Detection of *Tritrichomonas foetus* in the Bull

by

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Abstract

Bovine trichomoniasis is a venereal disease in cattle that causes severe reproductive loss and can have devastating economic impact on the cattle industry. Bovine trichomoniasis is caused by a protozoal organism that inhabits the reproductive tract of both female and male cattle. Infected females suffer clinically with inflammatory gynecologic disease and resultant reproductive losses. Bulls are considered clinically asymptomatic carriers. Infected bulls can spread the disease readily throughout cattle herds. Thus, it is important to ensure bulls are tested and determined to be negative for *Tritrichomonas foetus* (*T. foetus)* prior to being utilized for breeding purposes. The importance of being able to consistently identify *T. foetus* in breeding bulls is crucial for control and eradication of *T. foetus* from cattle herds.

The focus of this study was to determine if there are additional reservoir sites of *T. foetus* in portions of the reproductive tract, specifically the urethra and the secondary sex glands in bulls chronically infected with *T. foetus.* The identification of reservoir sites for the organism in the proximal reproductive tract of the bull has the potential to have detrimental effects on the possibility of a topical curative treatment in bulls.

Eight naturally infected *T. foetus* positive adult *Bos taurus* bulls ranging in age from four to 14 years of age were donated to Auburn University College of Veterinary Medicine (AUCVM) for research purposes. Five of the bulls were Angus and/or Angus cross bulls and three were Charolais. Upon admission to AUCVM, all bulls were tested for *T. foetus.* A preputial scraping using a Pizzle Stick (Lane Manufacturing, Denver, CO) was performed to obtain a reliable smegma sample for *T. foetus* testing. All smegma samples were placed in individual labeled vials containing Modified Diamond's Media (MDM) (Thermo Fisher Scientific; Waltham, MA) and submitted to the Thompson-Bishop-Sparks-Alabama State Diagnostic

Laboratory (TBSASDL) for testing for live organisms via culture, and testing for *T. foetus* DNA via Real Time Polymerase Chain Reaction (RTPCR) (VetMAX™-Gold Trich Detection kit, Thermo Fisher Scientific; Waltham, MA). All bulls were tested at least five times over the course of five months via culture and RTPCR to determine the chronicity of the *T. foetus* infection. Following testing, the bulls were sedated, euthanized, and submitted to the TBSASDL for necropsy. The reproductive tracts in their entirety were removed and placed on clean tables for sectioning. The tracts were dissected from the area of least potential contamination to the area of the most potential contamination. Sterile technique was used during dissection and sampling of the urogenital tracts. Three bulls had 1.0 cm x 1.0 cm sections cut from the right and left ampullae, right and left vesicular glands, the prostate, and sections of urethra, penis, and prepuce. The sections of the urethra included 5 cm proximal from the distal end of the urethra, 23 cm proximal from the distal end of the urethra, 5 cm distal to the last bend of the sigmoid, 13 cm proximal to the sigmoid at the level of the trigone of the bladder. The five other bulls had tissue collected via scrapings of the inside surface of the glands, urethra at the specified locations as described above, and the penis and the prepuce. Following the collection of the tissues, all samples were placed in individual vials of Modified Diamonds media (MDM) to remove the smegma and any tissue collected by the scraping. All samples were submitted to the TBSASDL for testing for *T. foetus* via culture for live organisms and RTPCR for *T. foetus* DNA. The prostate, vesicular glands, ampullae, bladder, and the urethra proximal to the sigmoid flexure were all found to be negative on culture for live organisms and for *T. foetus* DNA.

All bulls were determined to be chronically infected with *T. foetus*. All smegma samples from bulls were found to be positive for T. *foetus* on culture. All smegma from the preputial scraping, penis, and the urethral sections 5 cm proximal from the distal end of the urethra, and 23

cm proximal from the distal end of the urethra were all found to be positive for *T. foetus* DNA via RTPCR with a cycle threshold (CT) of 35.0, 33.5, 34.3, and 36.0, respectively for Bull 1 and 35.6, 30.6, 32.2 and 32.0 respectively for Bull 2 and 30.1, 30.6, 36.4, and 35.5, respectively for Bull 3. Bull 2 and Bull 3 were positive 5 cm distal to the last bend of the sigmoid with CT values of 37.4 and 35.0, respectively. Additionally, Bulls 6, 7, and 8 were determined to be positive for *T. foetus* DNA at the level of the distal urethra 5 cm from the distal end of the penis with CT values of 38.0, 33.8, and 35.0 with preputial scrapings having CT values of 29.6, 34.5 and 31.2, respectively. However, Bulls 4 and 5 were only found to be positive for *T. foetus* DNA only on preputial scrapings with CT values of 30.0 and 33.5, respectively with all other areas of the reproductive tract sampled negative for *T. foetus* DNA via RTPCR.

A total of six of the eight (75%) bulls sampled in this study were found to be positive for *T. foetus* DNA in the distal urethra. Three of the eight (37.5%) bulls had *T. foetus* DNA detected in the lower half of the urethra. Two of the eight (25%) bulls were positive for *T. foetus* DNA near the distal bend of the sigmoid flexure. Eleven of 24 tests for *T. foetus* DNA in the urethra of naturally infected *T. foetus* bulls were found to be positive. The significance of this finding was evaluated utilizing a one tailed, one sample proportion z test with an $\alpha = 0.05$, utilizing Excel 2016 (Microsoft Co; Redmond, WA). It was determined that the proportion of urethral samples positive for *T foetus* DNA was a significant finding ($p < 0.05$, $z = 2.441$). The study herein built upon the findings of Rush et al., and further documents *T. foetus* DNA in a more proximal location in the urethra in naturally infected *Bos taurus* bulls (Rush et al., 2020). There are few reports stating that *T. foetus* can be found in the distal urethral orifice of some bulls (Michi, 2016). These studies were performed prior to the development and wide use of RTPCRs so it is possible that false negatives and false positives may have occurred during testing procedures.

This study provides important information for researchers looking into the clearance of *T. foetus* from chronically infected bulls.

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List of Abbreviations

T. foetus	*Tritrichomonas foetus*
RTPCR	Real time polymerase chain reaction
QPCR	Quantitative PCR
PS	Pizzle Stick (Lane manufacturing)
MIP	Mare Infusion Pipette
TBSASDL	Thompson-Bishop-Sparks-Alabama State Diagnostic Laboratory
MDM	Modified Diamond's Media
CT	Cycle Threshold
TFCP	*Tritrichomonas foetus* cysteine proteinase
Cq	Quantification Cycle
CSS	Certified Semen Services

CHAPTER 1: INTRODUCTION

Tritrichomonas foetus is the causative agent of the venereal disease trichomoniasis in cattle. Trichomoniasis has been reported worldwide and is often economically devastating (Waters & Gard, 2021). The protozoan *T. foetus* is an extracellular flagellated parasite of the reproductive tract of the bovine and is a major cause of reproductive loss in cattle. The reproductive losses seen are amplified in naïve herds. The first identification of *T. foetus* in the United States was in 1932 in the state of Pennsylvania from the vaginal smears of two cows experiencing infertility (Emmerson, 1932; Rae, 2006). The organism was originally identified in 1888 by a French scientist Kunstler, however credit has historically been awarded to Mazzanti in 1900 (Morgan, 1947; Rae, 2006). At the time of discovery the disease Brucellosis was also identified and took the main focus of research pushing the uncovering of details about *T. foetus* back approximately 25 years (Morgan, 1947).

The spread of *T. foetus* is via coitus, where the bull serves as the chronically infected asymptomatic carrier and the female experiences the detrimental reproductive losses. Clinical signs seen in the infected female often occur within the first 95 days post infection with fetal loss as late as five to six months gestation (Ondrak, 2016). Reproductive disease is manifested in the infected female as abortion, early embryonic loss, post-coital pyometra, irregular return to estrus, cervicitis, vaginitis, and less frequently late term abortion (Morgan, 1947; Ondrak, 2016). While most females undergo transient infections, it is estimated that less than one percent of infected females become chronically infected (Skirrow, 1987). The lack of consistency in chronically infected females, the ease of sample collection, and availability of bulls during yearly breeding soundness examination make bulls the most reliable and convenient animals to test. During coitus, *T. foetus* can be transmitted from an infected bull to naïve cow, infected cow to naïve bull, and is possible that mechanical transmission can occur when a naïve bull spreads the

parasite from infected cow to naïve cow if the breeding takes place subsequently within 20 minutes post breeding (Clark, 1977; Rae, 2006, Ondrak, 2016; Jin, 2020).

There are currently no approved treatments for bovine trichomoniasis, emphasizing the importance of the identification and control of infected animals. Artificial insemination can serves as a means for control of *T. foetus* when proper testing prior to freezing is performed. Proper testing to ensure the bull is negative for T. foetus is vital for control as the trichomonad can survive the freezing process (Michi et al., 2016). The main means of control at this time is the ready identification and removal of infected animals. Currently there are no worldwide monitoring systems for the incidence and prevalence of bovine Trichomoniasis indicating that the suspected amount of cattle reproduction affected is underestimated (Michi et al., 2016). The lack of clinical signs in the bull emphasizes the importance of the efficacious identification the organism. Presently there are numerous ways to collect smegma for *T. foetus* testing, including the Pizzle stick (Lane manufacturing), mare infusion pipette with syringe attachment, TRICH IT (Morrison Livestock Products, Develan, WI), smegma soaked gauze, gentle scraping of the prepuce with a microscope slide, washing of the artificial vagina after semen collection, and preputial lavage. There is no current consensus on the most effective collection device procedure to obtain the smegma and the means of collection may largely be clinician dependent. Historically, the diagnostic test of choice for identification of *T. foetus* in the bovine was organism culture which included three cultures taken at intervals of a week to two weeks following a period of sexual rest. With the development of PCR and RTPCR, the gold standard of identification has shifted and the need for culture has lessened.

The focus of this study was to determine if there are any additional reservoir sites of *T. foetus* in portions of the reproductive tract specifically, the urethra and the secondary sex glands

in addition to the penis and prepuce in bulls confirmed to be chronically infected with *T. foetus*. The identification of the organism in the distal or proximal reproductive tract of the bull has the potential to have detrimental effects on the possibility of a topical curative treatment in bulls.

