PREVALENCE OF TRICHOMONIASIS IN ALABAMA BEEF BULLS

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PREVALENCE OF TRICHOMONIASIS IN ALABAMA BEEF BULLS

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PREVALENCE OF TRICHOMONIASIS IN ALABAMA BEEF BULLS

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VITA

Soren Piers Rodning, son of Charles Bernard Rodning and Mary Elizabeth Rodning, was born in Minneapolis, Minnesota on September 8, 1974. He received his Bachelor of Science (summa cum laude) in Animal and Dairy Sciences from the Auburn University College of Agriculture in June 1998. He subsequently attended the Auburn University College of Veterinary Medicine and was awarded the degree Doctor of Veterinary Medicine (cum laude) in May 2002. After graduation from veterinary school, he practiced veterinary medicine on large and small domestic animals in Haines City, Florida. In July 2003, Soren Rodning returned to the Auburn University College of Veterinary Medicine as a Theriogenology Resident in the Department of Clinical Sciences and enrolled in graduate school. Diplomate status in the American College of Theriogenologists was conferred to him on August 9, 2005. He married Kimberly Jean Rodning, daughter of Joe Bowyer Race and Carol Jean Race of Winter Haven, Florida, on December 30, 2000. His wife gave birth to their first daughter, Elizabeth Carol Rodning, on January 26, 2006.

THESIS ABSTRACT

PREVALENCE OF TRICHOMONIASIS IN ALABAMA BEEF BULLS

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Bovine trichomoniasis is a venereal disease caused by the protozoan *Tritrichomonas foetus*. Trichomoniasis is a major cause of fetal wastage throughout the world, and it causes substantial economic losses wherever natural breeding conditions exist. Several estimates are available regarding the prevalence of trichomoniasis in different regions of the United States and throughout the world. However, prior to this investigation, no estimates were available regarding the prevalence of trichomoniasis in Alabama beef cattle. Therefore, both a prospective survey and a retrospective analysis were conducted to estimate the prevalence of *T. foetus* in Alabama beef bulls.

The prospective survey included 240 Alabama beef bulls that were sampled between January 2005 and March 2006. Preputial smegma was collected from the 240 bulls with a dry pipette and cultured in an InPouch™ TF *Tritrichomonas foetus* culture pouch (BioMed Diagnostics; White City, OR). The samples were evaluated

microscopically once a day for six days for growth of any organism resembling *T. foetus*. To avoid false-positives due to fecal trichomonads, all suspect cultures were sent to both the Alabama State Diagnostic Laboratory and the Parasitology Laboratory at the Auburn University College of Veterinary Medicine for molecular-based confirmatory assays. Of the 240 bulls cultured in the prospective survey, three cultures (1.25 %) were considered suspect on microscopic evaluation. However, molecular-based assays were negative for *T. foetus*, suggesting that the samples most likely contained fecal trichomonads.

The retrospective analysis included 374 *T. foetus* cultures performed at the Alabama State Diagnostic Laboratory between October 2002 and March 2005. Of the 374 bulls included in the retrospective analysis, one bull (0.27 %) was confirmed positive by a molecular-based assay. Combining the 240 bulls from the prospective survey with the 374 bulls from the retrospective analysis gave an overall prevalence of 0.16 % (1/614) from October 2002 through March 2006.

A prevalence of 0.16 % is lower than expected, but it does indicate the presence of *T. foetus* in Alabama cattle. Alabama cattle producers and veterinarians should therefore work together to minimize the spread of trichomoniasis throughout the state, including testing for trichomoniasis when dealing with herd infertility problems. Also, despite the low prevalence of *T. foetus* in Alabama, producers should consider testing all new bulls because of the serious consequences of adding a positive animal to a herd.

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CHAPTER I

INTRODUCTION

Trichomoniasis, also known as trichomonosis, is a bovine venereal disease caused by the protozoan *Tritrichomonas foetus*. Infected bulls are often asymptomatic carriers of *T. foetus*. However, infected bulls are very efficient in transmitting the organism to a cow or heifer during coitus, and it is the female bovine that suffers the consequences of infection. Infections in cows and heifers can result in early embryonic death, abortion, pyometra, fetal maceration, or infertility [1-4], influencing the economic profitability of a cattle operation. *Tritrichomonas foetus* is sometimes called the silent calf thief because it can persist without detection in endemic herds for many years. Even when undetected, however, *T. foetus* has a substantial impact on a cattle producer's profits.

The economic impact of trichomoniasis is due to three factors: (1) reduced calf crop due to early embryonic loss or abortion; (2) reduced weaning weight due to delayed conception; and (3) culling and replacement of infected cattle. Since no legal treatment exists, preventive and control measures focus on testing and culling of all positive animals, administration of a killed vaccine, and producer and veterinarian education regarding risk assessment and herd biosecurity. Therefore, a major component of educating Alabama cattle producers and veterinarians is establishing the prevalence of trichomoniasis in Alabama to aid in risk assessment.

Prior to this investigation there were no reported prevalence rates of trichomoniasis in Alabama beef cattle. Therefore, based on epidemiological studies conducted in other states [5-10] that reported individual bull prevalence rates of 0 to 7.8 %, it was hypothesized that the prevalence of trichomoniasis in Alabama beef bulls was approximately 3 %. However, even at a prevalence of 3 % or less, trichomoniasis could still cost the Alabama beef industry millions of dollars a year.

Two approaches were undertaken to estimate the prevalence of *T. foetus* in Alabama beef bulls. First, a prospective survey of 240 Alabama beef bulls was conducted between January 2005 and March 2006. Preputial smegma was collected from 240 bulls with a dry pipette and cultured in an InPouch™ TF *Tritrichomonas foetus* culture pouch (BioMed Diagnostics; White City, OR). The samples were evaluated microscopically for growth of *T. foetus* over a six day period. To avoid false-positives due to fecal trichomonads, all suspect cultures were sent to both the Alabama State Diagnostic Laboratory and the Parasitology Laboratory at the Auburn University College of Veterinary Medicine for molecular-based confirmatory assays. The second part of the survey involved a retrospective analysis of *T. foetus* cultures performed at the Alabama State Diagnostic Laboratory. The retrospective analysis included 374 cultures performed between October 2002 and March 2005.

Of the 240 bulls cultured in the prospective survey, three cultures (1.25 %) were considered suspect on microscopic evaluation. However, molecular-based assays were negative for *T. foetus* at both the Alabama State Diagnostic Laboratory and in the Parasitology Laboratory of the Auburn University College of Veterinary Medicine. The

samples were therefore most likely contaminated by fecal trichomonads with similar motility and morphology as *T. foetus*.

Of the 374 bulls included in the retrospective analysis, one bull (0.27 %) was confirmed positive by a molecular-based assay. Combining the 240 bulls from the prospective survey with the 374 bulls from the retrospective analysis gave an overall prevalence of 0.16 % (1/614) from October 2002 through March 2006.

A prevalence of 0.16 % is lower than expected, but it does indicate the presence of *T. foetus* in Alabama cattle. Therefore, Alabama cattle producers and veterinarians should work together to minimize the spread of trichomoniasis throughout the state, including testing herds with poor reproductive performance for trichomoniasis. Also, despite the low prevalence of *T. foetus* in Alabama, producers should consider testing all new bulls due to the serious consequences of adding a positive bull to a herd.

CHAPTER II

LITERATURE REVIEW

Bovine Trichomoniasis

History of trichomoniasis

Trichomoniasis was first described in Europe in the late 19th century [11]. However, the discovery of trichomoniasis coincided with the discovery of brucellosis in 1897, and therefore very little research was conducted on trichomoniasis until the 1920s and 1930s [12]. In 1932, Emerson reported the first case of trichomoniasis in the United States in Pennsylvania, and since then trichomoniasis has been diagnosed throughout the United States [5-9,12,13] and in many parts of the world [14,15].

Taxonomy of *Tritrichomonas foetus*

Tritrichomonas foetus belongs to the phylum Parabasalia [16]. Parabasalids are anaerobic flagellates without mitochondria. Most parabasalids live as parasites in the alimentary or urogenital tract of vertebrates and invertebrates, with about half of the genera living in insects such as termites and roaches. Two free-living species also survive in stagnant water rich in organic matter, and a few species such as *Trichomonas vaginalis*, *Trichomonas gallinae*, *Histomonas meleafridis*, and *Tritrichomonas foetus* are pathogenic in the urogenital or alimentary tracts of various animals. The following

taxonomic classification (Figure 1) for *Tritrichomonas foetus* is adapted from Adl et al. [17] and Brugerolle and Lee [16]:

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

Figure 1. Taxonomy of *Tritrichomonas foetus*.

