific antibodies in preputial secretions of naturally infected bulls. *Vet Pathol,* **36**(5), 406-411.

Riley, D. E., Wagner, B., Polley, L. and Krieger, J. N. (1995). PCR-based study of conserved and variable DNA sequences of Tritrichomonas foetus isolates from Saskatchewan, Canada. *J Clin Microbiol,* **33**(5), 1308-1313.

Roberts, S. J. (1986). *Veterinary Obstetrics and Genital Diseases,* 3rd edn. Stephen J. Roberts, Woodstock, VT.

Sawyer, P. R., Brogden, R. N., Pinder, R. M., Speight, T. M. and Avery, G. S. (1976). Tinidazole: a review of its antiprotozoal activity and therapeutic efficacy. *Drugs,* **11**(6), 423-440.

Schmid, G., Narcisi, E., Mosure, D., Secor, W. E., Higgins, J. and Moreno, H. (2001). Prevalence of metronidazole-resistant Trichomonas vaginalis in a gynecology clinic. *J Reprod Med,* **46**(6), 545-549.

Schwebke, J. R. and Burgess, D. (2004). Trichomoniasis. *Clin Microbiol Rev,* **17**(4), 794-803, table of contents. doi: 10.1128/CMR.17.4.794-803.2004.

Schwebke, J. R. and Lawing, L. F. (2002). Improved detection by DNA amplification of Trichomonas vaginalis in males. *J Clin Microbiol,* **40**(10), 3681-3683.

Schönmann, M. J., BonDurant, R. H., Gardner, I. A., Van Hoosear, K., Baltzer, W. and Kachulis, C. (1994). Comparison of sampling and culture methods for the diagnosis of Tritrichomonas foetus infection in bulls. *Vet Rec,* **134**(24), 620-622.

Seidel, G. E. and Foote, R. H. (1970). Compartmental analysis of sources of the bovine ejaculate. *Biol Reprod,* **2**(2), 189-196.

Senger, P. L. (2012). The Organization and Function of the Male Reproductive System. In *Pathways to Pregnancy and Parturition* pp. 71-74. Current Conceptions Inc, Redmond, OR.

Shah, B. A., Hopwood, M. L. and Faulkner, L. C. (1968). Seminal Vesiculectomy in Bulls. *L Reprod Fert,* **16**, 171-177.

Skirrow, S., BonDurant, R., Farley, J. and Correa, J. (1985). Efficacy of ipronidazole against trichomoniasis in beef bulls. *J Am Vet Med Assoc,* **187**(4), 405-407.

Skirrow, S. Z. and BonDurant, R. H. (1988). Bovine Trichomoniasis. *Vet Bull,* **58**, 592-603.

Sood, S. and Kapil, S. (2008). An update on Trichomonas vaginalis. *Indian J Sex Transm Dis,* **29**(1), 7-14.

Soto, P. and Parma, A. E. (1989). The immune response in cattle infected with Tritrichomonas foetus. *Vet Parasitol,* **33**(3-4), 343-348.

Stark, J. R., Judson, G., Alderete, J. F., Mundodi, V., Kucknoor, A. S., Giovannucci, E. L., Platz, E. A., Sutcliffe, S., Fall, K., Kurth, T., Ma, J., Stampfer, M. J. and Mucci, L. A. (2009). Prospective study of Trichomonas vaginalis infection and prostate cancer incidence and mortality: Physicians' Health Study. *J Natl Cancer Inst,* **101**(20), 1406-1411. doi: 10.1093/jnci/djp306.

Strickland, L. G. (2010). Surface Architectural Anatomoy of the Penile and Preputial Epithelia of Bulls. In *Biomedical Sciences* pp. 62. Auburn University.

Sutcliffe, S. (2010). Sexually transmitted infections and risk of prostate cancer: review of historical and emerging hypotheses. *Future Oncol,* **6**(8), 1289-1311. doi: 10.2217/fon.10.95.

Sutcliffe, S., Giovannucci, E., Alderete, J. F., Chang, T. H., Gaydos, C. A., Zenilman, J. M., De Marzo, A. M., Willett, W. C. and Platz, E. A. (2006). Plasma antibodies against Trichomonas vaginalis and subsequent risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev,* **15**(5), 939-945. doi: 10.1158/1055-9965.EPI-05-0781.

Taylor, M. A., Marshall, R. N. and Stack, M. (1994). Morphological differentiation of Tritrichomonas foetus from other protozoa of the bovine reproductive tract. *Br Vet J,* **150**(1), 73-80. doi: 10.1016/S0007-1935(05)80098-3.

Tedesco, L. F., Errico, F. and Del Baglivi, L. P. (1979). Diagnosis of Tritrichomonas foetus infection in bulls using two sampling methods and a transport medium. *Aust Vet J,* **55**(7), 322-324.

Thomason, J. L. and Gelbart, S. M. (1989). Trichomonas vaginalis. *Obstet Gynecol,* **74**(3 Pt 2), 536-541.

Thornburn, A. L. (1974). Alfred Francois Donne, 1801-1878, Discoverer of *Trichomonas vaginalis* and leukaemia. *Brit J Vener Dis*, **50**, 377-380.

Trussell, R. E. (1947). *Trichomonas vaginalis and trichomoniasis,* 1st edn. C. C. Thomas, Springfield, Ill.

Wrobel, K.-H. (1998). Male Reproductive System. In *Textbook of Veterinary Histology* (eds. Dellman, H. D., and Eurell, J. A.), pp. 238-243. Williams & Wilkins, Baltimore, MD.

Appendix 1

Diamond's Media Formulation

Stock Solutions	(in 100 ml dH_2O)
1. K_2HPO_4	10 grams
KH_2PO_4	6 g
NaCL	20 g
2. Cysteine HCl	20 g
3. Ascorbic Acid	2 g
4. 20% Maltose (autoclaved)	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 Recipe

To a 500 ml bottle add:

1. 5.0 mls (K_2HPO_4/KH_2PO_4/NaCL)
2. 10 g Trypticase Peptone
3. 5.0 g Yeast extract
4. 2.0 g NaOH pellets
5. Adjust pH to 7.0
6. Autoclave and label "Base Media"
7. Store at room temperature

Diamond's Media

To a 500 ml bottle of Base Media add:

2.5 ml sterile Cysteine

5.0 ml sterile Ascorbic Acid

25 ml sterile 20% Maltose

5.5 ml sterile dH_2O

50 ml Fetal Bovine Serum

5.0 ml Pen/Strep

Store at 4˚C