CHAPTER 2: LITERATURE REVIEW

Description of protozoal organism: *Tritrichomonas foetus*

Tritrichomonas foetus is a flagellated, extracellular protozoan, able to penetrate the mucous barrier and is an obligate parasite of the reproductive tract of the bovine (Chapwanya, 2015). *Tritrichomonas foetus* belongs to the phylum *Parabasalia,* which is made up of the class amitochondriate, the order *Trichomonadea* and replicates via longitudinal binary fission (Pereira-Neves, 2009; Chapwanya, 2015; Collántes-Fernández, 2018).

Tritrichomonas foetus ranges in size from 10-25 μm in length and 3-15 μm wide (Yao, 2015). This protozoan exists in two distinct forms dependent on the environment it is currently inhabiting, the trophozoite and the multinucleated pseudocyst. When the environment is favorable for survival of the protozoan, the trophozoite will emerge. However, when conditions are unfavorable for survival the trophozoite, it will undergo specific changes, internalize its flagella, and complete its transformation into a pseudocyst (Pereira-Neves, 2009).

The trophozoite is comprised of a teardrop shaped cell containing one anterior nucleus, and spherical hydrogenosomes responsible for the metabolism of *T. foetus. Tritrichomonas foetus* exhibits three anterior flagella in addition to one posterior flagella (Rae, 2006; Yao, 2015; Dąbrowska, 2019). The single posterior flagella is located alongside a well-defined undulating membrane for approximately seventy five percent of the flagellar length and then terminates as a free flagellum on the posterior end of the body of the parasite (Dąbrowska, 2019**) (Figure 1).**

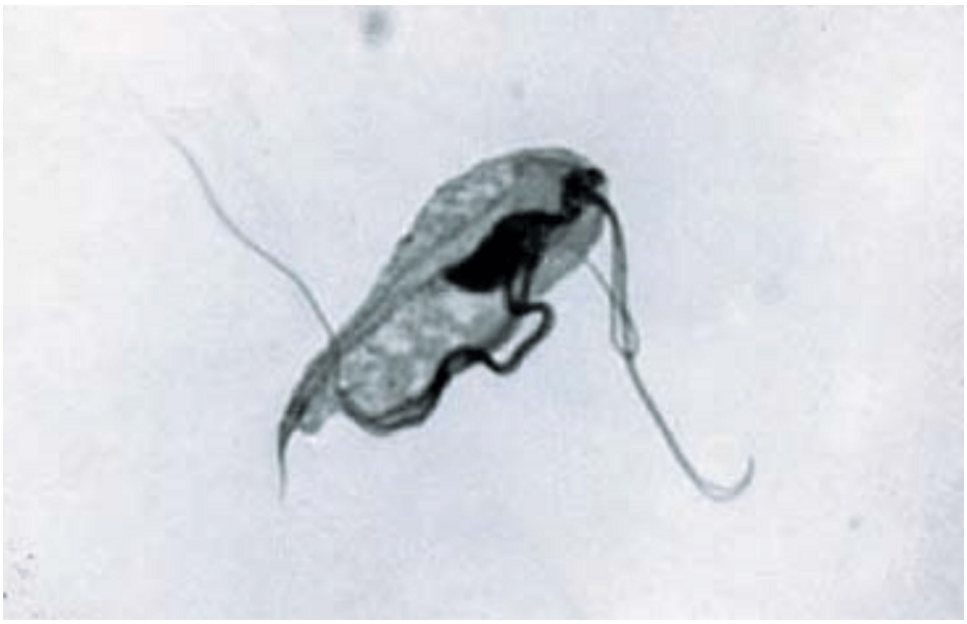

Figure 1. Image of *Tritrichomonas foetus* as seen on light microscopy. (Accessed on 4/2/2023 at https://www.vetlexicon.com/Vetstream/media/images/Bovis/Tritrichomonas-foetus-showing-three-anterior-flagella-and-trailing-posterior-flagellum.png?ext=.png)

The distinctive flagella seen are continuous with the cell membrane, speculating if the flagella are injured this could be detrimental to the cell membrane and the whole organism (Benchiomol, 1992; Granger, 2000). The undulating membrane demonstrates three to five waves along its length and is primarily responsible for the characteristic jerking movement of the parasite (Rae, 2006; Yao, 2015; Dąbrowska, 2019).

Tritrichomonas foetus exhibits a well-defined cytoskeleton that consists of an axostyle, pelta, and costa (Dąbrowska, 2019). The axostyle extends the length of the organism and projects posteriorly (Rae, 2006; Yao, 2015).

The pseudocyst form of the parasite lacks a cell wall (Pereira-Neves, 2003). While in the pseudocyst form, the parasites flagella will reside in the cell's cytoplasm (Granger, 2000; Mariante, 2004). The pseudocysts are multinucleated and contain up to eight nuclei, once the

environment is suitable for survival the nuclei will bud off as trophozoites from the pseudocyst (Pereira-Neves, 2009). Historically, it was believed that pseudocysts were an irreversible and degenerate form of *T. foetus*. However, a study by Pereira-neves et.al, proved that the multinucleated pseudocysts can undergo nuclear separation asynchronously or synchronously and revert to the trophozoite form (Pereira-Neves, 2009). This same study proved that nuclear separation is independent of cytokinesis. This specific type of mitosis is known as cryptopleuromitosis (Collántes-Fernández, 2018). These specific types of cell divisions allow further development and division of the nucleus but not the cell itself, leading to a polymastigont cell form (Pereira-Neves, 2009). Pereira-Neves, et al*.,* demonstrated that the pseudocyst form is capable of mitosis via a series of incubations where the parasite began in the trophozoite form transitioned to the pseudocyst form and then pseudocysts were transformed via a budding mechanism back to the trophozoite form. Trophozoite cultures grown for 36 hours at 37ºC and then decreased to 4ºC for 4 hours, when evaluated post incubation the pseudocyst form emerged with no observed change in the number of cells per milliliter or cell death (Pereira-Neves, 2009). In this same study, one group of the previously mentioned incubated pseudocyst samples were re-incubated at 37ºC for 4 hours, during this time the trophozoites reemerged and the number of cells per milliliter increased (Pereira-Neves, 2009).

Studies by Mariante et al., found that both the pseudocyst and trophozoite forms of *T. foetus* are capable of adhering to the host reproductive epithelial cells (Mariante et al*.,*2004). This study stated that the pseudocyst adhesion rate to the vaginal epithelium is significantly higher that of the trophozoite form (Mariante et al*.,* 2004). Additionally, the pseudocyst can cause significant cytotoxicity to the surrounding cells while in this form (Midlej and Benchimol

2010; Singh et al., 1999). This cytotoxicity mechanism is thought to involve phagocytic activity (Midlej and Benchimol 2010).

Three serotypes of *T. foetus* have been described, var *brisbane*, var *belfast,* and var *manley* (Rae, 2006). The current consensus is that the different serotypes do not play an important role in immunity against *T. foetus* as heifers that are immunized with one serotype strain develop resistance to the other serotypes as well (Rae, 2006; BonDurant, 1994). Although one serotype does not seem to be more significant than the other in stimulation of immunity, their importance lies in their ability to be cross protective (Rae 2006).

Pathophysiology of *T. foetus* in the female bovine

When a female bovine is infected with *T. foetus,* the disease can manifest as many forms of transient reproductive failure. The female is infected with *T. foetus* through coitus from an infected bull or through coitus with a bull serving as a vector that has bred an infected cow within the last 20 minutes (Clark, 1977; Rae, 2006, Ondrak, 2016). After insemination. *T. foetus* adheres to the vaginal epithelium, where it will enter the uterus via the cervix. Within one to two weeks, *T. foetus* will have colonized the entire reproductive tract including the cervix, uterus, and uterine tubes (Anderson, 1994; Rae, 2006, Chapwanya, 2015). *T. foetus* has been identified on the vaginal and endometrial epithelium, as well as the endometrial glandular lumen (Midlej & Benchimol 2010; Parsonson, 1974; Anderson, 1996; Rhyan, 1999). Transmission via fomites such as vaginal speculums and contaminated artificial insemination rods are possible, emphasizing the importance of equipment cleanliness. Fertilization often occurs even in the face of active infection and the conceptus will often live past the maternal recognition of pregnancy, leading to irregular inter-estrus intervals (BonDurant, 2007).

Once infected most female cattle undergo a transient period of infection. Experimental inoculation performed by Skirrow and BonDurant showed infection present up to 28 weeks post inoculation (Skirrow&Bondurant; Rae, 2006; Michi, 2016). An outbreak in Queensland, Australia managed by Alexander, revealed infection present in heifers up to 22 months post natural infection (Alexander, 1953). Less than one percent of infected females will become persistently infected with *T. foetus* (Rae, 2006).Persistently infected females are described as any female who carries the infection with *T. foetus* through gestation and into the subsequent breeding season (Skirrow, 1987; Rae, 2006). Chronically infected females have the potential to prevent the clearance of *T. foetus* from a positively infected herd. Chronically infected females

can serve as a nidus of infection for naïve animals or animals who have successfully cleared the infection and no longer have adequate immunity. Affected herds have the ability to be rehabilitated through the confirmation of a negative bull and the females being isolated and rolled into a closed herd with another breeding season. The cows should be confirmed pregnant at the end of the designated breeding season with the open cows being culled. Mature bulls confirmed negative for the presence of *T. foetus* should be utilized as a terminal sire, tested for the presence of *T. foetus* at the end of the breeding season and then culled. The presence and quantity of *T. foetus* in the cervico-vaginal mucous of the persistently infected female waxes and wanes during the estrous cycle, making diagnosis a difficult task (Yule, 1989). The most reliable time to sample the cervico-vaginal mucous is within several days of standing heat as the number of trichomonads present seem to be at their highest level (Yule, 1989).

Reproductive pathology in the female including embryonic loss, vaginitis, cervicitis, post-coital pyometra, endometritis, irregular inter-estrus intervals, and late term abortion (Michi, 2016). The most often missed clinical signs of infection are the vaginitis and cervicitis due to the lack of overt clinical signs. When observed these clinical signs are often manifested as a vaginal discharge except for pyometra.

Embryonic and fetal loss is the most well documented sequel in the female infected with *T. foetus* (Bondurant et al., 2007; Benchimol et al., 2007; Corbeil et al., 1998.; Waters & Gard 2021). The rate of infection clearance and return to estrus is depended on when the embryonic loss occurs and the immunity of the cow/heifer (Bondurant, 2007; Benchimol et al., 2007; Corbeil et al., 1998). In 1972, the Committee on Bovine Reproduction Nomenclature defined the death of a conceptus as the following: embryonic loss prior to day 42 of gestation, abortion as death after day 42 and before day 260 of gestation, and a still born death after day 260 of

gestation; as the fetus can survive if born at this time (Thurmond, 1990). If death of the conceptus occurs before the maternal recognition of pregnancy at 16-17 days, then it is possible the female could normal return to estrus in a normal timeframe (Anderson, 1994).