Morphology

Tritrichomonas means "three-haired single-celled protozoan", which accurately depicts some of the morphological characteristics of the organism as described by Taylor et al. [18]. *Tritrichomonas foetus* is a pyriform-shaped protozoan with a rounded anterior end and a pointed posterior end. Its size can vary from 10 to 25 μm in length and 5 to 10 μm in width. *Tritrichomonas foetus* has a single nucleus and four flagella. Three of the flagella are located on the anterior end, while the fourth extends backwards. One side of the organism has an undulating membrane with three to five waves and a characteristic vibrating movement.

Pathogenesis in the male

Tritrichomonas foetus is an obligate parasite of the bovine reproductive tract. Similar to most venereal diseases in domestic animals the male is an asymptomatic carrier, while the female suffers identifiable consequences of infection. *Tritrichomonas foetus* localizes in the smegma (secretions) of the epithelial lining of a bull's penis, prepuce, and distal urethra [19]. The organism does not readily invade the epithelium nor typically invoke an effective immune response in the bull [20]. *Tritrichomonas foetus* causes no penile or preputial lesions and does not affect semen quality or libido [19,21]. An infected bull, therefore, acts only as an asymptomatic carrier.

The asymptomatic, chronic carrier state associated with many *T. foetus* infections in bulls rarely clears regardless of time. The carrier state is apparently related to the depth of the preputial and penile epithelial crypts. Deeper epithelial crypts associated with older bulls provide the appropriate microaerophilic environment required for establishment of chronic infections [19,22-24].

Tritrichomonas foetus infections in younger bulls (less than 3-4 years of age) are more likely to be transient, with disease transmission only occurring if sexual contact with a noninfected cow occurs within minutes to days following breeding of an infected cow [25,26]. Transmission of *T. foetus* by a young bull is therefore more of a passive, mechanical transmission, as compared to transmission associated with a chronically infected older bull. Studies by Morgan [26] and Clark [25] indicated that clearance in a young bull is possible within 20 minutes after breeding an infected cow. However, any bull exposed to *T. foetus* in a natural breeding situation is capable of becoming chronically infected, regardless of age.

Pathogenesis in the female

Trichomoniasis in the cow occurs after coitus with an infected bull. The organism transverses the cervix during estrus and colonizes the entire reproductive tract within 1-2 weeks [27]. Pyometra and abortion are often the first physical signs of trichomoniasis that are noticed in a herd, but these signs occur in less than 5 percent of infected animals [21]. Infertility due to early embryonic or fetal death is the most economically damaging clinical sign and occurs in a much larger percentage of infected cows. However, often the only clinical sign associated with early embryonic death is an irregular return to estrus, which may go unnoticed in a large herd. The mechanism that leads to conceptus death is not known, but since the embryonic or fetal loss typically occurs after maternal recognition of pregnancy (days 15-17 of gestation), the dam's interestrus interval is prolonged [27].

Tritrichomonas foetus does not prevent conception, but as the organism multiplies in the uterus it can cause death of the embryo or fetus most commonly between gestational days 15 to 80 [11]. A small percentage of cows will abort in the second or even third trimester, and an even smaller number of cows (less than 1%) will maintain an infection through a normal gestation and deliver a live calf [28]. The few cows capable of maintaining a *T. foetus* infection throughout gestation are very damaging because they represent a source of reinfection for the herd during the following breeding season.

Unlike the bull, the cow is capable of mounting an effective immune response to *T. foetus* [29]. A humoral immune response can usually be found throughout the reproductive tract after the initial infection, but clearance of *T. foetus* from the cow's reproductive tract is quite variable.

Primary infections may be cleared from the reproductive tract in as little as 95 days [4] or as long as 22 months [30]. Subsequent infections are cleared in about 20 days, indicating an anamnestic response [20]. However, immunity does not persist, and the anamnestic response is only sufficient if the reinfection occurs within about 15 months of the primary infection [1,19]. A cow in a herd with a long breeding season could therefore become pregnant and infected with *T. foetus* early in the breeding season, lose that embryo, be infertile for several months, clear the initial *T. foetus* infection, rebreed, conceive, and as a result of her temporary immunity, carry a calf to term. The result is that more cows will calve later in the calving season than desired, and there is a resultant wide variety in weaning weights rather than just a reduced calving percentage. The later born calves are then marketed at lighter weights, or the cattle producer will incur increased feeding costs to achieve a desired market weight. In either case the cattle producer can sustain substantial economic loss.

Treatment

One of the complicating factors associated with bovine trichomoniasis is that there are currently no effective treatments with Food and Drug Administration approval [20]. Historically, topical as well as systemic therapies were used in bulls with a variety of results. Topical trichomonadicidal compounds include acriflavine and diminazene aceturate, Bovoflavin salve (trypaflavine), chlorhexidine, and metronidazole. Topical treatments were often expensive, labor intensive, and in some cases produced undesirable side effects [11,27,31]. The most successful treatment for bulls with trichomoniasis involved systemic treatment with nitromidazole derivatives such as dimetridazole, ipronidazole, and metronidazole [32-35]. However, the use of nitromidazole derivatives

is now illegal in food-producing animals because of their mutagenic and carcinogenic properties. No alternative treatments have been developed.

The lack of effective approved therapies for bovine trichomoniasis emphasizes the need for appropriate control measures through veterinary and producer education, testing, vaccination, and various herd management practices.

Vaccination

Immunization against *T. foetus* is an important management tool, especially in herds already infected or in herds at high risk of acquiring *T. foetus*. While avoiding the common risk factors associated with the introduction of *T. foetus* into a non-infected herd is superior to vaccination alone, research trials validate the use of *T. foetus* vaccination in certain situations [36-41]. Systemic vaccination does stimulate a mucosal antibody response [36,42], and Corbeil et al. [43] demonstrated that mucosal antibodies agglutinate and immobilize *T. foetus* as well as inhibit its adherence to vaginal epithelial cells. Such protection indicated that systemic immunization against *T. foetus* antigens could protect the bovine reproductive tract from a *T. foetus* infection.

In 1989, Hall et al. [38] initiated an immunogenicity trial at the University of Nevada, Reno, to determine the protective effects of a bovine *T. foetus* vaccine against fetal wastage. Heifers (n=90) were randomly divided into two groups of 45 heifers each. The groups were designated as mono and control. The mono group received two subcutaneous doses of an experimental *T. foetus* vaccine three weeks apart. The experimental *T. foetus* vaccine contained 5×10^7 killed *T. foetus* organisms per 2 ml dose, along with a Fort Dodge proprietary adjuvant. At the same time, but at different injection sites, both the control and mono groups were also vaccinated against *Clostridium*

chauvoei-septicum-haemolyticum-novyi-sordellii-perfringens types C and D, *Campylobacter fetus*, *Leptospira canicola-grippotyphosa-hardjo-icterohaemorrhagiae-pomona*, *Haemophilus somnus*, IBR, BVDV, and PI-3. The mono and control groups were managed as one contiguous herd, and breeding began 30 days after the second vaccinations. Eight *T. foetus*-infected bulls bred the heifers, and all heifers were also vaginally infused with 1×10^7 *T. foetus* organisms within 18 hours of each observed estrus. Conception rates were 72.50 % for the mono group and 86.36 % for the control group [38]. However, the calving rate for the heifers that initially conceived was 82.76 % for the mono group and 60.53 % for the control group, indicating that the experimental monovalent *T. foetus* vaccine reduced fetal wastage after *T. foetus* challenge.

Kvasnicka et al. [39] also conducted a clinical trial evaluating the efficacy of immunizing cattle with an experimental polyvalent vaccine that contained *Campylobacter fetus*, *Leptospira canicola-grippotyphosa-hardjo-icterohaemorrhagiae-pomona*, and 5×10^7 killed *T. foetus* organisms per dose. Heifers (n=130) were randomly assigned to either a vaccinated group or a non-vaccinated control group. The vaccinated group received two doses of the experimental polyvalent vaccine three weeks apart. The non-vaccinated control group did receive two doses of a control vaccine containing *Campylobacter fetus* and *Leptospira canicola-grippotyphosa-hardjo-icterohaemorrhagiae-pomona*, but not *T. foetus*. Both groups of heifers were then exposed to *T. foetus*-infected bulls for 15-day breeding periods. Conception rates were similar between groups, 89.2 % for vaccinated heifers and 85.9 % for non-vaccinated control heifers 30 days post-breeding [39]. Pregnancy examinations conducted 65 days post-breeding revealed that approximately 80 % of the non-vaccinated controls and 90 %

of the vaccinated heifers that originally conceived remained pregnant. However, over the next four months the pregnancy rates decreased to approximately 30 % in the non-vaccinated control group and 60 % in the vaccinated group. At the end of the trial, 62.1 % of the vaccinated heifers produced calves versus a 31.5 % calving rate in the non-vaccinated control heifers.

Prevalence

Several estimates are available regarding the prevalence of trichomoniasis in different regions of the United States. In 1964, Johnson reported a 7.5 % prevalence in western range bulls [7]. More recent studies from Florida [5], Oklahoma [9], and California [6] found prevalence rates of 7.3, 7.8 and 4.1 %, respectively. The Florida and Oklahoma studies sampled bulls from sale barns or abattoirs, while the California study sampled bulls from randomly selected herds.