Despite the presence of active infection, fertilization often occurs, and the conceptus survives through the time of maternal recognition of pregnancy (BonDurant, 2007). It is common that the females will have an irregular return to estrus. This prolonged return to estrus can be interpreted as a presumed pregnancy leading to economic loss for producers (BonDurant, 2007). Studies have shown that in vitro embryos are cultured in combination with *T. foetus* that there is little to no impact on the conceptus through the blastocyst hatching stage (BonDurant, 2007). Although, other studies involving in vitro evaluation of oocytes and cumulus cells have shown *T. foetus* to cause severe damage to these cells (Benchimol, et al*.,* 2007). When *T. foetus* is present in vivo in the uterine lumen, the organisms will surround the conceptus as the extra fetal membranes form (BonDurant, 2007). *Tritrichomonas. foetus* will then be entrapped in the amniotic membrane encasing the fetus allowing the fetus to eventually ingest the protozoan, allowing the identification of the organism in the abomasal fluid of the conceptus (BonDurant, 2007). Although less common, abortion beyond four months of gestation has been observed (Anderson, 1994). Retained deceased fetuses can be observed in up to five percent of exposed females (Anderson, 1994). Lesions have been identified in both the extra-fetal membranes, and the fetus from *T. foetus* abortions (Dąbrowska, 2019). Reported placental lesions include placental edema, placentitis, lymphocytic and histiocytic chorionitis, along with trophoblast necrosis (Dąbrowska, 2019). Rhyan et al., demonstrated the presence of *T. foetus* in the chorionic stroma of the placenta (Rhyan, 1988). The most commonly observed *T. foetus* fetal lesions are

pyogranulomatous bronchopneumonias with trichomonads present in the bronchioalveolar fluid (Rhyan, 1988; Dąbrowska, 2019).

Post coital pyometras occur in less than five percent of infected females however, pyometras and abortions are often the first seen clinical signs (Rae, 2006). The diagnosis of pyometra is made during trans-rectal palpation at the time of pregnancy evaluation. The findings are as follows; a purulent fluid filled uterus identified via palpation and/or ultrasound of the uterus. It is possible to find trichomonads present in the purulent vaginal fluid (Bondurant, 1997). However, the destructive nature of the bacteria present may interfere with the identification of the parasite (Clothier et al., 2019; Clothier et al., 2015). Metritis and/or vaginitis can be seen and is manifested as a vaginal discharge and will often lead to a reproductive examination of the animal. Macerated fetuses are occasionally found on transrectal palpation and ultrasound (Michi 2016; Bondurant 2007; Rae 2006; Anderson 1994; Dabowska 2019).
The immune response of the female reproductive tract to exposure of *T. foetus* has been extensively evaluated (Michi 2016; Rae 2006; Bondurant, 2007: Skirrow, 1990; Corbeil 1998; Corbeil, 2005; Corbeil, 2008, Singh, 2004). However complete understanding is relatively illusive. Female cattle exposed to *T. foetus* experience an antigen specific antibody response in the caudal reproductive tract (Michi, 2016). Skirrow et al., identified as few as $7x10^6$ organisms inoculated in the female reproductive tract induced production of *T. foetus* specific IgG1 and IgA antibodies in the vaginal mucous (Skirrow, 1990. Michi, 2016) Peak production of the antibodies differed depending on the location they were present in the reproductive tract. Vaginal and cervical IgA and IgG1 peaked at 7-9 weeks post infection, whereas the uterine IgA and IgG1 levels peaked at 10-12 weeks post infection (Skirrow, 1990. Michi, 2016). It is speculated that the immune response is due to a masking of the local immunity via the parasites ability to mask

antigens or digest both specific innate and acquired immunity proteins (Michi, 2016). *T. foetus in vitro* released the extracellular proteinase virulence factor, *T. foetus* cysteine proteinase (TFCP),that is responsible for the digesting of the host's reproductive tract immunity proteins (Thomford, 1996). Immunity acquired by inoculated or natural infection provides decreased length and severity of subsequent disease but does not reliably prevent reproductive failure (Edmondson, 2016; Michi, 2016, Oretag-Mora et al*.,* 2022). The immune response seen with vaccination against *T. foetus* is primarily seen as increase the serum and genital secretion level of IgG (Cobo, 2011)

Infertility is another negative effect of *T. foetus* infection in the female bovid. However, the mechanism underlying has not always been clear. Infection of the uterus and the uterine tubes and the cytotoxic and hemolytic effects induced by *T. foetus* have been thought to be the responsible for the cases of infertility in the female (Anderson et al., 1996; Benchimol, et al., 2007, Burguess et al., 1990). Benchimol et al., looked further at the causes of T. *foetus* induced infertility by assessing *T. foetus* interactions *in vitro* with bovine cumulus cells and oocytes (Benchimol, et al., 2007). Scanning electron microscopy (SEM) revealed that exposure of oocytes to *T. foetus* caused rapid adhesion of the trichomonads to cumulus cells and the zona pellucida of the oocytes followed by penetration of the ZP (Benchimol, et al., 2007). The induction of apoptosis including chromatin condensation, and cytoplasmic vacuolization seen in *T. foetus* infected cumulus cells and oocytes could explain the infertility associated with *T. foetus* infections in the female (Benchimol, et al., 2007).

Pathophysiology of *T. foetus* in the male bovine

Bulls infected with *T. foetus* are chronic, asymptomatic carriers of the venereal disease Trichomoniasis. Bulls are infected as a result of coitus with an infected female. Neither chronically infected or transiently infected bulls exhibit any penile or preputial lesions, nor are there reports of a negative impact on libido (Parsonson, 1974; Waters & Gard, 2021). Although there are no outward lesions or clinical signs of infection, Ribeiro et.al, showed that *T. foetus* has detrimental effects on spermatozoa (Ribeiro, 2010). This study identified extracellular products excreted by *T. foetus* had a significant negative impact on sperm motility. The extracellular substances did not initially lead to outright loss of sperm viability. However, the results of the Annexin-V/propidium iodide assay and culture indicated cellular damage to the sperm cells due to *T. foetus* (Ribeiro, 2010). Benchimol et al., evaluated the susceptibility of bovine sperm to the presence of *T. foetus* (Benchimol, 2008). This study revealed that when exposed to *T. foetus*, the sperm undergo an initial tropism, followed by adhesion of the trophozoite to sperm cells, along with severe sperm agglutination (Benchimol, 2008). This same work showed that the sperm attached to the trichomonads were eventually phagocytized by the parasite into an intracellular vacuole and digested via lysosome degradation (Benchimol, 2008).

Transmission from an infected female to a naïve bull can require up to six breedings whereas, an infected bull can infect a naïve female with only one breeding (Jin, 2020). Reports as high as 95% of naïve cows bred by chronically infected bulls one time become infected (Dewell, 2016). The possibility of bull-to-bull spread has been discussed, as young bulls express homosexual mounting of other bulls. It is possible smegma could be left behind on the hair of the mounted bull and if mounted by another naïve bull the organism could be spread (BonDurant, 2007). In a study by Parker et al., virgin bulls were co-mingling with *T. foetus* positive bulls

(Parker et al., 2003a). *Tritrichomonas. foetus* was diagnosed in a virgin bull following introduction to *T. foetus* positive bulls (Parker et al*.,* 2003a). It was not known if the virgin bull was positive prior to co-mingling with positive bulls (Parker et al., 2003). Additional studies are needed to determine if this is a real concern. This concern in bull studs is combated by the disinfecting of the rump of the teaser animal. It is unlikely that this pathway of transmission is seen in a natural breeding setup as the time of exposure of the organism to the environment and ultraviolet light would most likely kill the parasite (BonDurant, 2007). This transmission is even less likely in certified semen services (CSS) approved bull studs as there is current rigorous *T. foetus* testing performed during isolation and prior to semen collection.

Tritrichomonas foetus is harbored on the non-keratinized, stratified squamous epithelium of the bull's reproductive tract, specifically the penis and the prepuce (BonDurant, 2007). *T. foetus* has been identified to inhabit the distal urethra (Parsonson, 1974). However, the secondary sex glands including the more proximal urethra, testes, and epididymis from these 15 naturally infected *T. foetus* bulls were culture negative for *T. foetus* (Parsonson et al., 1974). This study was completed prior to the use of PCRs (Parsonson, 1974). Rhyan et al., used immunohistochemical staining in evaluation of the epididymides, prostate and seminal vesicles for *T. foetus* but was not able to detect the protozoan's presence in this study (Rhyan et al.,1999). In a recent study by Lovelady (Master's Thesis), all the accessory sex glands from 20 *T. foetus* naturally infected bulls were tested for *T. foetus* via culture for live organisms and PCR for *T. foetus* DNA (Lovelady, 2013). All tissues were collected at an abattoir. All accessory sex glands were sampled, with two tissue samples taken from each gland. All samples were placed in MDM. One of samples was utilized for culture and was observed daily for *T. foetus* over four days with no detection (Lovelady, 2013). The second tissue sample was macerated prior to DNA

extraction and then underwent PCR, and gel electrophoresis. A single prostate sample was identified as positive for *T. foetus* using this PCR and on comparative DNA sequencing (Lovelady, 2013). There was not a reported CT value for this study as it was run out on a polyacrylamide gel. It could be this was accidental contamination due to sampling error or a laboratory error. If the CT value lies in the range of 38-40 then it is considered a weak reaction indicative of minimal amounts of target nucleic acid, which can represent possible infection or environmental contamination. However, this was not available in this study. It is important to note that the maceration procedure prior to DNA extraction could have been damaged the DNA and more samples could have been positive. Additionally, the PCR procedures have improved since this study, so this could have affected the ability to detect *T. foetus* DNA in the accessory sex glands in the bulls tested.

Unlike what is reported in bulls, aside from Lovelady's findings, the urethra and the prostate are reservoir sites for men infected with *Trichomonas vaginalis* (*T. vaginalis*) (Van der Pol et al., 2021; Lovelady, 2013: Gardner et al., 1986; Rhyan 1999; Parsonson 1974). Testing modalities including the urine of men have been utilized with success (Van der Pol et al., 2021). A multicenter, prospective study evaluated the performance of the cobas *T. vaginalis*/*Mycoplasma genitalium* (TV/MG) assay for detection of *T. vaginalis* DNA in symptomatic and asymptomatic patients (Van der Pol et al., 2021). In men, the cobas TV/MG assay was 100% sensitive for the detection of *T. vaginalis* in both male urine samples and meatal swabs, with specificity of 98.4% in urine samples and 92.5% in meatal swabs (Van der Pol et al., 2021). It could be a similar urine test could be made in bulls for *T. foetus*. However, collection of urine would not be as easily obtained as a preputial scraping, but it might be that an additional testing modality might prevent false negatives from occurring when testing for *T. foetus* in bulls.

Historically, it was believed that the prepuce of the bull contained crypts on the mucosal surface of the prepuce and as the bull increased in age the crypts became larger and more cavitated allowing *T. foetus* to inhabit the tract undetected (Strickland et al., 2014; Beckett & Wolfe, 1998; Hammond & Bartlett 1993; Hafez, 1993). However, it has been since proven that the prepuce is not a mucosal surface but instead has epithelium that is consistent with skin and there are no preputial crypts present (Strickland, 2014). A study by Strickland et al., found that there were no defined preputial crypts present on the penis and prepuce of the bulls. This study evaluated the structural differences in the epithelium of bulls two years of age and mature bulls greater than five years of age. This group identified that there were no differences in any of the following areas including the area encompassed by epithelial folds, number of epithelial folds, depth of epithelial folds, or the surface area of the penile and preputial epithelium (Strickland, 2014).

Similar to the immunologic response of the female, the bull experiences elevated levels of specific immunoglobulins when compared to naïve controls (Rhyan, 1999). In confirmed infected smegma, the highest identified concentration of a specific immunoglobulin was IgG1. IgM and IgA levels were approximately the same concentration, and a small amount of IgG2 response was reported (Rhyan, 1999). *Tritrichomonas. foetus* is consistently found on the epithelial surface of the reproductive tract but is unable to penetrate the basement membrane of the tissue (Ryhan, 1999; Michi, et al., 2016). Numerous studies have given support to restriction of *T. foetus* to mucosal surfaces and does not have the ability to invade tissues (Michi et al., 2016). However, studies have shown the ability of *T. foetus* antigens in epithelial cells to interact and react with stromal antigen presenting cells (Michi et al., 2016). It is interesting in that *T.*

foetus can consume sperm but not invade the basement membrane of the reproductive tract (Benchimol et al., 2008; Parsonson et al., 1974).

It is well documented that bulls greater than four years of age are more likely to become chronic carriers, and young bulls less than 4 years of age are often able to clear the infection (Collántes-Fernández, 2018). The mechanism by which the organism is cleared is still not well understood. However, a recent study by Rush et al., different immune cell populations were evaluated, and significant differences were found between young and mature bulls (Rush et al., 2019). Twelve *Bos taurus T. foetus* negative bulls were utilized in the study. Six young bulls, 14-24 months of age, and six mature bulls, five years and older, had biopsies taken three locations along the penis (Rush et al*.,* 2019). Slides of the biopsies were stained with hematoxylin and eosin and immunohistochemistry was performed using markers IBA1+, CD79a + and CD3+ for identification of macrophages, T-lymphocytes, and B-lymphocytes, respectively (Rush et al., 2019). Inflammation scores marginated neutrophil infiltration scores, CD3+ T cell numbers, CD3+ T cells numbers around vessels, CD79a+ B cell infiltration scores within lymphoid nodules, IBA1+ cell numbers in the epidermis, IBA1+ cells numbers in the superficial dermis, epidermal dermal junction basement membrane disruption scores, and epidermal junction cellular hyperplasia scores were all found to be statistically different ($p<0.05$) between the two groups (Rush et al*.,* 2019). Young bulls had an increased presence of inflammatory cell infiltrate when compared to the mature group ($p<0.05$) (Rush et al*.,* 2019). The mature bulls had greater presence of B-lymphocytes (CD79a+) and macrophages (Iba1+) than (what was seen in) the young bulls ($p<0.05$) (Rush et al*.,* 2019). These results suggest an age-associated change in the number and type of inflammatory cells within the penile and preputial epithelium in bulls. These cellular differences may be key in clearance of *T. foetus* from the penis and prepuce in bulls.

The ability of the trophozoite form of *T. foetus* to adapt and transform into the pseudocyst form is vital for survival. The normal pH of the bovine reproductive tract is 7.4-7.8. This neutral to more basic environment is ideal for the growth of *T. foetus.* Morin-Adeline et al., showed that a slight acidic change in the pH of the environment pH of 7 to a pH of 6 caused significant decreases in both the viability of the trophozoite itself, along with an increase in cell granularity p-value <0.003 and <0.00002, respectively (Morin-Adeline et al., 2015). This same study also revealed that there was a distinct lack of the pseudocyst form in this acidic environment (Morin-Adeline et al., 2015).

Review of sampling methods for *T. foetus*

Identification of *T. foetus* in the infected bovine is performed by the sampling of smegma from the preputial cavity of bulls, or the cervico-vaginal mucus of the female (Parsonson, 1976; Collántes-Fernández, 2018). In the female positive samples can be obtained as early as two weeks post-infection however, the number of parasites present fluctuates with the stage of estrus (Collántes-Fernández, 2018). In the bull, the sample of choice for the diagnosis of *T. foetus* is preputial and penile smegma. The collection of smegma from the male is more reliable as hormone fluctuations and cervical patency do not play a role in presence or quantity of the parasite as is seen in the female.

Most commonly smegma samples are obtained at the time of the recommended yearly Breeding Soundness Examination approximately sixty days prior to the beginning of breeding season. Sampling at this time allows the producer time to identify sub-fertile or infectious bulls in their herd, as well as adequate time to identify a new herd bull if required. Often at this time the bulls are not housed with cows which aids in the recommendation that regardless of the chosen collection device, the bull undergo one to two weeks of sexual rest prior to collection for the detection of *T. foetus* (Peter, 1997; Ondrak, 2010). Additionally, a study by Parker et al., found that smegma collected from the right side of the bull's prepuce had a higher sensitivity (96.1%) when compared to collection from the left side of the bull (88.8%) on smegma culture (Parker, 2003). This study possessed limitations that included that PCR was not available at the time and only cultures were performed. The reasoning for the difference in positive testing for the side of collection was not determined. However, there are many speculations for the result, such as the anatomy of the bull penis. The bull's penis includes a counter-clockwise turn to the right (Parker, 2003). This may increase the likelihood of the protozoan inhabiting that portion of

the tract and that all the samples were taken by a veterinarian who was right-handed (Parker, 2003). The speculation was made that sample collection with the collector's dominant hand yields a better sample (Parker 2003). The speculation behind this suggests that the dominant hand will have a more controlled, firm, and consistent collection technique (Parker, 2003). This same study by Parker et al., saw no statistical difference when the number of preputial scrapings were increased from 10 to 20 applications along the prepuce (Parker et al., 2003).

Through the years numerous smegma collection methods and devices have been used with equivocal results. Although many sampling options are described, all the sampling techniques utilize the concept of the scraping/wiping of the penile and preputial epithelium for the collection of smegma and the accompanying *T. foetus* organisms (Parker, 2003). At this time there, no specific sampling method has been deemed the gold standard. Available options for the collection of smegma from the bull include; preputial smegma aspiration and scraping utilizing: infusion pipette with attached syringe, Pizzle stick (Lane Manufacturing, Denver CO), Tricamper™ (Queensland DPIF, Brisbane QLD), TRICHIT™ (Morris Livestock Products, Delavan, WI); preputial scraping/exfoliation utilizing: an microscope slide, or metal pipette (Sutka, 1969); preputial swabbing with a 16-ply gauze sponge (Dewell, 2016) or cotton tip applicator (Fitzgerald, 1952); and artificial vaginal post collection washing (Parsonson et al., 1976; Gregory et al., 1990).

Once smegma is collected, the sample is then placed in Modified diamond's media, or phosphate buffered saline and submitted for PCR with or without culture prior to PCR. With the development of each collection method there have been various studies to help in identifying the most reliable means of collection (Parker 2003; Dewell et al., 2016; Lammers 2015).

Reliable preputial smegma collection is the goal of all of the devices and methods used. When scraping the prepuce with or without aspiration, it is recommended to collect the animal from the side of the chute that correlates with the sampler's dominant hand (Parker, 2003). When utilizing devices such as the Pizzle stick or an infusion pipette with a syringe, the thumb of the non-dominant hand is placed in the ventral aspect of the preputial orifice and gentle pressure is used to extend down the haired portion of the prepuce and allowing the thumb to be a guide to the collection device. Once the thumb is in place and the sheath somewhat taut, the collection device is placed over the thumb and gently advanced to the level of the preputial fornix. Once the collection device is at the level of the fornix, the non-dominant hand is placed on the haired prepuce at the level of the proximal end of the collection device and gentle massage of the preputial area is performed. Current with the massaging of haired prepuce with non-dominant hand, the dominant hand is applying gentle movements to the collection device proximal and distally to the device along the prepuce 5-10 times while aspirating if required for the specific device.

Smegma collection devices vary in the way the collection is performed, however all have the main concept of the collection of smegma through the gentle scraping of the prepuce. Several of the preputial scraping devices such as the Pizzle stick and Tricamper™ can be utilized successfully with or without the use of a syringe for aspiration as they both possess corrugated blunt ends that successfully collect smegma in the grooved end of the device. One collection device that relies almost solely on syringe for aspiration is the infusion pipette. The pipette is a smooth, opened ended plastic tube that has a rubber stopper on the distal end that allows the connection of the syringe for aspiration, 15 milliliters of air aspiration with each pass of the pipette is recommended (Parker, 2003). Preputial scraping/exfoliation of the extended penis can

be performed with a gauze pad, microscope slide or with a cotton tip applicator. Dewell et al., validated the collection of smegma for testing of T. foetus by PCR testing via the 16- ply gauze sponge, by utilizing 111 bulls in a high-risk area with known *T. foetus* outbreaks. The bulls were sampled with both an infusion pipette and a gauze sponge with penis extended for sample collection (Dewell, 2016). This study found no significant difference between the 2 sampling modalities with a sensitivity for preputial scraping and penile sponging of 92% (CI: 69-98), and 95% (CI: 82-99) respectively (Dewell, 2016). The limiting factor for this sampling technique is that extension of the penis is required and if performed too aggressively then preputial or penile irritation can occur. The required extension of the penis is not a major downfall if done at the time of breeding soundness examination as full extension of the penis is required. However, if the bull only requires testing for *T. foetus* then extension of the penis using an electro-ejaculator or digital massage (which is not always a reliable means of penile extension) of the accessory sex glands must be utilized. Lammers et al., examined the PCR sensitivity of two collection methods after smegma collection using a dry infusion pipette with attached syringe to aspirate and the use of a smegma swabbing with a gauze while the penis was at full extension (Dewell 2016; Lammers, 2015). The results from this study showed that there was not significant difference in the collection techniques sensitivity ($p>0.05$) (Lammers, 2015).