Rae et al. [8] conducted a more recent epidemiological survey of *T. foetus* in Florida between 1997 and 1999. They randomly selected natural service beef herds in Florida and reported a 6 % overall prevalence for *T. foetus* infected bulls (3.1 % in north Florida and 6.8 % in south Florida). The overall herd infection rate was 30.4 %, with 11.1 % in north Florida and 39.5 % in south Florida. Medium sized herds were less likely to be infected (100-499 cows, 10 %) than large herds (500+ cows, 53.9 %).

In other parts of the world, Riley et al. [15] reported a 6 % prevalence in bulls in Saskatchewan, Canada, and Erasmus et al. [14] reported a 7 % prevalence in the North Western Cape Province, Western Transvaal, and the Orange Free State in South Africa.

Economic impact

The economic impact of trichomoniasis is due to: (1) reduced calf crop from early embryonic loss or abortion; (2) reduced weaning weight due to delayed conception; and (3) culling and replacement of infected cattle. Fitzgerald et al. [44] estimated that in 1958 each infected bull in a large herd was responsible for an $800 loss per year. Wilson et al. [9] estimated a $2.5 million annual calf loss in 1979 due to trichomoniasis in Oklahoma replacement heifers alone. In 1986, Fitzgerald estimated that the total economic impact in the USA was $65 million annually [2]. These estimates are several years old, however, and current losses are probably substantially higher.

More recently, Rae developed a computer simulation model to study the impact of trichomoniasis on a cow-calf producer's profitability [45]. The model estimated a 14 to 50 % reduction in annual calf crop if *T. foetus* infections were present in 20 to 40 % of the bull population, and the net return per cow exposed to an infected bull decreased by 5 to 35 % [45].

If a single cow is infected with *T. foetus* and as a result does not deliver and wean a 500-pound calf, then a cattle producer potentially loses $500 if calf prices are $1/pound. In a herd of 100 cows, if 50 % do not deliver and wean a calf the potential loss is $25,000. If that same cow calves 4 months later than expected due to a *T. foetus* infection early in the breeding season, her calf could be 240 pounds lighter (at 2 pounds average daily gain) than expected at weaning time, with a potential loss of $240. In either case, the economic loss is substantial.

The economic impact of trichomoniasis can be so devastating that several western states consider trichomoniasis a reportable disease and require bull testing prior to sale or

before the use of public land. None of these western states have more than an 8 % prevalence of trichomoniasis in their bull population, but they mandate testing of bulls because one bull can infect many cows, leading to severe economic consequences.

Diagnosis of Bovine Trichomoniasis

Diagnosis of trichomoniasis relies primarily on microscopic identification of *T. foetus* in preputial smegma or cervicovaginal mucus (CVM). Identification of *T. foetus* is performed either via direct examination of preputial smegma or CVM, or after cultivation of diagnostic specimens in selected culture media. However, fecal trichomonads can confound microscopic evaluation of specimens. Therefore, research has recently focused on molecular-based confirmatory assays.

Sampling techniques in the male

Several sampling techniques have been utilized for obtaining diagnostic specimens in the bull including: 1) a swab technique; 2) a dry pipette technique; 3) a wet pipette technique; 4) the douche technique; and 5) a metal brush technique. Each technique focuses on recovering preputial smegma for either direct microscopic evaluation or in vitro cultivation.

Morgan first described the swab technique in 1946 [12]. A cotton swab is rotated across the prepuce several times and then cultured for *T. foetus*. Fitzgerald et al. [46] 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. The swab technique is therefore rarely used in the United States.

The dry pipette technique involves a dry, plastic infusion pipette with a 12 or 20 mL syringe attached as an aspiration device. The pipette is inserted into the preputial fornix and then scraped vigorously across the penile and preputial epithelium as suction is applied with the syringe [20,47]. Samples are often slightly blood-tinged. Any preputial smegma recovered is then placed directly into the preferred transport or culture medium.

The wet pipette technique is similar to the dry pipette technique but involves the infusion of 5 to 10 mL of physiologic saline or transport/culture medium into the bull's preputial cavity [48]. One hand holds the preputial orifice closed to keep the flush fluid in the preputial cavity while the other hand vigorously massages the sheath. The fluid is then aspirated with the pipette and syringe. Depending on the amount of fluid recollected, the sample may be centrifuged prior to microscopic examination or culture inoculation.

The douche method involves infusing the preputial cavity with 100 to 200 mL of physiologic saline [48]. The preputial orifice is occluded while the sheath is vigorously massaged. The flush fluid is then recollected into an appropriate container. The fluid is centrifuged before the sediment is microscopically examined or inoculated into the preferred culture medium.

Tedesco et al. [49] and Parker et al. [50] both evaluated the efficacy of scraping a bull's prepuce with a metal brush to improve *T. foetus* recovery rates. Neither group reported any advantage with the metal brush when used with traditional in vitro culture systems. Metal brushes are therefore not used to scrape a bull's prepuce to collect diagnostic specimens.

The dry pipette technique is the preferred sampling method in the United States, while the douche method is the preferred technique in Europe [47]. Schönmann et al. [47] reported that the two methods are not statistically different.

Regardless of the technique used, it is generally recommended that bulls be sexually rested 1-2 weeks before testing for *T. foetus*; otherwise, false-negative results are more likely because breeding removes many of the organisms from a bull's penis and prepuce. Given the sensitivity of *T. foetus* cultures, false-negative results are also possible even if a bull has been sexually rested. Only with three negative tests at weekly intervals (Figure 2) can a cattle producer be 99 % sure that a bull is *T. foetus* negative [51].

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

Figure 2. Sensitivity (in series) of *T. foetus* cultures [51].

Sampling techniques in the female

Researchers investigating diagnostic sampling methodologies for *T. foetus* have focused primarily on optimizing sample collection and culture from bulls. Also, since the majority of female cattle do eventually clear a *T. foetus* infection [4,27], the time of sampling relative to exposure can have a significant impact on diagnostic results. Therefore, it is not surprising that isolation of *T. foetus* from female cattle is less sensitive

when compared to the bull [35,52]. In fact, culture of cervicovaginal mucus from cows/heifers only has a sensitivity of 58 to 75 % [35].

The technique most commonly used to sample female cattle for *T. foetus* is a dry pipette technique [20,23,48]. An infusion pipette is used to aspirate cervicovaginal mucus (CVM) from the vaginal fornix or near the external cervical os. Alternatively, in the case of a post-coital pyometra, an infusion pipette can also be used to aspirate some of the contents of the pyometra. Either sample is then examined directly or placed into the preferred culture medium and evaluated microscopically for up to six days.

Direct examination

Preputial smegma or cervicovaginal mucus can be examined directly for the presence of *T. foetus*. An undiluted sample of preputial smegma/cervicovaginal mucus or a centrifuged preputial wash can be examined at 100× and then 400× magnification with bright-field microspcopy. The sample is first examined at 100× magnification to identify motile organisms. If motile organisms are seen then the sample is examined at 400× magnification to identify the characteristic morphology and motility of *T. foetus*. *Tritrichomonas foetus* is identified as having three anterior flagella, one posterior flagellum, and an undulating membrane, as well as a characteristic rolling, jerky motility [18,23]. However, since several protozoal organisms resembling *T. foetus* can contaminate the bovine reproductive tract [18,53-56], diagnosis of *T. foetus* should be confirmed using various staining techniques [57] or DNA-based assays [15,55,58,59].

In vitro culture

Direct microscopic examination of specimens for *T. foetus* is diagnostic, but a far more sensitive method for the detection of *T. foetus* is in vitro culture of preputial

smegma or cervicovaginal mucus in a selective nutrient medium for up to a week [35,49,60]. In vitro culture allows the proliferation of *T. foetus* to more readily detectable levels. Cultures are evaluated daily or every other day in a manner similar to direct 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.

In vitro culture media

Various culture and transport media systems have been used including Kupferberg medium and broth, Claussen's medium, Sutherland medium, trypticase-yeast extract-maltose (TYM) medium, Diamond's medium, and most recently the InPouch™ TF *Tritrichomonas foetus* culture pouch (BioMed Diagnostics; White City, OR). For many years, the standard medium in the United States was TYM. However, Diamond's medium, produced by adding 1 % agar to TYM [23,61,62], eventually became the preferred medium. The agar trapped many contaminants in the upper layers of a 10 mL tube of medium, allowing the facultatively anaerobic *T. foetus* to grow extremely well at the bottom of the tube where the redox potential was the lowest [23].

BioMed Diagnostics introduced a commercially available culture system in 1990 called the InPouch™ TF *Tritrichomonas foetus* culture pouch (BioMed Diagnostics; White City, OR). The InPouch™ TF is a self-contained transport and culture medium that is selective for the transport and growth of *T. foetus* while inhibiting the growth of some contaminating microorganisms. The pouch is constructed with a clear plastic film with water vapor and oxygen-barrier qualities that help maintain the proper microaerophilic environment.