Description of Proper Sample Handling

Historically, *T. foetus* was diagnosed based on the presence of the protozoan and its characteristic jerking movement (Rae, 2006). The culture of *T. foetus* utilized a modified diamonds media to support the parasite will in transit (BonDurant, 1997; Diamond, 1957). While this media was the transportation method of choice, the way in which the sample was handled could have detrimental effects on the results leading to higher incidences of false negatives (Mukhufhi, 2003). For the most accurate results, it is recommended that the culture be placed in the incubator as close to collection as possible, however this is seldom a direct occurrence. As small as a delay as 24 hours, there can be up to a 10% loss of sensitivity of the sample due to the death of the parasite when utilizing culture testing alone (Mukhufhi, 2003). Mukhufhi et al., speculates that the degradation enzymes possessed by the parasites, can cause deterioration of the nucleic acid of the parasite, resulting in decreased sensitivity of the PCR and leading to false negative results (Mukhufhi, 2003). The same group performed a study that looked at the sensitivity of PCR and culture of *T. foetus* they found that after 5 days of storage there was a significant decrease in sensitivity (Mukhufhi, 2003).

Proper storage is vital in successful identification of *T. foetus* no matter the media that is being utilized. After collection of the smegma, the sample should be stored at room temperature, roughly 22ºC and out of direct sunlight, the sample should not be placed on ice as improper handling can kill the parasite or allow contaminants to grow in the media (Cobo, 2007). In recent studies by Clothier et al., concurrent bacterial contamination and biological materials were shown to affect the ability to detect *T. foetus* in cultures and in PCR (Clothier et al., 2015; Clothier et al., 2019). Improvement of molecular techniques have resulted in the development of a PCR that does not require culture medium (Summarrell et al., 2018). The ability to test

smegma for *T. foetus* reliably without the use of a culture media, has the ability to decrease the incidence of false negatives due to fewer errors of sampling handling.

Available diagnostic testing for T. foetus

Diagnostic testing for *T. foetus* includes culture of the organism followed by direct identification via light microscopy, staining of the organism to evaluate anatomic characteristics and/or molecular testing via PCR. Use of RTPCR provides the ability to identify the organismal DNA, regardless of whether the organism is alive or dead. The ability to identify the organism via the presence of specific DNA greatly improves both the sensitivity and specificity of our diagnostic tests.

For numerous years, repeat cultures on smegma samples placed in Modified Diamond's Media was the standard protocol for a definitive negative diagnosis of *T. foetus* (Cobo et. al, 2003). Culture of the sample allows the organism to replicate and thus increase the number of identifiable parasites present, allowing for a more accurate result of the diagnostic test. Lovelady et al. described the results of a negative *T. foetus* culture to mean one of the following: the bull is not infected with *T. foetus*, no organisms were identified but were actually present and the bull is truly infected, no organisms were collected at the time of sample collection, or the organisms collected failed to replicate and were not identified at the time of the test results (Lovelady, 2013).

Bondurant et al. identified the ability of a false negative utilizing only culture and the jerky motion of the visualized trichomonad (Bondurant et al., 1999). The aforementioned study combined the identification of the jerky movement along with morphologic evaluation of the trichomonad via Diff Quick™ staining. The results showed that the trichomonads identified possessed anatomic characteristics that were not consistent with bovine *T. foetus* and suspected the presence of fecal trichomonads (Bondurant et al., 1999). Cobo et al. utilized scanning electron microscopy and PCR to analyze trichomonads found in the preputial smegma of bulls,

finding that the trichomonads varied morphologically and possessed differing DNA from *T. foetus* (Cobo et al, 2003).

Currently, RTPCR is the most commonly utilized diagnostic test for detection of *T. foetus*.. Often one negative result is deemed appropriate for the conformation of a negative animal. Benefits of the RTPCR over culture include a faster turnaround time as the sample does not always have to include culture and that detection of nuclei acid is not dependent on if the organism is alive or dead if present in high enough amounts.

Researchers recently developed a direct reverse-transcription quantitative real-time PCR (direct RT-qPCR (Summarell et al*.,* 2018). An automated nucleic acid purification method was utilized in this diagnostic test which reduces human labor and enables high-throughput sample processing (Summarell et al*.,* 2018). The direct RT-qPCR exhibited 100% diagnostic sensitivity and 100% specificity, whereas the currently employed qPCR (culture qPCR), which utilizes cultured samples, exhibited 95% diagnostic sensitivity and 100% specificity (Summarrell et al*.,* 2018). More positive samples with lower quantification cycle (Cq) values were reported, direct RT-qPCR Cq range = 14.6–32.3 vs. culture qPCR Cq range = 18.7–37.4) (Summarell et al*.,* 2018). This technology would be very applicable in research applications as well as providing faster more reliable results.

Prevention of Bovine trichomoniasis

A with standard health maintenance a biosecurity protocol is necessary for control and prevention of *T. foetus*. Current prevention protocols include testing all new bulls prior to being introduced with the herd, vaccination of heifers in areas where *T. foetus* is prevalent, and controlled breeding seasons and pregnancy evaluations 3 days post breeding season to identify any subfertility in the herd (Waters, 2021). Vaccination of heifers was proven by Edmondson et al., who showed that 2 doses of a killed vaccine, Trichguard®, (Boehringer Ingelheim, Vetmedica, Inc., St. Joseph, MO, USA).), administered 14 days apart 60 days prior to the breeding season (Edmondson 2016). Females inoculated with the killed vaccine resulted in decreased time to clearing of the infection and improved conception rates in the late breeding season post infection when compared to placebo vaccinated heifers (Edmondson, 2016). There was a significant difference (P = 0.048) in live births between the sham-vaccinated heifers (20%) compared with (50%) of the vaccinated heifers (Edmonson, 2016). Embryonic or fetal loss was detected in 47% of vaccinated heifers and 71% of sham-vaccinated heifers but a significant difference was not detected between the two groups (P = 0.153) (Edmondson, 2016). However, there was a 24% increase in embryonic and fetal losses in the sham-vaccinated group which could have potentially been significant if larger numbers of animals were utilized in this study.

A new non-commercially available vaccine, Trichobovis®, against *Tritrichomonas foetu*s infections in cattle was tested in a recent study (Ortega-Mora, et al., 2022). The vaccine is made of trophozoites that have been inactivated by lyophilization and Quil-A-adjuvanted (Ortega-Mora, et al., 2022). The study compared Trichobovis® (CZ VETERINARIA and SALUVET-Innova; Madrid Spain) to TrichGuard® (Boehringer Ingelheim). Cows immunized with Trichobovis® (CZ VETERINARIA and SALUVET-Innova) cleared the infection faster than the

non-immunized/challenged group (27-28 vs. 60 days; $P < 0.05$) but there was not a significant difference between the two vaccines (Ortega-Mora, et al., 2022). The new vaccine stimulated high serum anti-*T. foetus* IgG and genital IgA levels and generated an IgG booster effect similar to what is seen with vaccination with TrichGuard® (Boehringer Ingelheim) (Ortega-Mora, et al., 2022). IgA levels were associated with significantly earlier genital clearance of *T. foetus* in cows immunized with Trichobovis® (CZ VETERINARIA and SALUVET-Innova) or TrichGuard® (Boehringer Ingelheim) versus negative controls (Ortega-Mora, et al., 2022). The Trichobovis® (CZ VETERINARIA and SALUVET-Innova) vaccine was investigated in a *T. foetus* positive herd. The Trichobovis® (CZ VETERINARIA and SALUVET-Innova) vaccinated group improved reproductive performance in the *T. foetus* positive herd by significantly reducing calving intervals by 45.2 days ($P < 0.05$), with calves more calves born earlier (28 days) in the calving season ($P < 0.05$) and an increase of 8.7% in the calving rate ($P > 0.05$) (Ortega-Mora, et al., 2022). Hence, the use of vaccines can be beneficial in reduction of losses associated with *T. foetus* in cattle herds.

Control of Bovine trichomoniasis

Currently, there is no approved treatment for *T. foetus* in the USA. It has been shown in a recent study, that metronidazole can clear infection from a positive bull (Love, et al., 2017). In this study, the five pre-treatment cultures were positive, and all five post-treatment cultures were negative resulting in a significant difference ($P < 0.05$). The issue is that metronidazole is prohibited for use in food animals (Love, et al., 2017). *In vitro* studies involving formulas of benzimadazoles and pluronic lecithin organogels (PLOs) have shown marked success by killing *T. foetus* in culture (Niehaus et al., 2013: Koziol, et al., 2017). In recent clinical studies, six *T. foetus* naturally infected bulls that were topically treated with the patented formulas (US patent 11,160, 867, continuation 17/487,078) of benzimidazole PLO paste applied to the penis and prepuce (Duran and Schnuelle et al., 2022 and unpublished studies). Concurrent to topical treatment, simultaneous orally administration of Synanthic® (Boehringer Ingelheim), an approved cattle dewormer consisting of 22.5% oxfendazole, at a dosage of 4.5 mg/kg was given (Duran and Schnuelle et al., 2022 and unpublished studies). The bulls were determined to be culture negative for live organisms on weekly cultures for six weeks and one bull was culture negative for 11 months (Duran & Schnuelle et al., 2022 and unpublished studies). The results from these recent clinical trials were similar to the study by Love et al., but longer cure rates were achieved (Love, et al., 2017; Duran & Schnuelle et al., 2022 and unpublished studies et al., 2022). The issue of potential reservoir sites of *T. foetus* creates reinfection of the penis and prepuce and complicates the ability to completely clear *T. foetus* in bulls (Parsonson et al., 1974; Lovelady 2013; Rush *et. al.,* 2020; Duran & Schnuelle et al., 2022 and unpublished studies). Lowering the pH into the acid range (pH = 6) has been found to significantly affect *T. foetus* growth (Morin-Adelin, et al., 2015). Hence, feeding ammonium chloride to lower the urine pH

along with the application of recent therapies utilizing benzimidazoles and PLO gels might have promising results in the removal an elimination of *T. foetus* from the urogenital tract of chronically infected bulls. However, more clinical trials are necessary to determine if indeed *T. foetus* can be eliminated from the urogenital tract of bulls.

Utilizing current technologies involving nanoparticles holds promise for elimination of *T. foetus* from the urogenital tract of bulls. In a recent study by Fang et al., metronidazole-loaded nanoparticles were tested *in vitro* and *in vivo* (Fang et al., 2022). The *in vitro* trials comparing metronidazole standard solution to the formulation with metronidazole nanoparticles, the metronidazole nanoparticle formulation zone of inhibition was over five times that of metronidazole, 44.25 m and 8.54, respectively. The metronidazole nanoparticle formulation out preformed metronidazole standard solution treatment in *in vivo* trials in mice (Fang et al., 2022). The metronidazole nanoparticle formulation resulted in a complete cure of *T. vaginalis* within three days of the onset of treatment, but the metronidazole standard solution did not result in a cure (Fang et al., 2022). The use of the of the metronidazole nanoparticle formulation may be able to reduce more resistant strains of *T. vaginalis* from developing due to a greater drug utilization (Fang et al., 2022). The use of nano-chitosan technologies may hold the key to clearance of *T. foetus* in cattle in the future.

The current standard for the control of Trichomoniasis is identification and removal of positive animals. Each state possesses their own regulations for the control and movement of animals over the age of 18 months of age with an emphasis on non-virgin bulls. The current regulation for non-virgin bulls and bulls greater than 18 months of age entering the state of Alabama is that the animal must have tested negative for *T. foetus* on PCR within 30 days prior

to entry, in addition to a Certificate of Veterinary Inspection signed by an accredited veterinarian with the associated negative results (Waters & Gard 2021).

Due to the lack of an approved treatment, bulls that test positive are culled and sent to slaughter. In large naïve herds which have experienced detrimental reproductive losses due to the introduction of a *T. foetus* bull, unpublished results by Hopper et al., have showed promising results for recovery of the females in the herd by repetitive induction of estrus via administration of Lutalyse® (Zoetis; Parsippany, New Jersey) prior to breeding. One large cattle operation post *T. foetus* outbreak was managed via the combination of any open female that were housed in the same pasture as a *T. foetus* positive identified bull. These females were kept the duration of a 90-day breeding season and housed with an older *T. foetus* negative bull. At the end of the breeding season the cows were evaluated for pregnancy and had satisfactory pregnancy results. At this time any open cows and the bulls that were housed with the females were culled. The pregnancy females continue to be housed together and an older terminal sire is used each year for this herd. This is only reasonable in herds where the genetics of the herd are worth saving, ample pasture and/or cheap feed is available to feed open animals. In most cases, culling open or infected animals is still the best option.

Chapter 3

Detection of *Tritrichomonas foetus* DNA in the Urethra of Bulls Naturally Infected with *Tritrichomonas foetus*

3.1 Abstract

Eight chronically and naturally infected *Tritrichomonas foetus* positive adult *Bos taurus* bulls were donated to Auburn University College of Veterinary Medicine (AUCVM). Upon admission to AUCVM, all bulls were again tested for *T. foetus* via culture followed by Real Time Polymerase Chain Reaction (RTPCR). All bulls were confirmed to be positive for *T. foetus* DNA and on culture of live organisms. The bulls were euthanized, and their urogenital tracts were removed and sectioned. The tracts were dissected from the area of least potential contamination to the area for the most potential for contamination. Sections and or scrapings of the ampullae, vesicular glands, prostate, proximal urethra, middle urethra, distal urethra, penis, and prepuce, were collected utilizing sterile technique. The sections and scrapings were placed in Modified Diamond's Media and submitted to the Thompson Bishop Sparks Alabama State Diagnostic Laboratory for *T. foetus* DNA testing via RTPCR.

All bulls were determined to be chronically infected with *T. foetus*. All smegma samples from bulls were found to be positive for T. *foetus* DNA and culture of live organisms. The prostate, vesicular glands, ampullae, bladder, and the urethra proximal to the sigmoid flexure were all found to be negative on culture for live organisms and for *T. foetus* DNA. A total of six of the eight bulls (75%) sampled in this study were found to be positive for *T. foetus* DNA in the distal third of the urethra with CT values ranging from 32.0 to 38. Eleven of 24 tests for *T. foetus* DNA in the urethra of naturally infected *T. foetus* bulls were found to be positive. The significance of this finding was evaluated utilizing a one tailed, one sample proportion z test with an $\alpha = 0.05$, utilizing Excel 2016 (Microsoft Co; Redmond, WA). It was determined that the proportion of urethral samples positive for *T foetus* DNA was a significant finding ($p < 0.05$, $z = 2.441$). This is the first documentation of *T. foetus* DNA in a more proximal location in the

urethra in *Bos taurus* bulls. All other areas of the reproductive tract sampled were found to be negative for *T. foetus* DNA via RTPCR. This study sheds light on alternative reservoir sites of *T. foetus* in the urogenital tract of chronically infected bulls.

3.2 Introduction

Bovine Trichomoniasis is a venereal disease in cattle that causes severe reproductive losses (Edmondson et al., 2016). It has a devastating economic impact on the cattle industry (Edmonson et al., 2016; Waters & Gard 2021). Bovine Trichomoniasis is a protozoal organism that inhabits the reproductive tract of both female and male cattle (Michi et al., 2016). Infected females suffer clinically with inflammatory gynecologic disease and resultant reproductive losses (Benchimol et al., 2007; Corbeil et al., 1998; Michi et al., 2016; Edmondson et al., 2016). However, bulls are considered clinically asymptomatic carriers (Michi et al., 2016; Waters & Gard 2021). Infected bulls can spread the disease radially through a naïve herd. Thus, it is important to ensure bulls are tested and determined to be negative for *T. foetus* prior to being utilized for breeding purposes, the importance of being able to consistently identify *T. foetus* in breeding bulls is crucial for control and eradication of *T. foetus* from cattle herds. Prevention of *T. foetus* from entering the herd by testing bulls prior to comingling with the herd has provided the best means of control of *T. foetus* in cattle herds (Waters & Gard 2021). Treatment of *T. foetus* has not been an option due to the inability to be able to use metronidazole and other drugs in the same class to clear infections in bulls (Love et al.). In recent *in vitro* studies, new treatments have shown promise (Koziol, et al., 2017; Neihaus et al., 2013). However,

determination if reservoir sites occur within the urogenital tract of bulls infected with *T. foetus* is key in the development of viable *in vivo* treatments.

3.3 Statement of Research Objectives

The objective of this study was to determine if there are additional reservoir sites of *T. foetus* in bulls chronically infected with *T. foetus.* The identification of reservoir sites for the organism in the proximal reproductive tract of the bull has the potential to have detrimental effects on the possibility of a topical and systemic curative treatment in bulls. In addition, identification of reservoir sites might shed light onto new theories of why some bulls can clear infection and some become chronic carriers. Hence, the hypothesis of this study was that there are additional reservoir sites in the urogenital tract of bulls naturally, and chronically infected with *T. foetus.*

3.4 Materials and Methods

Animal ethics approval

All procedures were performed in accordance with the Institutional Animal Care and Use standards under an approved protocol.

Animals

Eight adult chronically infected *T. foetus* positive *Bos taurus* bulls ranging in age four to 14 years of age were purchased or donated to Auburn University College of Veterinary Medicine (AUCVM) for research purposes. Five of the bulls were Angus cross bulls and three were

Charolais. All bulls were naturally infected with *T. foetus* and came from cattle herds having a current *T. foetus* outbreak. The number of animals necessary was calculated with a power analysis utilizing a power of 80% (1.0364), alpha=0.05, standard deviation of 0.5, effect of 0.4, for a one tailed test (1.65) equals the sample number (n) of 6.716 rounded to 7. An additional bull was added encase a bull had to be removed from the study.

Establishment of chronicity of infection with *T. foetus*

Upon admission to AUCVM, all bulls were tested for *T. foetus* via culture for live organisms followed by Real Time Polymerase Chain Reaction (RTPCR) (VetMAX™-Gold Trich Detection kit, Thermo Fisher Scientific; Waltham, MA) on at least five different occasions over a five-month period to determine if the bulls were chronically infected. All bulls underwent sexual rest for at least two weeks rest prior to smegma sampling to ensure a greater chance of detection of *T. foetus* in smegma samples (Peter, 1997; Ondrak, 2010, Waters & Gard 2021; Irons et al., 2002; Mukhughi *et.al*.,2003).

Smegma collection

Smegma was collected by performing a preputial scraping using a Pizzle Stick (Lane Manufacturing, Denver, CO) to obtain a reliable smegma sample for *T. foetus* testing. A 20 ml plastic BD syringe (Becton Dickinson; Franklin Lakes, NJ) was attached to the end of the Pizzle Stick (Lane Manufacturing) to aid in the aspiration of the preputial smegma. The Pizzle Stick (Lane Manufacturing) was placed into the preputial orifice and slowly moved to the fornix of the prepuce. The Pizzle Stick's corrugated surface was scraped against the prepuce five times. The plunger of the 20 mL syringe (Becton Dickinson) was pulled to create a suction to aid in the

collection of the smegma. All smegma samples were placed in individual labeled vials containing Modified Diamond's Media (MDM) (Thermo Fisher Scientific; Waltham, MA) and submitted to the Thompson-Bishop-Sparks-Alabama State Diagnostic Laboratory (TBSASDL) for testing for live organisms via culture, and testing for *T. foetus* DNA via Real Time Polymerase Chain Reaction (RTPCR) (VetMAX™-Gold Trich Detection kit, Thermo Fisher Scientific). Prior to euthanasia, an additional smegma was collected via a preputial scraping utilizing a Pizzle Stick and 20 mL syringe, as previously described, and placed in labeled vials of MDM (Thermo Fisher Scientific).

Sedation and Euthanasia Collection of urogenital tissues for testing

The bulls were sedated with 500 mg of xylazine (Rompun®) (Dechra; Northwich, United Kingdom) intravenously (IV) followed by IV administration of 1mL of Beuthanasia® (Merck; Rahway, NJ) per 0.45 kgs of body weight. The bulls and the smegma samples were submitted to the TBSASDL for necropsy and culture followed by RTPCR for *T. foetus*, respectively.