In vitro cultivation using either Diamond's medium or the InPouch™ TF system is currently the most common and efficient method to diagnose *T. foetus*. Both culture systems are fairly equal in sensitivity, 81 to 95 % [23,47,63,64]. However, BioMed's InPouch™ TF is somewhat more convenient than Diamond's medium [65]. The InPouch™ TF has a 12-month shelf-life at room temperature, compared to a two to three week shelf-life for Diamond's medium in a refrigerator. Also, the plastic pouch design of the InPouch™ TF system is less likely to break or leak than tubes containing Diamond's medium. Unfortunately, the InPouch™ TF system is more expensive than Diamond's medium.

For many years, cultivation of microorganisms with motility and morphology resembling *T. foetus* in either the InPouch™ TF or Diamond's medium was considered to be 100 % specific. However, accurate microscopic identification of *T. foetus* has since been shown to be complicated by the presence of other contaminating trichomonadid protozoa [18,53-56]. All cultures containing organisms resembling *T. foetus* should therefore be confirmed with appropriate molecular-based assays.

<u>In vitro culture contamination</u>

Most protozoan species in the order Trichomonadida live in the gastrointestinal tract of their host, while only a few are associated with the urogenital tract [66]. Several protozoan species in the family Trichomonadidae are found in the bovine digestive tract such as *Tritrichomonas enteris*, *Tetratrichomonas pavlovi*, *Tetratrichomonas buttreyi*, and *Pentatrichomonas hominis*, as well as other flagellates from the orders Trichomonadida, Diplomonadida, and Oxymonadida [66-68]. Many of these protozoa are considered non-pathogenic, commensal organisms [66]. However, the presence of non-*T.*

foetus protozoa in preputial smegma or cervicovaginal mucus can confound the diagnosis of *T. foetus*. Such protozoa may have motility and morphology similar to *T. foetus* and can therefore be incorrectly identified as *T. foetus* on microscopic evaluation. There are several reports of non-*T. foetus* trichomonads cultured from the preputial smegma of virgin bulls [53,54,69].

BonDurant et al. [53] described trichomonad isolates from 14 virgin bulls submitted to the California Veterinary Diagnostic Laboratory for confirmation of trichomoniasis. Bright-field microscopy revealed many single-celled motile organisms about 12 μm X 9 μm. The organisms had multiple anterior flagella, one posterior flagellum, an axostyle, and an undulating membrane. Their motility was jerky and rolling similar to *T. foetus*. Culture smears were air-dried and stained with Giemsa or Diff-Quick/iodine. The stained slides revealed organisms similar to *T. foetus*, but slightly more rounded. Several of the stained organisms also appeared to have four anterior flagella. Scanning electron microscopy verified the presence of four anterior flagella, and also showed the presence of an axostyle that was consistently longer than normally seen with *T. foetus*. Polymerase chain reaction (PCR) utilizing primers specific for the 5.8S ribosomal RNA genes of *T. foetus* was used to further identify the protozoan. No amplification product was detected in any of the 14 virgin bull isolates, while the positive control *T. foetus* isolates all produced the appropriate sized amplicon. BonDurant et al. therefore concluded that the protozoan isolated from the 14 virgin bulls was in fact not *T. foetus*. They speculated that the protozoan was probably a commensal organism of the lower bowel.

Cobo et al. [54] also described an unusual tetratrichomonad species isolated from the preputial smegma of 6 virgin bulls. Using scanning and electron microscopy, Cobo et al. [54] identified a protozoan with four anterior flagella of unequal length and a recurrent flagellum as the undulating membrane. A PCR-based assay was also used to further identify the organism. Amplification products were only seen with primers TFR1 and TFR2 (specific to trichomonads), but not with TFR3 and TFR4 (specific to *T. foetus*). On the basis of their morphologic and genetic evaluations, Cobo et al. concluded that the protozoan was not *T. foetus*. Instead, they speculated that the organism was *Tetratrichomonas buttreyi*.

In a report by Campero et al. [69], approximately 8.4 % of 567 preputial samples from one to two year old virgin Angus and Hereford bulls were culture positive for a protozoan resembling *T. foetus*. Similar to the results of BonDurant et al. [53] and Cobo et al. [54], further evaluation of the organisms via bright-field microscopy and scanning electron microscopy revealed motility similar to *T. foetus*, but also revealed four anterior flagella on most organisms and an axostyle that was longer than that associated with *T. foetus*. A PCR-based assay indicated the organism was a trichomonad, but not *T. foetus*.

Attempts to experimentally infect bull and heifer reproductive tracts with *Tetratrichomonas* sp. isolated from preputial smegma of virgin bulls have been unsuccessful, suggesting that such trichomonads are non-pathogenic in the bovine reproductive tract. Cobo et al. [70] inoculated nine heifers and four bulls with 7×10^6 *Tetratrichomonas* sp. in the vaginal lumen or preputial cavity, respectively. Two heifers and two bulls were also experimentally infected with *T. foetus* as positive controls. *Tetratrichomonas* sp. were only isolated in vaginal mucus from 7/9 heifers at 6 hours

post-inoculation. The heifers were subsequently slaughtered in groups of three on days 2, 9, and 21 post-inoculation. No vaginal, uterine, or oviductal samples collected at slaughter contained any *Tetratrichomonas* sp., and all bulls challenged with *Tetratrichomonas* sp. remained culture negative. Cobo et al. [70] thus concluded that the *Tetratrichomonas* sp. used in their experiment is non-pathogenic in the male and female bovine reproductive tract.

Several non-pathogenic protozoa are normal inhabitants of the bovine gastrointestinal tract [66-68], and therefore proper cleaning of the preputial orifice and proper sampling techniques are critical to avoid fecal contamination of diagnostic samples. The presence of non-pathogenic protozoan organisms can result in false-positive cultures if diagnosis is based solely on microscopic evaluation. No legal, effective therapy exists for cattle that are chronically infected with *T. foetus*, so it is therefore imperative that cultures suspected of being *T. foetus* be confirmed with an appropriate technique such as a molecular-based assay or culture stain.

Molecular diagnostics

Diagnosis of *T. foetus* has traditionally relied upon microscopic identification of key morphological characteristics in various culture media. Such characteristics include three anterior flagella, one posterior flagellum, and an undulating membrane resulting in a jerky movement pattern. However, accurate microscopic identification of *T. foetus* can be complicated by the presence of other trichomonadid protozoa [18,53-56]. Contamination of the preputial orifice, prepuce, or penis with fecal material probably explains the presence of these opportunistic trichomonads. None of the contaminating trichomonads, however, results in reproductive pathology in cows or bulls [70].

Given the lack of legal therapy for bulls infected with *T. foetus*, the only reasonable course of action is to slaughter a bull with trichomoniasis. It is therefore imperative to correctly identify *T. foetus* infected bulls and not misdiagnose based on the presence of non-pathogenic fecal trichomonads. The first DNA-based assay was developed in 1991 to help verify the presence of *T. foetus* [71].

Personnel in Veterinary Molecular Biology at Montana State University developed the first DNA-based test for *T. foetus* [71]. The test was designed to extract nucleic acid material, bind the nucleic acids to filters, and then probe for the presence of nucleic acid sequences specific for *T. foetus*. Three probes were initially designed. The first probe was a universal probe designed to react with nucleic acids from *T. foetus*, *Trichomonas vaginalis*, and bovine cells. The second probe was a trichomonad probe that reacted only with *T. foetus* and *T. vaginalis*, and the third probe reacted specifically with strains of *T. foetus*. The DNA-probe was initially very promising, but subsequent field tests indicated inadequate sensitivity when compared to traditional culture systems [63]. Polymerase chain reaction-based assays using different primers have therefore been developed to amplify various DNA sequences of *T. foetus* to aid in diagnosis [15,55,58,59].

Polymerase chain reaction (PCR) is a fast and inexpensive technique described by the National Human Genome Research Institute as molecular photocopying [72]. Substantial amounts of a DNA sample are necessary for molecular and genetic analyses, therefore PCR is used to amplify small segments of DNA for analysis. To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures into two pieces of single-stranded DNA. Next, an enzyme called Taq polymerase synthesizes two

new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Each of these strands can then be used to create two new copies. The cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA segment [72]. The amplified target sequence, which may have been present as only a single copy in the original mixture, can then be detected by electrophoresis. The entire cycling process of PCR is automated and is directed by a machine called a thermocycler. The thermocycler is programmed to alter the temperature of the reaction every few minutes to allow DNA denaturing and synthesis. The entire PCR process is complete in just a few hours.