Collection of urogenital tracts

At necropsy, the postmortem removal of the urogenital tracts including the prepuce, penis, accessory sex glands, urethra from the glans penis to the trigone of the bladder were taken out en bloc with care taken to not enter the reproductive tract. The tract was placed on clean tables for sectioning.

Sectioning and sampling of urogenital tracts

The outside of the tracts were rinsed with 70% isopropyl alcohol (McKesson Medical; Irving, TX) in order to remove accidental contamination of the tract during handling. The tracts were dissected from the area of least potential contamination to the area of the most potential contamination, starting with the ampullae, vesicular glands, prostate, bladder, proximal urethra (before the sigmoid flexure), middle urethra (near the distal bend of the sigmoid flexure), distal urethra, prepuce, and penis. Sterile technique was used during dissection of the reproductive tracts. Sterile gloves and sterile, stainless-steel, number 10 Bard Parker® scalpel blade (Bard Parker: New Providence, NJ) were utilized during the procedure. Gloves and blades were changed following the dissection of each of the separate glands from the surrounding tissue, when making a cut to open the glands, urethra, and again each time the glands, urethra and penis and prepuce were sampled. Three bulls had 1.0 cm x 1.0 cm sections cut from the right and left ampullae, right and left vesicular glands, the prostate, bladder (trigone area) and sections of urethra, penis and prepuce (Figure 2., Table 1. And Table 2.). The sections of the urethra for these bulls were located at the specified locations; 5 cm proximal from the distal end of the urethra, 23 cm proximal from the distal end of the urethra, 13 cm proximal to the sigmoid **(Figure 2 and Table 1).**. The remaining five bulls had tissue collected via scrapings of the inside surface of each of the ampullae, vesicular glands, and the split surface of the prostate, bladder (trigone area), inside surface of the urethra at the specified locations as described above, and the penis and the prepuce were collected using the sharp edge of a sterile, stainless steel, number 10 Bard Parker® scalpel blade (Bard Parker) (Figure 1, Table 1. And Table 2.). A new pair of gloves and a new scalpel blade were utilized for the scrapings as described. Three scraping motions were made from the internal surface of each gland, and the urethral sections, and the outside surface of the penis and the prepuce. Following the collection of the tissue, the blade was

swirled around in the vial of Modified Diamonds media (MDM) to remove the smegma and any tissue collected by the scraping. All samples were submitted to the TBSASDL for testing for *T. foetus* via culture and RTPCR.

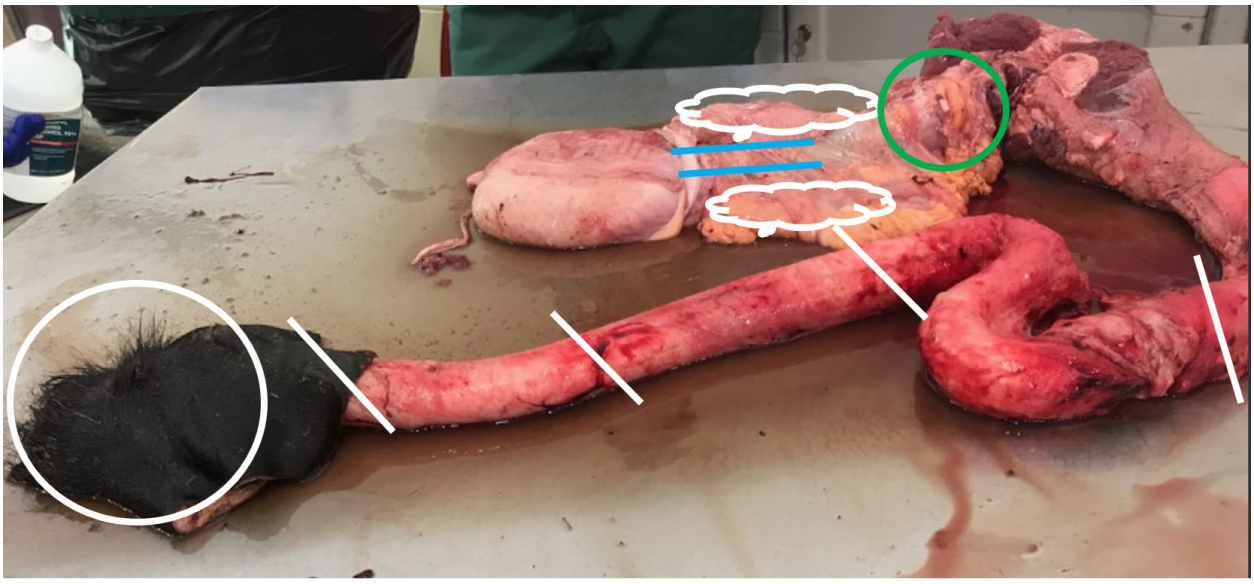

Figure 2. Image of a bull's urogenital tract and the location of the sampling sites utilized in this study. . White lines correlate to locations of samples along the urethra. The smooth white circle represents the prepuce and glans penis samples. The accessory sex glands are represented as follows: blue line are paired ampulla, white scalloped circles represent the paired vesicular glands, and the green circle represents the prostate.

Table 1: The location of the urethral sites that were sampled and tested for the presence of *Tritrichomonas foetus* DNA.

Specific urethral locations:	5 cm proximal from distal end of urethra
	23 cm proximal from distal end of urethra
	5 cm distal to distal bend of sigmoid flexure

	13 cm proximal to proximal bend of sigmoid flexure

Table 2: The list of the sampling methods utilized and the sites that were sampled for *Tritrichomonas foetus* DNA.

3 Bulls: 1x1 cm sections	5 Bulls: Tissue Scrapings
Right and left ampullae	Right and left ampullae
Right and left vesicular glands	Right and left vesicular glands
Prostate	Prostate
Specific distances along urethra	Specific distances along urethra
Glans penis	Glans penis
Prepuce	Prepuce

Culture of smegma and tissue samples

Culture of smegma and all tissue samples for *T. foetus* was carried out in an aerobic non-CO_2 incubator. Approximately 1 mL of each collected sample was immediately suspended in 2 mL of MDM (Themo Scientific) and incubated at 35-37◦C for five to seven days in accordance with the standard operating procedures of the TBSASDL. Individual wet mounts were made from each cultured sample and inspected on days 4 and 7 post-arrival for *T. foetus* via light

microscopy (200x screening and 400x for confirmation). Smears were made utilizing 25µL drops of sample media, air dried and then stained with RAL Diff Quick™ (Siemens; Munich, Germany). Smears were examined for identifying morphology for *T. foetus*. Samples were classified as positive based on the presence of motile *T. foetus* trophozoites with concurrent positive morphological assessment via smears.

Molecular testing for *Tritrichomonas foetus*

DNA extraction

Samples were incubated at 37◦C in an aerobic non-CO_2 incubator for 48 hours following submission to the TBSASDL. Following culture, 1 mL of sample was removed from the tube and place in a 1.5 mL labeled tube (Eppendorf; Enfield, CT) and vortexed vigorously. Next, 300 µL of the vortexed sample was utilized for the extraction of *T. foetus* DNA. The magnetic extraction kit BioSprint 96 One-For-All Vet Kit (Cat. No.: SP947057) (Indical Bioscience; Leipzig, Germany) was used in accordance with the standard operating procedures of the TBSASDL for extraction of *T. foetus* DNA.

Real Time – PCR for *Tritrichomonas. Foetus*

Following *T. Foetus* DNA extraction, 8µL were pipetted from the extracted samples and combined with 17µL of the RT PCR master mix which included *T. foetus* primer probe mix, provided by in the Trich kit (VetMaxGold) (Thermo Fisher Scientific) on a 96 well plate (Thermo Fisher Scientific). The positive control was the T.foetus-Xeno™ Control DNA Mix

(1,000 copies/µL) and the extraction control was the Mock-purified 1x phosphate buffered saline sample (PBS). The experimental type is standard curve run in the standard mode. The ROX™ passive reference dye was included in the 2xqPCR master mix along with TaqMan® probe reporter dyes and quenchers. The RT PCR was run in accordance with standard operating procedures of the TBSASDL.

3.5 Results

All smegma samples collected from the bulls were found to positive on culture for live organisms and RTPCR for *T. foetus* DNA consistent with chronic infections in bulls. The cycle threshold (CT) values of all chronically infected *T. foetus* bulls in this study ranged from 25.8 to 36.8. The prostate, vesicular glands, ampullae, bladder, and the urethra proximal to the sigmoid flexure were all found to be negative on culture for live organisms and on analysis for *T. foetus* DNA. The bulbourethral glands were not evaluated in any of the bulls in the present study. Smegma from the preputial scrapings, penis, and the urethral sections 5 cm proximal from the distal end of the urethra, and 23 cm proximal from the distal end of the urethra were all found to be positive for *T. foetus* DNA via RTPCR with a cycle threshold (CT) of 35.0, 33.5, 34.3, and 36.0, respectively for Bull 1 and 35.6, 30.6, 32.2 and 32.0 respectively for Bull 2 and 30.1, 30.6, 36.4, and 35.5, respectively for Bull 3. Bull 2 and Bull 3 were positive 5 cm distal to the last bend of the sigmoid with CT values of 37.4 and 35.0, respectively. Additionally, Bulls 6-8 were determined to be positive for *T. foetus* DNA at the level of the distal urethra 5 cm from the distal end of the penis with CT values of 38.0, 33.8, and 35.0 with preputial scrapings having CT values of 29.6, 34.5 and 31.2, respectively. However, Bulls 4, and 5 were only found to be positive for *T. foetus* DNA only on preputial scrapings with CT values of 30.0, and 33.5, respectively with all other areas of the urogenital tract be sampled negative for *T. foetus* DNA via RTPCR. A total of six of the eight bulls (75%) sampled in this study were found to be positive for *T. foetus* DNA in the distal urethra. Three of eight (37.5%) had *T foetus* DNA detected in the lower half of the urethra. Two of the eight (25%) bulls were positive for *T. foetus* DNA near the distal bend of the sigmoid flexure. Eleven of the 24 tests for *T. foetus* DNA in the urethra of naturally infected *T. foetus* positive bulls were found to be positive. The significance

of this finding was evaluated utilizing a one tailed, one sample proportion z test with an $\alpha = 0.05$, utilizing Excel 2016 (Microsoft Co; Redmond, WA). It was determined that the proportion of urethral samples positive for *T. foetus* DNA was a significant finding ($p < 0.05$, $z = 2.441$). The range of CT values reported from the smegma testing of all the bulls in the study was found to be in the range consistent with an abundant to moderate amounts of *T. foetus* DNA. All the CT values of all the positive urethral samples were consistent with abundant to moderate amounts of *T. foetus* which is not commonly seen with contamination (reference). Three off the positive bulls utilized the sectioning of the tissues to gather samples and the other three positive bulls used the scraping of the tissue to gather samples for testing of *T. foetus* DNA. There was no significant difference ($p > 0.05$) when comparing the two methods employed for collection of tissues for diagnostic testing.