Ho et al. [59] developed the first DNA-probe used in conjunction with PCR amplification. The 0.85-kb *T. foetus* DNA probe was developed through identification of conserved DNA sequences from *T. foetus* isolates collected in California, Idaho, Nevada, and Costa Rica. The probe specifically identified *T. foetus* regardless of geographic origin, but it did not hybridize to DNA preparations of *T. vaginalis*, bovine cells, or a variety of bacteria from cattle. To maximize sensitivity, a partial sequence of the probe was used to identify two oligonucleotide primers (TF1 and TF2) that were then used to amplify a 162-bp product from *T. foetus* DNA by PCR. Ho et al. [59] then hybridized a chemiluminescent internal *T. foetus* sequence probe to Southern blots of the amplification products.

The DNA-probe developed by Ho et al. [59] was able to detect one *T. foetus* organism in culture or 10 organisms in bovine preputial smegma. In their analysis of 52 clinical samples collected from both infected and uninfected bulls, 47 (90.4 %) out of 52

were correctly identified with no false-positive reactions. Traditional culture diagnostic methods correctly identified 44 (84.6 %) out of 52 samples. The sensitivity of the PCR-based test was 88.6 % (39/44), while the sensitivity of traditional culture methods was 81.8 %. However, Ho et al. [59] acknowledged that the sensitivity of their PCR-based test was perhaps lower than anticipated because of inhibition of *Taq* DNA polymerase by various inhibitors present in bovine smegma [73-75]. While this new PCR-based *T. foetus* test was very promising, other researchers [58,69] using the same methods as Ho et al. [59] reported false positives due to amplification of non-specific amplicons, as well as some cross-reactivity with other Trichomonads.

Between May 1993 and April 1994, 65 (6 %) of 1,048 bulls from Saskatchewan, Canada were culture positive according to the Provincial Veterinary Laboratory and the Western College of Veterinary Medicine Laboratory [15]. Riley et al. [15] maintained 16 of these positive cultures by serial passage for further PCR investigation of the *T. foetus* field isolates. DNA was purified from two *T. vaginalis* isolates, all 16 Saskatchewan field isolates, and an American Type Culture Collection Austrian isolate, 50151. The *T. foetus*-specific single-band PCR test described by Ho et al. [59] was then conducted on the purified DNA. The two *T. vaginalis* isolates were PCR negative, while 15 of the 16 Saskatchewan field isolates and the American Type Culture Collection Austrian isolate, 50151, strongly amplified the expected 162-bp product [15]. The remaining field isolate only produced a weak 162-bp product, suggesting that the isolate was either not *T. foetus* or that the isolate perhaps contained some genetic variation in the region amplified by primers TF1 and TF2. To further investigate the identity of the unknown isolate, Riley et al. [15] conducted additional DNA fingerprinting of all the *T. foetus* isolates with both

T17 PCR and randomly amplified polymorphic DNA PCR. Their research revealed genetic variation (polymorphism) among *T. foetus* isolates, indicating the need for further investigation into DNA-based diagnostic assays for *T. foetus*.

Felleisen et al. [55] reported more recent progress in developing an effective *T. foetus* PCR-based assay in 1998. Felleisen had previously described highly conserved sequences involving the 5.8S ribosomal RNA (rRNA) gene and the flanking internal transcribed spacer regions (ITSRs) ITS1 and ITS2 of *T. foetus* isolates originating from different geographic regions [76]. The PCR amplification process developed by Felleisen et al. [55] was therefore based upon the primers TFR3 and TFR4 directed at the rRNA gene units of *T. foetus*. TFR3 is complimentary to the ultimate 5′ end of the 28S rRNA gene, and TFR4 is located at the border of the 18S rRNA gene and ITS1. By using the TFR3 and TFR4 primers, Felleisen et al. [55] incorporated both the 5.8S rRNA and the ITS1/ITS2 regions previously reported to be unique to *T. foetus* [76]. Both sequences were reportedly different from those of other related trichomonads [76], and specificity was confirmed using genomic material from related trichomonads.

The PCR-based assay developed by Felleisen et al. [55] detected a single organism in diagnostic culture or about 50 organisms per milliliter of preputial wash fluid. The decreased sensitivity in preputial wash fluid was thought to be due to the presence of inhibitory substances in preputial smegma that could not be removed. However, Felleisen et al. suggested that despite the slight decrease in sensitivity associated with preputial wash fluid, PCR-based tests should still be evaluated as direct *T. foetus* screening tests.

Another problem frequently encountered in PCR-based diagnostic tests is the presence of unwanted amplification products. To avoid the potential carryover contamination of byproducts from previous amplification reactions, Felleisen et al. [55] adapted the use of the uracil DNA glycosylase system described by Longo et al. [77] in 1990. The success of their newly developed PCR-based test for *T. foetus* led Felleisen et al. [55] to propose that their test be used either as (1) a confirmatory test following microscopic diagnosis via in vitro culture; or (2) a direct *T. foetus* screening test.

A frustrating situation with the previously described PCR-based assays for the confirmation of *T. foetus* infections is that negative results present as null data, indistinguishable from a failed PCR during *T. foetus* specific amplification [78]. Thus, a negative PCR assay has three potential meanings: (1) the sample is *T. foetus* negative, (2) the sample is *T. foetus* negative, but the PCR amplification failed, or (3) the sample is *T. foetus* positive but reported as negative because of a failed PCR amplification [78]. While the first two scenarios are harmless, the third is potentially devastating. In an effort to address this problem, Hayes et al. [56] and Grahn et al. [78] developed different strategies to eliminate false negatives and positively identify the causative agent involved in a bovine trichomonad infection.

Hayes et al. [56] developed an approach for differentiating three common types of trichomonads found in the bovine preputial cavity. They specifically focused on *T. foetus*, *Pentatrichomonas hominis*, and a *Tetratrichomonas* species. The process involved PCR followed by restriction fragment length polymorphism (RFLP) analysis. Universal trichomonad primers, TFR1 and TFR2, amplified the 5.8S rRNA gene and the ITS1/ITS2 regions. The amplification products were then digested with the restriction enzyme

*Hpy*CH4IV, followed by RFLP. The RFLP analysis was effective in differentiating between *T. foetus*, *Pentatrichomonas hominis*, and a *Tetratrichomonas* species. This technique eliminated the need for costly and time-consuming DNA sequencing previously needed to identify the cultured trichomonad, but only for three common types of trichomonads found in the bovine preputial cavity.

Grahn et al. [78] improved the previously developed PCR-based assays by analyzing diagnostic size variants from the ITS1 region located between the 18S rRNA and 5.8S rRNA subunits. Samples of sequence-verified DNA from various trichomonad species were tested including *T. foetus*, *Tritrichomonas suis*, *Trichomonas vaginalis*, *Trichomonas gallinae*, *Pentatrichomonas hominis*, and a *Tetratrichomonas* species, as well as sequence-verified isolates from the pigeon, house finch, crow, scrub jay, and domestic duck. The newly developed PCR-based assay [78] successfully amplified trichomonad DNA from many genera and positively identified the infectious agent.

At present, PCR-based assays are most commonly used as confirmatory tests for bovine trichomoniasis because of the relatively low cost of in vitro cultivation compared to PCR-based assays. However, PCR-based assays are currently very effective in diagnosing human trichomoniasis caused by *Trichomonas vaginalis*, with a sensitivity of 95 % and a specificity of 98 % [79]. It is therefore very likely that in the future the preferred diagnostic test for bovine trichomoniasis will be a PCR-based assay. Some researchers have already advocated the use of molecular-based assays as independent diagnostic tests for bovine trichomoniasis [78,80].

Staining techniques

Suspect specimens can also be stained to evaluate the cultured organism's morphology to help differentiate *T. foetus* from non-*T. foetus* protozoa. Non-*T. foetus* protozoa are similar in size and shape to *T. foetus*, but they have varying numbers of flagella (1-5) and their undulating membranes are either absent or not as well developed [18]. The primary purpose of staining is to optimize the visualization of key anatomic structures to aid in accurate identification of the organism [57].

A simple and rapid staining technique reported by Lun and Gajadhar [57] involves Diff-Quick® and Lugol's iodine solution. Their staining procedure allows microscopic identification of several key morphological structures of *T. foetus*. Three anterior and one posterior flagella stain purple. The undulating membrane also stains purple with 2-5 waves. The axostyle stains translucent purple, while the nucleus appears dark purple. The blepharoplast/pelta and costa stain purple, whereas the cytoplasm stains blue and contains translucent vacuoles. Several organisms on a stained slide are usually examined before visualizing all of the important structures of *T. foetus*.

Serologic tests

The "gold standard" for diagnosis of trichomoniasis in either male or female cattle is the culture of live *T. foetus* organisms from either preputial smegma or cervicovaginal mucus, respectively. Ideally, a positive culture would be confirmed with a PCR-based assay. A single culture of preputial smegma from a bull has a relatively high sensitivity between 80 to 90 % depending on culture conditions [48,64]. However, culture of cervicovaginal mucus from cows/heifers only has a sensitivity of 58 to 75 % [35]. Culturing females is further complicated by the fact that most female cattle will clear

themselves of a *T. foetus* infection in two to five months [81]. In an effort to improve screening herds for *T. foetus*, attempts have therefore been made to develop serologic tests for evidence of herd exposure. Serological tests could also help potentially identify previously exposed females prior to purchase, indicating the need for multiple negative cultures prior to their entry into a new herd.

Robertson [82] first suggested that serum antibodies formed in response to a venereal infection, and could perhaps be used to screen for trichomoniasis. However, the presence of nonspecific serum antibodies in uninfected females inhibited the diagnostic applicability of such an assay. The cross-reacting antibodies were possibly the result of either an immune response to normal gut flora, including members of the family Trichomonadidae [22], or the result of nonspecific binding of immunoglobulin to the parasite surface [83].

Virgin heifers experimentally infected with *T. foetus* do develop anti-*T. foetus* antibodies (IgA, IgG1, IgG2, and IgM) in vaginal, cervical, and uterine secretions [29,36,84-86]. Such experiments, however, have detected little if any serum IgG1or IgG2 response to infection. In an effort to improve serological based detection of *T. foetus*, BonDurant et al. [81] developed a serological assay for the detection of: (1) an erythrocyte-adhering molecule shed by *T. foetus*; and (2) serum antibodies to the erythrocyte-adhering molecule in exposed cattle. The sensitivity and specificity of the assay in known infected and uninfected cows/heifers was 94 and 95.6 %, respectively. While the newly developed assay was effective in determining exposure to *T. foetus* antigens in female cattle, the assay could not differentiate between antigens derived from

natural exposure or vaccination. However, the assay is still valuable if herd vaccination status is known. Unfortunately, the test is not commercially available.

The serological assay developed by BonDurant et al. [81] was also evaluated in 14 chronically infected bulls. Only six (43 %) of the bulls showed a positive hemolytic response. The poor response in bulls is presumably a consequence of the more superficial nature of *T. foetus* infections in bulls [23].

In summary, cows and heifers infected with *T. foetus* do develop detectable systemic and local antibodies to various antigens of *T. foetus*. However, despite research advances in developing serologic assays for *T. foetus*, there are no effective, commercially available serologic tests for *T. foetus*.

Sample handling

Improper handling of diagnostic specimens can impede diagnosis of *T. foetus*. Skirrow et al. [64] reported that storage of preputial smegma in lactated Ringer's solution at 5 ºC for 24 hours before inoculation into Diamond's medium resulted in a 14 % loss in sensitivity. The decrease in sensitivity may have resulted from the low storage temperature, the use of a non-nutrient storage medium, or both.

Bryan et al. [87] conducted an experiment to evaluate the effects of temperature on the survival of *T. foetus* in Diamond's and InPouch™ TF media. Regardless of whether Diamond's or InPouch™ TF media was used, all the cultures were positive when kept for up to four days at either 22 ºC or 37 ºC. Those results agreed with previous studies that showed *T. foetus* would grow well in Diamond's and InPouch™ TF media when incubated at 37 ºC [63,65]. In contrast, all the samples maintained at –20 ºC for more than one hour in Diamond's medium or more than 3 hours in InPouch™ TF medium

were negative. Depending on geographical location, it is therefore important to consider the effects of extremely cold temperatures on *T. foetus* survival. Wide variations in temperature can also occur with commercial air carriers.

Refrigeration of diagnostic samples reduces the growth of some bacterial and fungal contaminants [87]. However, the success of *T. foetus* cultures maintained at 4 ºC for 12 to 48 hours has varied greatly, with sensitivities ranging from 68.8 to 100 % [64,88-90]. Some of the variation is possibly due to the genetic variability of *T. foetus* isolates [15]. Another possibility is that the phase of the growth cycle of *T. foetus* may be a factor in low temperature survival [90].

Research supports the conclusion that time, temperature, *T. foetus* isolate, type of medium, sunlight, and possibly phase of the growth cycle may all have an impact on the sensitivity of in vitro *T. foetus* cultures. Therefore, the general recommendation is that diagnostic specimens be placed in Diamond's or InPouch™ TF media immediately. The inoculated culture should then be placed in an incubator between 22-37 ºC.

Prevention and Control of Bovine Trichomoniasis

Prevention and control of bovine trichomoniasis follow many of the same management strategies, and to a large extent focus on herd biosecurity. Ideally, every cattle operation should focus on the prevention of bovine trichomoniasis.

Preventing introduction of *T. foetus* into a cattle herd

Preventing the introduction of *T. foetus* into a cattle herd requires the application of basic biosecurity protocols such as:

1. Avoid grazing cattle on public lands where both bulls and cows have a much greater risk of exposure through coitus with other *T. foetus* infected animals [91].
2. Utilize artificial insemination when possible [20].
3. Use a 60-90 day breeding season. Cull all cows and heifers that are not pregnant after the breeding season. A long breeding season not only allows propagation of *T. foetus*, but it may also hide production losses due to reduced weaning weights because of delayed conception [92].
4. Control animal movement into a herd. Maintain good fences to prevent *T. foetus* infected animals from inadvertently entering a herd, or to prevent uninfected animals from temporarily entering a *T. foetus*-infected herd and then returning with *T. foetus* to their clean herd.
5. Purchase virgin bulls and heifers as replacements. Buying older bulls and cows as replacements greatly increases the chance of purchasing a *T. foetus*-infected animal. While older bulls are much more likely to become chronically infected with *T. foetus* than cows, a small percentage of cows will also become chronically infected [28].
6. Test all bulls for *T. foetus* at least once before introducing them into a new herd [20]. The test should be performed after two weeks of sexual rest. Ideally, a bull should have three negative cultures at weekly intervals.

7. Maintain as young a bull battery as possible. Older bulls are much more likely to be chronically infected with *T. foetus* [19,93].
8. Breed purchased cows and heifers in a separate herd. Cull all the cows and heifers that are not pregnant after the breeding season. Ideally, continue to keep the pregnant animals segregated from the rest of the herd through the next breeding season [48].

Control of *T. foetus* in an infected cattle herd

Since no legal treatment for *T. foetus* exists in the United States [20], veterinarians and cattle producers must rely on various management strategies to control and eradicate *T. foetus* from an infected herd. In addition to following all of the previously mentioned prevention strategies for *T. foetus*, producers and veterinarians trying to control *T. foetus* in an infected cattle herd should focus on the following recommended practices:

1. Test and cull all infected bulls. Infected bulls should be sold for slaughter only.
2. Decrease the number of bulls per breeding unit. Single-sire herds offer the lowest exposure potential. However, single-sire units may not always be practical.
3. Reduce the average age of the bull herd.
9. Only purchase bulls from herds known to be free of *T. foetus*. All purchased bulls must pass a breeding soundness evaluation and have a least one negative *T. foetus* culture before being allowed into the herd. The test should be performed after two weeks of sexual rest. Ideally, three negative cultures at weekly intervals would be obtained.
4. Utilize artificial insemination when possible [20].

5. Reduce the breeding season to 90-120 days. Pregnancy exams should be performed 45-60 days after the breeding season. Open cows and heifers should be culled. If there are too many open cows for culling to be economically feasible, then these animals should at least be separated into a high-risk herd.
6. Culture all pyometras diagnosed in cows or heifers during pregnancy examinations.
7. Submit all aborted fetuses and placental tissue to a diagnostic laboratory.
8. Vaccination of all breeding age females against trichomoniasis will raise a herd's immunity. Vaccination does not offer complete protection, but it does reduce the duration of infection therefore mitigating the reproductive wastage caused by *T. foetus* [38-40,94].

TrichGuard® (Fort Dodge Animal Health) is the only vaccine for *T. foetus* available in the United States. The vaccine requires an initial subcutaneous dose followed by a booster dose two to four weeks later. The second injection should precede the breeding season by 4 weeks. Annual revaccination four weeks prior to the breeding season is recommended.

CHAPTER III

METHODS AND MATERIALS

Prospective Survey of Trichomoniasis in Alabama Beef Bulls

The research design (Figure 3) for the prospective survey of trichomoniasis in Alabama beef bulls focused primarily on sampling bulls throughout Alabama that presented to veterinarians for routine breeding soundness evaluations. Owners volunteered to have each bull cultured for *T. foetus* at no additional cost.