3.6 Discussion

It was important to establish the chronicity of infection of *T. foetus* in the bulls used in this study prior to evaluation of the urogenital tract for presence of *T. foetus*. False positives can occur due to accidental contamination of collection media during collection and transfer of smegma into collection vials or accidentally in the laboratory leading to erroneous results. Hence, repetitive sampling and testing of the bulls and analysis of multiple sections of the urethra and tissues helped to prevent the occurrence of a type 1 error in this study. A larger sample size of bulls would have been beneficial to further reduce a chance of a type 1 error.

There are few reports stating that *T. foetus* can be found in the distal urethral orifice of some bulls (Michi, 2016; Parsonson et al., 1974; Rush et al., 2020). Fecal trichomonads can

cause false positive results on culture alone (Michi 2016). The use of molecular diagnostics, PCR, aids in prevention of false positives from fecal trichomonads and should be used concurrently with culture to provide a more reliable diagnosis of disease (Summarell et al*.,* 2018). Some of these early studies were performed prior to the development and wide use of RTPCRs (Parsonson et al., 1974; Michi 2016). So, it is possible that false negatives and false positives may have occurred during testing procedures.

Laboratory error can also occur as small amounts of nucleic acid extracted samples are utilized in PCR testing for *T. foetus* DNA. In the study herein, only 8µL of the extracted RNA/DNA sample is used in the running of a PCR for *T. foetus*. So, there is a reasonable chance of a false negative result if a low concentration of *T. foetus* was in the sample. This may have occurred in this study, resulting in false negative is the accessory sex glands and in the more proximal sections of the urethra and bladder. The bulls did undergo sexual rest to ensure a greater chance of detection of *T. foetus* in samples, (Peter, 1997; Ondrak, 2010;Waters & Gard 2021; Irons et al., 2002; Mukhughi *et.al*.,2003).

Additionally, pH has been found to significantly affect *T. foetus* growth and detection (Morin-Adelin, et al., 2015). In a recent study by Morin-Adelin et al*.,* pH tolerance of bovine and feline isolates of *T. foetus* was evaluated *in vitro* over a range of physiologically relevant pH *in vitro* using flow cytometry and electron microscopy. Following exposure to mild acid stress (pH 6), the bovine *T. foetus* isolates showed a significant decrease in cell viability and increased cytoplasmic granularity (p-value < 0.003, p-value < 0.0002) compared to pH 7 and 8 (p-value > 0.7) (Morin-Adelin et al., 2015). The pH of the urine and the urogenital tract was not tested for the bulls in this study. However, the urogenital tract of the bull has been found to be slightly alkaline in the range of 7.4–7.8, perfect for *T. foetus* survival (Morin-Adelin et al., 2015).

It could be that the bulls that were negative for *T. foetus* DNA in their urethra had a more acidic pH in their urogenital tract leading to absent or low concentrations of *T. foetus* resulting in negatives and or false negatives, respectively. Lowering of the pH of the urine through feeding of feed grade ammonium chloride might aid in the removal of *T. foetus* from the urogenital tract of bulls along with systemic and topical therapies that have been recently studied (Kosiol et al., 2017, Neihaus et al., 2013; Duran and Schnuelle et al., 2022 and unpublished studies).

The results of our study are more reminiscent of what is seen in men (Van Der Pol et al., 2021). In men, *T. vaginalis* is known to be a usual inhabitant of the urethra (Van Der Pol et al., 2021). Men who are positive for *T. vaginalis* are known to have pain on urination but can be asymptomatic carriers as is seen in bulls (Waters and Gard, 2021; Van Der Pol et al., 2021). Studies to determine if bulls have pain on urination are lacking. In this study, we did have a bull that showed signs of pain on urination, but this was attributed to the presence of seminal adenitis (vesiculitis). Another, bull in the study had pain that was attributed to osteoarthritis and had infection of the ampullae to the point of blockage but did not to seem to have pain on urination. Both of these bulls were determined to be positive for *T. foetus* in the urethra. It is possible that infection and inflammation in these glands interfered with detection of *T. foetus* DNA. However, none of the bulls were found to be positive for *T. foetus* DNA in any of the accessory sex glands or the proximal portions of the urethra that were tested in this study. The bulborethral glands were not directly sampled because the urethra distal and proximal to the sigmoid flexure was sampled. The results of this study are consistent with past studies evaluating the accessory sex glands in the bull (Parsonson et al., 1974; Rhyan et al., 1999; Rush et al*.,* 2020). There is one exception, besides the anecdotal reports detailed is a recent study by Lovelady (Lovelady, 2013). Lovelady found one positive prostate sample that tested positive for *T. foetus* DNA from a

population of 20 bulls naturally infected with *T. foetus*. In Lovelady's study, all tissues were collected from an abattoir. The prostate, bulbourethral glands, ampullae and the vesicular glands were sampled from each bull. Two tissue samples were collected from each gland and all samples were placed in MDM. From the pair of samples, one was tested via culture of live organisms and the other was tested for *T. foetus* DNA via a PCR. The culture samples were observed daily for *T. foetus* for four days with no detection of organisms from any of the samples collected (Lovelady, 2013). The sample that was utilized for the PCR underwent maceration prior to DNA extraction. Following DNA extraction, the sample was tested via PCR with gel electrophoresis (Lovelady, 2013). A single prostate sample was identified as positive using this PCR protocol (Lovelady, 2013). Prior to analysis via PCR, the sample was macerated, and the DNA was extracted. Following DNA extraction, the sample was tested via PCR with subsequent gel electrophoresis (Lovelady, 2013). Using comparative DNA sequencing, the positive sample was verified to be *T. foetus* (Lovelady, 2013). It was thought that this might have been accidental contamination due to cross contamination during sampling or a laboratory error. Since, gel electrophoresis was utilized there was not a CT value reported. The CT value might have given weight to the possibility of contamination. Since CT values in the range of 38-40 are considered suspect, and indicative of minimal amounts of target nucleic acid consistent with contamination events. On the other hand, the maceration procedure utilized prior to the DNA extraction could have damaged the DNA and more samples could potentially have been positive. Molecular procedures have improved considerably since this study, so the molecular techniques available could have been a limiting factor in detecting *T. foetus* DNA in the accessory sex glands in Lovelady's study as highlighted in a recent study (Summarell et al., 2018). Lovelady's study did not focus on the urethra. With the improvement in molecular techniques, and the recent report of

the presence of *T. foetus* in the urethral of a naturally infected bull (Rush et al., 2020), the study herein chose to more closely evaluated the urethra as reservoir site for *T. foetus.*

Two types of sampling of tissues were employed to determine if one sampling might be beneficial over the other. However, a significant difference in the sampling method employed was not found ($p>0.05$). There might have been greater chance for contamination of *T. foetus* of the lower urethra when harvesting 1.0 cm by 1.0 cm sections when compared to scraping the internal portion of the urethra and the other tissues due to the larger portion of tissue harvested. On the other hand, there may be a greater chance in detection of *T.foetus* in tissues if more tissue is sampled. However, more tissue sampled brings the chance of greater bacterial contamination of the sample (Mukhufhi, 2003; Cobo 2007, Clothier et al*.,* 2015; Clothier et al., 2019)). In recent studies by Clothier et al., concurrent bacterial contamination was shown to affect the ability to detect *T. foetus* in cultures and in PCR (Clothier et al., 2015; Clothier et al., 2019). The RTPCR utilized by the TBSASDL relies on culture of samples prior to nucleic acid extraction. In the study herein and in the study by Lovelady, bacterial contamination could have played a role in not detecting *T. foetus* in the accessory sex glands and the more proximal urethra (Lovelady, 2013). New molecular techniques that do not rely on culture prior to extraction have shown great success (Summarell et al*.,* 2018). Researchers recently developed a direct reverse-transcription quantitative real-time PCR (direct RT-qPCR) utilizing smegma, eliminating the use of culture (Summarell et al*.,* 2018). An automated nucleic acid purification method was utilized in this which reduces human labor and enables high-throughput sample processing (Summarell et al*.,* 2018). The direct RT-qPCR exhibited 100% diagnostic sensitivity and 100% specificity, whereas the currently employed qPCR (culture qPCR), which utilizes cultured samples, exhibited 95% diagnostic sensitivity and 100% specificity (Summarell et al*.,* 2018). More positive samples with

lower quantification cycle (Cq) values were reported, direct RT-qPCR Cq range = 14.6–32.3 vs. culture qPCR Cq range = 18.7–37.4) (Summarell et al*.,* 2018). The direct RT-qPCR would have been beneficial to use in this study. It may have resulted in more positive samples but was not readily available to the researchers.

The study herein built upon the findings of Rush et al., and further documents *T. foetus* DNA in a more proximal location in the urethra in naturally infected *Bos taurus* bulls (Rush et al., 2020). The results of this study do lead to the assumption that the urethra is a reservoir site for *T. foetus* as is seen with *T. vaginalis* in men. This study provides important information for researchers looking into the clearance of *T. foetus* from infected bulls as topical treatments of just the penis and prepuce may not be curative in bulls. *In vitro* and clinical trials are promising but addressing but may lend to greater success in utilizing topical and systemic therapies (Niehuas et al., 2013; Koziol et al., 2017; Duran & Schnuelle et al., 2022). Treatments eliminating *T. foetus* from the urethra as well as the penis and prepuce looks to be necessary to prevent reinfection of the penis and prepuce resulting in a true cure. Lowering the pH of the urinary tract might provide an added benefit to current therapies in clearance of *T. foetus* from the urogenital tract of chronically infected bulls as well.

Declaration of competing interests

Monies from the Auburn University Launch Grant were utilized to pay for this project. However, the financial support received by Auburn University did not influence the outcome of this project. Hence, there are no known conflicts of interest associated with this publication.

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