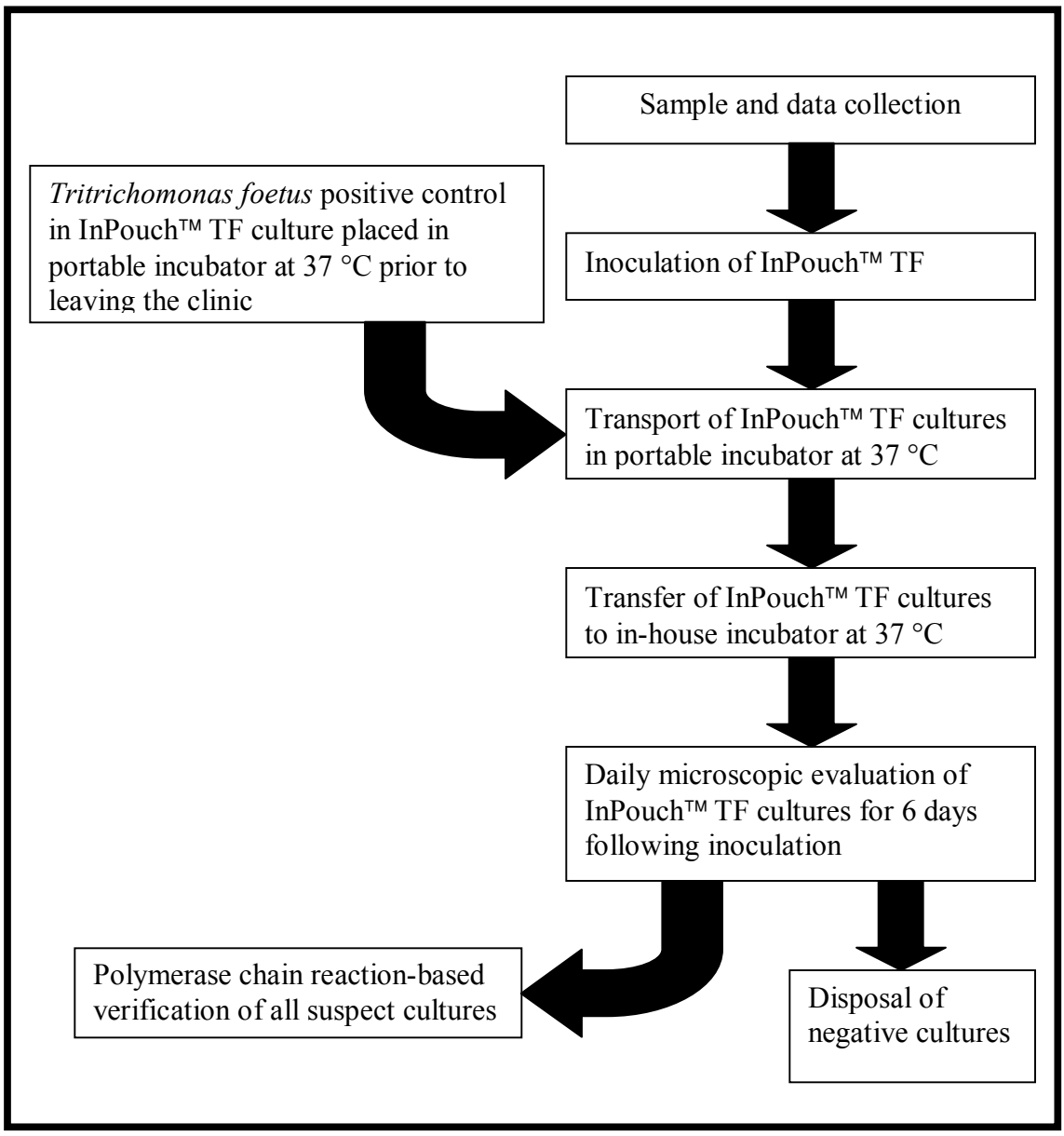

Figure 3. Research Design.

Sample size determination

According to the Alabama Agricultural Statistics Bulletin there were approximately 724,000 beef cows in Alabama in 2005 [95]. Based on that figure, and assuming a bull to cow ratio of 1:20, there were approximately 36,200 herd bulls in

Alabama in 2005. The sample size was then calculated using the Epi Info™ Version 3.3 software from the Centers for Disease Control and Prevention. Given an estimated prevalence of 3 % (and not less than 0.1 %), a confidence level of 95 %, and a population of 36,200 herd bulls, 132 bulls were needed to determine the preliminary prevalence of *T. foetus* in Alabama beef bulls.

Survey animals

Bulls were sampled from January 2005 to March 2006. A bull's individual identification, age, breed, location, and herd size were recorded at the time of collection. All owners volunteered to have their bulls tested for *T. foetus*. Of the 240 bulls sampled, 148 presented to the Auburn University College of Veterinary Medicine Large Animal Clinic for routine breeding soundness evaluations, 76 presented to private Alabama veterinarians for routine breeding soundness evaluations, and 16 presented to a private Alabama veterinarian due to a herd reproductive problem.

Sampling technique

Bulls were properly restrained in a livestock chute to avoid injury to the bulls or personnel and then sampled as described by Peter [20]. The external preputial area was cleaned with disposable paper towels without soap or disinfectants. A new pair of exam gloves was used for each bull, and a sterile, dry, plastic infusion pipette with a 12 mL or 20 mL syringe attached to one end was placed into the preputial fornix. The pipette was scraped vigorously across the preputial epithelium without aspiration, and then negative pressure was applied with the syringe to collect some of the preputial smegma. The negative pressure was released before removing the pipette from the sheath to avoid unnecessary aspiration of urine or other contaminants. After removing the pipette from

the sheath the sample was placed immediately into the transport/culture medium as described below. A new syringe and pipette was used for each bull.

Culture media

The selected transport and culture medium for this research was the self-contained InPouch™ TF *Tritrichomonas foetus* culture pouch (BioMed Diagnostics; White City, OR). The InPouch™ TF medium contains trypticase, proteose peptone, yeast extract, maltose and other sugars, amino acids, salts, antifungal and antimicrobial agents in a normal saline phosphate buffer. The medium is therefore selective for the transport and growth of *T. foetus* while inhibiting the growth of contaminating microorganisms. The pouch is constructed with a clear plastic film with water vapor and oxygen-barrier qualities that help maintain the proper microaerophilic environment. The pouch contains two V-shaped chambers that are separated by a channel that allows liquid to pass through if pressure is applied. An inoculum of 100 or fewer microorganisms is sufficient to result in a positive test [65].

The pouch was inoculated as described by Borchardt et al. in 1992 [65]. The InPouch™ TF medium was manually expressed to place approximately 1 mL (out of 4 mL total) in the upper chamber. The pouch was then opened at the notch just above the closure tape, and the pipette tip inserted into the upper chamber. Approximately 0.5 mL and no more than 1.0 mL of the preputial sample was inoculated into the upper chamber. If the preputial smegma adhered to the wall of the pipette then a small amount of medium was used to rinse the contents of the pipette into the upper chamber. The upper chamber was then manually expressed into the lower chamber, and the top of the pouch folded

down several times until the closure tape was at the top of the label. Wire tabs were folded to lock the pouch and maintain the proper microaerophilic environment.

Transport

The inoculated InPouch™ TF culture pouch was placed directly into a 37 °C portable incubator in a vertical position for transport.

Portable incubator model

The portable Deluxe ThermoTote Incubator from Scientific Device Laboratories (Des Plaines, IL) was used for all sample transport.

Incubation

The samples were maintained in the original InPouch™ TF and transferred in a vertical position to an in-house incubator at 37 °C. The samples remained in the incubator at all times except for periodic microscopic evaluations.

Positive control

A culture of *T. foetus* (Beecham strain) was purchased from BioMed Diagnostics, Inc. and maintained in an InPouch™ TF culture pouch for use as a positive control. The culture was maintained through weekly subcultures into a fresh InPouch™ TF. As a positive control, a new InPouch™ TF was inoculated with a small amount of *T. foetus* on the day of testing and placed in the portable incubator with the other test samples. Upon returning to the clinic, both the positive culture and the test samples were transferred to the in-house incubator. The positive control was microscopically evaluated at the same time as the test samples. The positive control was verified as *T. foetus* by a PCR-based assay at the Alabama State Diagnostic Laboratory.

Sample analysis

Microscopic evaluation of the InPouch™ TF culture was conducted daily for 6 days following inoculation. Prior to evaluation, the pouch was pulled approximately five times across the edge of a table for mixing. The bottom of the lower chamber was then placed on the raised platform of the InPouch™ TF microscope clip. The microscope clip was locked over the pouch and then evaluated first at 100X magnification. Any suspect organisms were evaluated at 400X for positive morphological identification.

Polymerase chain reaction-based verification

Accurate microscopic identification of *T. foetus* can be complicated by the presence of other trichomonadid protozoa [18,53-56]. Contamination of the preputial orifice or cavity with feces probably explains the presence of these opportunistic trichomonads. None of the contaminating trichomonads, however, results in reproductive pathology in cows or bulls [70]. To avoid false-positive results due to fecal contamination, all suspect cultures on microscopic analysis were submitted to the Alabama State Diagnostic Laboratory and the Parasitology Laboratory at the Auburn University College of Veterinary Medicine for PCR-based verification.

Polymerase chain reaction (PCR) is a fast and inexpensive technique described by the National Human Genome Research Institute as molecular photocopying [72]. Significant amounts of a sample of DNA are necessary for molecular and genetic analyses, therefore PCR is used to amplify small segments of DNA for analysis. To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures into two pieces of single-stranded DNA. Next, an enzyme called Taq polymerase synthesizes two new strands of DNA, using the original strands as templates. This

process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Each of these strands can then be used to create two new copies. The cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA segment [72]. The amplified target sequence, which may have been present as only a single copy in the original mixture, can then be detected by electrophoresis. The entire cycling process of PCR is automated and is directed by a machine called a thermocycler. The thermocycler is programmed to alter the temperature of the reaction every few minutes to allow DNA denaturing and synthesis. The entire PCR process is complete in just a few hours.

Retrospective Analysis of *Tritrichomonas foetus* Cultures Performed at the Alabama State Diagnostic Laboratory

The Alabama State Diagnostic Laboratory generated a computer record for all *T. foetus* cultures performed between October 2002 and March 2005. The record included each animal's county of origin, but no information was available regarding animal breed, age, or herd size.

CHAPTER IV

RESULTS

Prospective Survey Prevalence

The prospective survey was performed from January 2005 to March 2006. A total of 240 preputial samples were collected from bulls in 21 herds located in 11 Alabama counties (Table 1). Of the 240 bulls sampled, 148 bulls presented to the Auburn University College of Veterinary Medicine Large Animal Clinic for routine breeding soundness evaluations, 76 bulls presented to private Alabama veterinarians for routine breeding soundness evaluations, and 16 bulls from one herd presented to a private Alabama veterinarian because of herd reproductive problems. All owners volunteered to have their bulls tested for trichomoniasis.

The 240 bulls represented approximately 0.7 % of the estimated 36,200 bulls in Alabama in 2005. Angus bulls represented 59.6 % of the sample population, and the average age for all bulls tested was 4.4 years (Table 2). Herds were classified as small, medium, or large based on the number of breeding age females. Small herds contained 1-99 breeding age females; medium herds contained 100-499 breeding age females; and large herds contained 500+ breeding age females. The survey included 8 small herds, 9 medium herds, and 4 large herds (Table 3).

Alabama County	Number of herds	Number of bulls
Bullock	1	10
Chambers	6	82
Chilton	1	1
Escambia	1	38
Hale	1	16
Lee	3	9
Lowndes	1	6
Macon	3	9
Monroe	1	22
Tallapoosa	2	31
Wilcox	1	16
11 counties	21 herds	240 bulls

Table 1. Number of herds and bulls sampled in various Alabama counties as part of prospective survey.

Bull breed	Number of bulls	Percent	Mean age (Years)
Angus	143	59.6	4.5
Brangus	9	3.8	3
Charolais	26	10.8	4.5
Gelbveih	2	0.8	3
Hereford	23	9.6	4.2
Limousin	2	0.8	7
Red Angus	7	2.9	3.7
Simmental	28	11.7	4.3
Total	240	100	4.4

Table 2. Bull breed representation and mean age in prospective survey.

Herd size	Number of herds
Small (1-99 breeding age females)	8
Medium (100-499 breeding age females)	9
Large (500+ breeding age females)	4

Table 3. Number of herds of varying sizes included in prospective survey.

Of the 240 preputial samples, three cultures (1.25 %) contained organisms resembling *T. foetus* (Table 4). However, the three suspect cultures were all negative for *T. foetus* by PCR-based assays performed by the Alabama State Diagnostic Laboratory and in the Parasitology Laboratory at the Auburn University College of Veterinary Medicine. The positive control *T. foetus* isolate yielded the appropriate sized PCR amplification products. Therefore, no bulls cultured positive for *T. foetus*.

Number of bulls sampled	Number of suspect samples	Number of PCR positive samples	Number of bulls positive for *T. foetus*
240	3 (1.25 %)	0	0

Table 4. Prospective survey culture results.

The three suspect cultures all came from the same herd and were collected on the same day. The suspect cultures were collected from a 5 year-old Simmental bull, a 3 year-old Angus bull, and a 4 year-old Angus bull. The suspect organisms had a jerky and rolling motility, and they had visible undulating membranes and multiple anterior flagella. The organisms were approximately the same size as *T. foetus*, but they were more rounded than the *T. foetus* positive control. The suspect organisms also did not grow as readily as the *T. foetus* positive control in the InPouch™ TF medium, and they did not survive the subculture process into a new InPouch™ TF culture pouch.

Retrospective Analysis of Alabama State Diagnostic Laboratory Records

The retrospective analysis of Alabama State Diagnostic Laboratory records contained *T. foetus* cultures performed from October 2002 through March 2005. The

analysis included 374 bulls from 19 herds located in 14 Alabama counties (Table 5). No data was available regarding bull breed or age. The reason for testing was also unavailable. Of the 374 bulls, one bull (0.27 %) was confirmed positive for *T. foetus* by a PCR-based assay (Table 6). No data was available as to the number of suspect samples.

Alabama County	Number of herds	Number of bulls
Autauga	1	37
Chilton	1	8
Coffee	1	1
Covington	1	94
Crenshaw	1	41
Dallas	1	10
Greene	1	2
Hale	1	5
Lee	1	1
Lowndes	5	145
Marengo	1	1
Mobile	1	5
Monroe	1	15
Montgomery	2	9
14 counties	19 herds	374 bulls

Table 5. Number of herds and bulls analyzed in a retrospective analysis of *T. foetus* cultures performed at the Alabama State Diagnostic Laboratory.

Number of bulls sampled	Number of suspect samples	Number of PCR positive samples	Number of bulls positive for *T. foetus*
374	Data not available	1	1 (0.27 %)

Table 6. Retrospective analysis of Alabama State Diagnostic Laboratory culture results.

Combined Results of Prospective Survey and Retrospective Analysis of Alabama State Diagnostic Laboratory Records

Combining the results of the prospective survey and the retrospective analysis of the Alabama State Diagnostic Laboratory records, a total of 614 bulls were tested from October 2002 through March 2006. The 614 bulls came from 40 herds in 20 counties (Table 7). Of the 614 bulls tested, one bull (0.16 %) cultured positive for *T. foetus* and was confirmed by a PCR-based assay (Table 8).

Alabama County	Number of herds	Number of bulls
Autauga	1	37
Bullock	1	10
Chambers	6	82
Chilton	2	9
Coffee	1	1
Covington	1	94
Crenshaw	1	41
Dallas	1	10
Escambia	1	38
Greene	1	2
Hale	2	21
Lee	4	10
Lowndes	6	151
Macon	3	9
Marengo	1	1
Mobile	1	5
Monroe	2	37
Montgomery	2	9
Tallapoosa	2	31
Wilcox	1	16
20 counties	40 herds	614 bulls

Table 7. Combined number of herds and bulls in the prospective and retrospective study.

Number of bulls sampled	Number of PCR positive samples	Number of bulls positive for *T. foetus*
614	1	1 (0.16 %)

Table 8. Combined results of prospective survey and retrospective analysis of Alabama State Diagnostic Laboratory cultures.

CHAPTER V

DISCUSSION

The prevalence of trichomoniasis in Alabama beef bulls, based on a prospective survey of 240 Alabama bulls and a retrospective analysis of 374 *T. foetus* cultures performed at the Alabama State Diagnostic Laboratory, was 0.16 % from October 2002 through March 2006. A prevalence of 0.16 % is less than the prevalence of trichomoniasis reported in many western states (4-7 %) [5-7,9] and Florida (6 %) [8], but it does indicate the presence of *T. foetus* in Alabama. Therefore, Alabama cattle producers and veterinarians should work together to minimize the spread of trichomoniasis throughout the state, including testing herds with poor reproductive performance for trichomoniasis. Also, despite the low prevalence of *T. foetus* in Alabama, producers should consider testing all new bulls due to the serious consequences of adding a positive bull to a herd.

Of the 240 bulls included in the prospective survey, 226 bulls (94 %) originated from herds with no known reproductive problems. These 226 bulls were from well-managed herds presenting their bulls for routine breeding soundness evaluations. The remaining 16 bulls were from one herd with a history of infertility. The prevalence could therefore be significantly higher in Alabama if a known high-risk population of bulls were sampled. Age and herd size are both considered risk factors for a bull acquiring a

chronic *T. foetus* infection. The mean age in the prospective survey was only 4.4 years, and 17 out of the 21 herds (81 %) contained less than 500 breeding age females. In the epidemiological survey conducted by Rae et al. [8], medium sized herds were much less likely to be infected (100-499 cows, 10 %) than large herds (500+ cows, 53.9 %).

The 374 cultures included in the retrospective analysis did presumably contain many bulls from herds with reproductive problems. However, no data regarding bull age, breed, or reason for testing was available, so the risk status of those 374 bulls was unknown. Therefore, it is possible that many of the cultures were performed routinely prior to sale, prior to purchase, or prior to breeding, with no indication of reproductive problems in the herd of origin.

The fact that 3 (1.25 %) of the 240 cultures performed as part of the prospective survey contained fecal trichomonads supports the recommendation that a PCR-based confirmatory assay is essential for any culture suspected of being *T. foetus*. Also, a clean sampling technique is important to minimize fecal contamination of *T. foetus* cultures.

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